ISOLATION AND IDENTIFICATION OF BEHAVIOURALLY ACTIVE SEMIOCHEMICALS FROM HUMAN FOOT ODOUR AS ATTRACTANTS FOR AFRICAN MALARIA VECTORS

BY

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DECLARATION

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

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DEDICATION

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LIST OF ABBREVIATIONS

CDC----------Centers for Disease Control

CFG----------Counter Flow Geometry

CI----------Confidence Interval

DCM----------Dichloromethane

DDT----------1,1,1-Trichloro-2,2-*bis*-(*p*-chlorophenyl)ethane

EAD----------Electro-antennal Detector

EAG----------Electro-antennogram

EIMS----------Electron Impact Mass Spectrometry

FID----------Flame Ionization Detector

GC----------Gas Chromatography

GC-MS----------Gas Chromatography – Mass Spectrometry

GC-EAD----------Gas Chromatography – Electro-antennogram detector

GM----------Genetically Modified

HP----------Hewlett Packard

IDRC----------International Development Research Centre

IGRs----------Insect Growth Regulators

IVM----------Integrated Vector Management

LA----------Least Attractive

LSD----------Least Significant Difference

M/Z----------Mass to Charge ratio

MA----------Most Attractive

MS----------Mass Spectrometry

NMR----------Nuclear Magnetic Resonance

p-ABA----------Para aminobenzoic acid

SAS----------Statistical Analysis System

SIT----------Sterile Insect Technique

TLC----------Thin Layer Chromatography

UK----------United Kingdom

W/W----------Weight by Weight

WHO----------World Health organization

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ABSTRACT

Despite their small size and short lifetime, mosquitoes (Diptera: Culicidae) are vectors of diseases of vast economic and medical importance. They transmit life threatening human and animal diseases, in addition to their buzzing, biting and annoyance. Questions on why mosquitoes are attracted to certain hosts and what attractants are responsible for their odour mediated behaviour remain unanswered. There is a plethora of evidence suggesting that mosquito host-seeking behaviour is mediated by a wide range of info-chemicals. Mosquito chemical ecology remains poorly understood. With improved knowledge of the role of the chemical cues in mosquito behaviour, new methods of mosquito control based on behaviour-modifying cues could be implemented, thus leading to effective and improved control of the spread of the dangerous pathogens such as, the malaria causing parasites (*Plasmodium* spp). Various human volatiles (kairomones) have been observed to mediate the host-seeking process in laboratory settings, yet no effective bait has been identified for field application. In this work, 11 Electro-antennogram (EAG) active compounds were identified from the human foot odour. Subtraction bio-assays under laboratory and semi-field conditions of the EAG active compounds revealed that 3 out of the 11 were allomones, while the remaining 8 were important kairomonal blend components of the human foot odour to *Anopheles gambiae sensu sitricto* mosquitoes. The results show that these compounds are effective as a blend and that the differential attractiveness of the malaria vectors to human feet is due to quantitative differences in the production of these compounds. *Anopheles gambiae s.s.* and *An. funestus* have been trapped with human foot odour baited Counter Flow Geometry (CFG) traps under semi-field conditions in western Kenya. Several entomopathogens have been shown to be effective against mosquitoes, but their application is difficult. The use of attractants could improve the efficacy of entomopathogens as means of controlling anopheline mosquito population and hence malaria transmission.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria parasites have been with us since the dawn of time. They probably owe their origin to Africa (along with mankind). Fossils of mosquitoes up to 30 million years old show that the vectors for malaria were present well before the earliest known history of man (Wigglesworth, 1976). From their origins in Africa, early trans-pacific voyagers possibly brought *Plasmodium vivax* and *P. malariae* to the New World, and this trend of imported malaria continues to this day (Katinka, 1999). *P. falciparum* may have come in consignments of slaves bound for the Spanish colonies (Wigglesworth, 1976; Service, 1986). The *Plasmodium* parasites are highly specific, with vertebrates as the only hosts and *Anopheles* mosquitoes as the only vectors (Anderson & Morales, 1993).

Hippocrates was the first to describe the manifestations of the disease, and relate them to the time of the year and where the patients lived. Before this, the supernatural was blamed. The association of malaria with stagnant waters led the Romans to begin drainage programs as the first control strategy against malaria (Wigglesworth, 1976). The first recorded treatment dates back to 1600, where the native Peruvian Indians used the bitter bark of the cinchona tree. By 1649, the bark was available in England, as 'Jesuits powder' (Barry *et al.*, 1995) so that those suffering from 'agues' might benefit from the quinine in it. Malaria in UK (known as agues) would have been clustered around stagnant marshes, and the invading Roman soldiers would certainly have brought the disease with them (Wigglesworth, 1976).

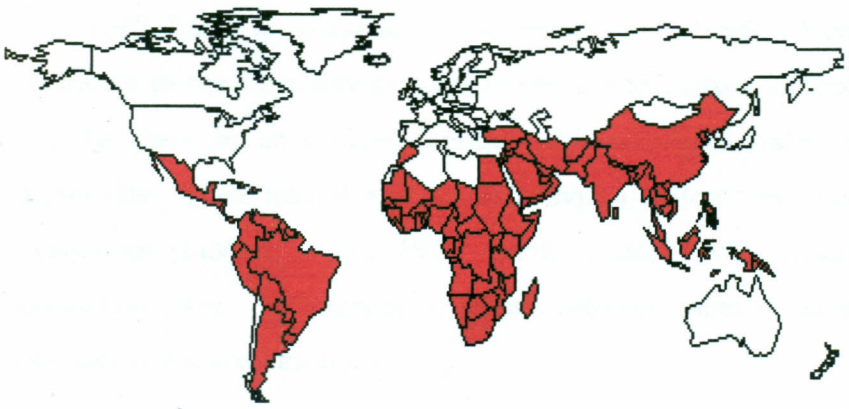
It was not until 1880 that Laveran described the protozoan cause responsible for malaria, from his work in Algeria, and in 1897 *Anopheles* mosquito was demonstrated to be the vector for the disease (Ross, 1897; Bastianelli *et al.*, 1898). Epidemiology of malaria was clear at this point and implementation of control measures started.

Global eradication of malaria seemed possible with the discovery of DDT in 1942 and its first use in Italy in 1944 (Anderson & Morales, 1993). Widespread systematic control measures such as the spraying with DDT, coating marshes with paraffin (to block *Anopheles* mosquito larvae spiracles), draining stagnant water and the use of cheap, effective drugs such as chloroquine were implemented, with impressive results. Despite initial success, there was a complete failure to eradicate malaria in many countries due to many factors. Although technical difficulties such as insecticide and drug resistance have played a part, the main failure to reduce the disease is probably due to socio-political factors preventing efficient implementation of control measures (Anderson & Morales, 1993).

1.2 Distribution of malaria

Globally, an estimated 200-300 million people are affected by malaria, of which 1.5-2.7 million die each year (WHO, 1997; Breman, 2001). Ninety percent of these cases occur in sub-Saharan Africa and children under five years are worst hit by the disease (WHO, 1998). There are an estimated 2.4 billion people at risk of malaria, which has been recorded as far north as 64° latitude and south as 32° latitude. Malaria occurs 400 m below sea level in the Dead Sea area and 2800 m above sea level in Cocha-Mbamba, Bolivia. Within these limits of latitude and altitude there are large areas free of malaria, which is essentially a focal disease (Fig. 1).

Figure 1: The distribution of malaria in the world (WHO, 1997)



Malaria is common in sub-Saharan Africa and throughout the tropics in Africa, Papua New Guinea, Asia and Haiti, where *Plasmodium falciparum* is the most predominant species (Powells, 1989). *P. vivax* has the widest geographical range but is more common in Central America and parts of South America, North Africa, Middle East, Indian sub-continent and North-east Asia (Wigglesworth, 1976). *P. falciparum* and *P. vivax* are approximately equal in their distribution in other parts of South America, South East Asia and Oceania (Peters, 1987). *P. malariae* is patchily present over the range of *P. falciparum* but much less common and relatively uncommon outside Africa (Wigglesworth, 1976). *P. ovale* is found mainly in tropical Africa, rarely outside West Africa and occasionally in the West Pacific (Anderson & Morales, 1993).

The variation in the range of malaria distribution reflects a complex interaction between human, biological and environmental factors (Martens *et al.*, 1997; Githeko *et al.*, 2000; Patz *et al.*, 2000). The most important of these being vector efficiency, resistance to insecticides, parasite resistance to drugs, rainfall, numbers of mosquito breeding sites, access to health care (both curative and preventive), measures and adequacy of control programs (Anderson & Morales, 1993; Mouchet *et al.*, 1998; Githeko *et al.*, 2000; Patz *et al.*, 2000).

The occurrence of clinical malaria cases may depend on the parasite virulence and cytoadherence among other factors (Ferreira *et al.*, 1998; Modiano *et al.*, 1991; Terrenato *et al.* 1988; Rooth & Bjorkman, 1992; Genton *et al.*, 1998). Vector behaviour may also contribute to the variability in human exposure to malaria infections. Large differences may be there in anopheline densities and inoculation rates between localities and households. There may also be innate individual differences among people in attracting mosquitoes (Lindsay *et al.*, 1993; Knols *et al.*, 1995), which affect the individual inoculation rates. Differences in human behaviour and occupation also affect man's exposure to malaria infections.

1.3 Economic costs

In spite of all control measures available, malaria is still a major health problem in tropical countries. The burden it imposes on the economy is substantial. The high morbidity and mortality in some countries with intense transmission has reduced annual per capita income by up to 1.3%. In fact, a 10% reduction in malaria has been associated with higher economic growth (Gallup & Sachs, 2001). The cost of treatment and prevention alone is vast. Furthermore, many man-hours are lost each day from those suffering from malaria or looking after such patients. Many pregnant women suffer severe anaemia with up to 800,000 infantile mortalities, a substantial number of miscarriages, and very low birth weight (VLBW) babies, annually, as well as high risk of death due to the disease (Greenwood *et al.*, 1987; Greenwood *et al.*, 1992). Additionally, malaria in childhood is thought to affect cognitive and behavioural development, although the long term effect of this remains unclear (Holding & Snow, 2001).

The economic costs involved as a result of deaths from malaria are high, not to mention the pain and suffering associated with the disease. In addition, the spread of drug resistant malaria strains substantially raise the cost of treatment (Greenwood *et al.*, 1987; Molyneux *et al.*, 1999). In the long term, malaria negatively affects flows of trade and foreign investments, such as tourism in regions with a high risk of infection (Gallup & Sachs, 2001).

1.4 Resurgence of malaria

Malaria epidemics have been linked to climatic changes like the *el nino* weather phenomenon, global warming and interference of man with the environment (Githeko, 2000; Patz *et al.*, 2000; Mouchet *et al.*, 1998; Martens *et al.*, 1997; Bouma *et al.*, 1997; 1996; Lindsay & Birley, 1996; Jerten *et al.*, 1996), as well as drug and insecticide resistance (WHO, 1999). Man-made irrigation schemes, dams, and other development projects such as agroforestry, mining, urbanization, deforestation and road construction have provided new habitats for *Anopheles* mosquitoes resulting in 'man-made' malaria (Lindsay & Martens, 1998; Sharma & Mehrotra, 1986).

The extension of urban areas has led to epidemics in the peripheries of the growing cities. Mass migrations of non-immune populations into endemic areas for political reasons have also led to increased transmission of the disease (Mouchet *et al.*, 1998). The growing interchange of populations between malaria-endemic and malaria-free countries is responsible for the continuous increase in the number of imported malaria cases in European countries, and causes serious concern because of possible epidemic focal resurgence in receptive areas such as the Mediterranean (Bouma *et al.*, 1996). Besides, malaria has re-emerged in certain locations in Africa that previously had effective control programs, such as Madagascar, South Africa and Zanzibar (Bouma *et al.*, 1996; 1997; Lindsay & Birley, 1996).

Since 1976, several new pockets of malaria transmission have evolved. A WHO report (1984) recommended that countries, which had become malaria-free, should maintain at least one malaria vigilance unit. In many regions, malaria control programs have deteriorated or been abandoned due to high costs of sustaining them. Renewed efforts in malaria control are now needed than ever before.

1.5 African malaria vectors

The majority of malaria cases in sub-Saharan Africa are transmitted by mosquitoes of the *Anopheles gambiae* Giles complex and *An. funestus* Giles (White, 1974; Gillies & Coetzee, 1987; Katinka, 1999). Hunt *et al.* (1998) found that *An. gambiae* complex (also referred to as *An. gambiae sensu lato* or *An. gambiae s.l.*) comprises of 7 morphologically identical sibling species. In West Africa, *An. gambiae sensu stricto* consists of several ecophenotypes, which are strongly associated with specific habitats (Coluzzi *et al.*, 1985). As a result of their different biology, the species of the *An. gambiae* complex differ in their ability to transmit malaria (White, 1974; Gibson, 1996). In fact, some of its members are among the most efficient vectors of malaria parasites (Coluzzi, 1964; Service, 1985).

An. gambiae s.s and *An. arabiensis* Patton are widespread over the African continent, although the distribution of the latter species extends into more arid areas. In areas where

their distributions overlap, they can be found in small, transient habitats and have similar larval habitat requirements (Service, 1970; Service *et al.*, 1978; Service, 1993; Gimnig *et al.*, 2001). Populations of *An. arabiensis* survive the dry season better, while that of *An. gambiae s.s.* peak shortly after the onset of the rainy season (White *et al.*, 1972; Petrarca *et al.*, 1987; Githeko *et al.*, 1996; Lemasson *et al.*, 1997).

The ancestral species of the complex, *An. quadriannulatus* (Theobald), has recently been split to *An. quadriannulatus* species A, occurring in South Africa, Zimbabwe and Mozambique and *An. quadriannulatus* species B, which is found in Ethiopia (Hunt *et al.*, 1998). It is not considered a vector of malaria, as it exclusively feeds on animals, although some exceptions may occur (Pates *et al.*, 2001). Two other species of the complex, *An. merus* Dönitz and *An. melas* Theobald, are of local importance in malaria transmission, since they breed in saline waters along the East and West African coast, respectively. The distribution of *An. bwambae* White is restricted to a forested area around geothermal springs in Bwamba County, Uganda (White, 1974; Harbach *et al.*, 1997). *Anopheles pharaoensis* is another vector common in Egypt in areas near the river Nile. This species require extensive vegetation cover; inhabit swamps and relatively permanent water bodies with organic material (WHO, 1982).

An. funestus has a similar distribution in Africa as *An. gambiae s.l.*, but prefers to breed in large, permanent water bodies. Therefore, adult densities of this species are less affected by rainfall than *An. gambiae s.l.* and may be responsible for malaria transmission during dry seasons (Githeko *et al.*, 1996).

Anopheles larvae are found in different habitats such as fresh and salty water marshes, mangrove swamps, and rice fields, edges of streams, pools and ponds (Manson & Bell, 1987; Katinka, 1999). They are also found in small temporary waters such as found in tree holes, puddles, hoof prints, tyres prints, wells, waste containers, old tyres, and sometimes in water storage pots (Service, 1977; 1993; Manson & Bell, 1987).

Larval development takes 7 days for tropical mosquitoes and the pupal stage lasts 2-3 days (Gillies & De Meillon, 1968). Both sexes of adult mosquitoes feed on nectar and other plant fluids. The female ingests blood from vertebrates (Tempelis, 1975). One blood meal is enough for a female mosquito to lay a batch of 30-150 eggs (Service, 1986; 1993). Among the culicidae, *Anopheles* species have the most regular genotrophic cycle (blood feeding and egg laying). In the tropics, it takes 2-3 days depending on temperature, while in temperate areas it may take several weeks. Each species has its own cycle of activity. Some attack at dusk, during daytime and others at mid-night. Most species are nocturnal. Athropophilic and athropophagic species are those showing strong attraction to and feeding on human blood, respectively. Zoophilic and zoophagic species prefer animals to man. Endophilic and exophilic species rest indoors and out-door, respectively, while endophagy and exophagy refer to in-door and out-door feeding, in that order. Some species feed indoors on man and fly outside to rest before ovoposition (Garett-Jones *et al.*, 1980).

Anopheles gambiae s.s. is highly anthropophilic (almost exclusively bites humans), whereas *An. arabiensis* is more opportunistic and as a result feed on humans as well as on animals, depending on the availability of both hosts (White, 1974). Consequently, the proportion of the vector population infected with *P. falciparum* sporozoites is generally much higher for *An. gambiae*. After blood feeding, majority of *An. gambiae* s.s. females rest indoors (endophily), while *An. arabiensis* seek shelter outdoors (exophily) (White, 1974; Gibson, 1996). Like the *An. gambiae s.s.*, *An. funestus* are highly anthropophilic (Gillies & Coetzee, 1987). *Anopheles gambiae s.s.*, *An. arabiensis* and *An. funestus* are the principal vectors of malaria parasite in East Africa (White, 1974; Gillies & Coetzee, 1987; Katinka, 1999).

1.6 The malaria parasite

Malaria is caused by a protozoan *Plasmodium* (Laveran, 1880). About 120 species of *Plasmodia* have been identified but, only four are capable of infecting the humans. These are *P. vivax*, *P. malariae* (Laveran), *P. faliciparum* and *P. ovale* (Stephens), which cause

benign tertian malaria, quartan malaria, malignant subtertian malaria and a mild form of benign tertian malaria, respectively (Ridley, 1997). *Plasmodium faliciparum* is the most virulent species and predominates in Africa, eastern Asia, Oceania and the Amazons (WHO, 1997; Ridley, 1997). *Plasmodium ovale* occurs in Central, West Africa and sporadically in West Pacific regions (Powells, 1989).

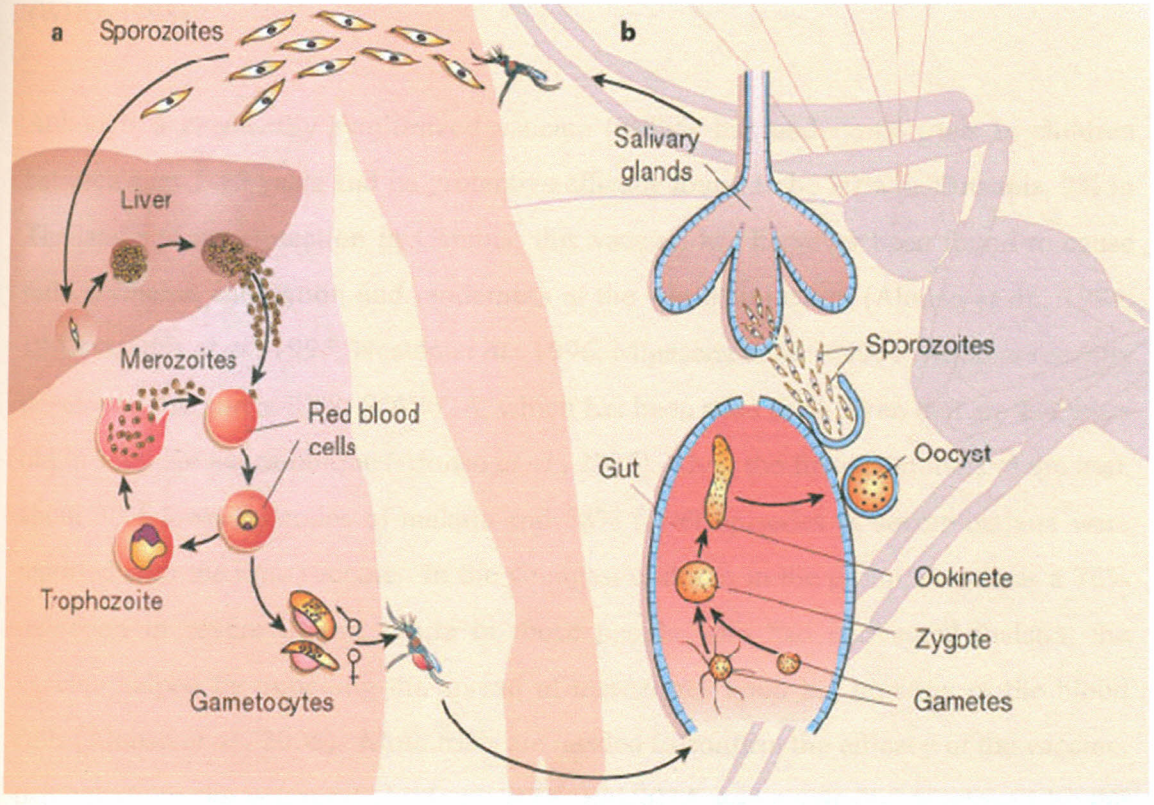
After the first observations of oocysts by Ross (1897) and of all stages of the parasite in a mosquito, Bastianelli *et al.* (1898) described the different developmental stages of the human malaria parasite in *Anopheles claviger* (Meigen). Developmental cycles of *P. falciparum* Welch and *P. vivax* were described by Grassi *et al.* (1899). Grassi (1890) demonstrated that only *Anopheles* spp are capable of transmitting human malaria.

The life cycle of the parasite consists of two phases, the sexual phase (sporogony) in female anopheline mosquito and the asexual phase (schizogony) in man (Fig. 2). During sporogony, a mosquito feeding on blood takes up the gametocytes, which would fuse, and form a zygote (Rosenberg *et al.*, 1990; Beier *et al.*, 1991ab; Ponnudurai *et al.*, 1991). The zygote penetrates the stomach of the mosquito to form an oocyst (Ganham, 1966; Shahabuddin & Kaslow, 1994). Within the oocyst, large numbers of sporozoites develop (Pringle, 1965; Rosenberg & Rungsiwongse, 1991). The sporozoites pass through the body cavity and some enter the salivary glands. Sporozoites are inoculated into a new host when an infected female mosquito takes blood, thereafter disappearing from the blood stream within one hour and invade the liver (Fairley, 1947; Bruce-Chwatt, 1985). The time from infection until the appearance of parasites in the blood varies with the species of parasites; *P. falciparum*, 5-7 days; *P. vivax*, 6-8 days; *P. ovale*, 9 days; *P. malariae*, 12-16 days, (Manson & Bell, 1987).

The duration of the extrinsic incubation period in the mosquito varies according to the temperature and the parasite species. Below temperatures of 16 and 18 °C *P. vivax* and *P. falciparum*, respectively, cannot complete their developmental cycle (Wernsdorfer &

MacGregor, 1988). Knowledge of the parasite life cycle is important in the design of effective anti-malarial vaccines and chemotherapeutic or chemoprophylactic agents.

Figure 2: Life cycle of *Plasmodium falciparum* (http://encarta.msn.com/media)



1.7 Malaria control strategies

Generally, malaria control has been achieved through chemoprophylaxis; chemotherapy and vector control (Collins & Paskewitz, 1995). Vaccination has also been attempted and is still under active research and development (Alonso *et al.*, 2005; Bojang *et al.*, 2001).

1.7.1 Malaria vaccine

Over the past six decades, substantial progress has been made in the search for a malaria vaccine (Cohen & Mitchell, 1978; Alonso *et al.*, 2005; Bojang *et al.*, 2001). The three major types of vaccines being developed are: anti-sporozoite vaccine, which are designed to prevent infection (Franke *et al.*, 1999); anti-asexual blood state vaccines, designed to

prevent severe manifestations of the disease (Cowman *et al.*, 2002); and the transmission-blocking vaccines designed to arrest the development of the parasite in the mosquito (WHO, 1998).

Although, a chemically synthesized vaccine (SPf66) has undergone trials in children between ages 1-15 years and its protective efficacy found to be 30% in Tanzania, 9% in Thailand and no protection in Gambia; this vaccine has however been found to cause mild erythema, induration and tenderness at the site of injection (Alonso *et al.*, 1994; D'Alessandro *et al.*, 1995; Nosten *et al.*, 1996; Migasena *et al.*, 1997). Another recently developed vaccine is RTS, S/AS02A, which has been tried in children that are 1-4 years old in southern Mozambique (Alonso *et al.*, 2005). Over the first six months of the trial, about 30% fewer episodes of malaria and 58% fewer episodes of severe malaria were reported with the new vaccine. In the youngest children in the group, there was a 78% reduction in severe illness, while in those people who had contracted malaria, the vaccine helped by impeding the spread of merozoites upon the bursting of the blood cells (Alonso *et al.*, 2005). More trials are needed to confirm the efficacy of the vaccine, particularly in the youngest children. RTS, S/AS02A was originally developed by the U.S. military and consists of a genetically engineered molecule that combines a key protein at one stage of the parasite's development with a protein that coats the hepatitis B virus (Alonso *et al.*, 2005). The combination stimulates a counterattack by the immune system (Alonso *et al.*, 2005). Peptide-based vaccines have successfully been used though they face the challenge of toxic adjuvants, which are critical for immunogenicity of synthetic peptides (BenMohamed *et al.*, 2002). Other candidate trial vaccines include nucleic acids targeting asexual stage (Doolan & Hoffman, 2002) and pre-erythrocytic stage (Ballou *et al.*, 2002).

Many factors have made the vaccine development complicated, difficult and thus challenging. First, the size and genetic complexity of the parasite mean that each infection presents thousands of antigens to the human immune system. Forty promising antigens have so far been identified. Understanding these is important for vaccine development.

Second, the parasite changes through several life stages even while in the human host, presenting a different subset of molecules for the immune system to combat at each stage. As a result of this, they conceal themselves from the immune responses that might otherwise be stimulated by a vaccine. Third, the parasite has evolved a series of strategies enabling it to confuse and misdirect the human immune system. Finally, it is possible to have multiple malaria infections of not only different species, but also of different strains at the same time (Hoffman *et al.*, 1996).

Mapping of the malaria parasite genome has opened more rational ways of discovering new vaccines (Collins & Paskewitz, 1995; WHO, 1998; Cowman *et al.*, 2002). Based on this knowledge, scientists from several organizations worldwide have been working together to develop a multistage, multigene DNA-based vaccine against *P. falciparum* malaria. This collaborative vaccine development effort is called "multi-stage DNA-based malaria vaccine operation" (Kumar *et al.*, 2002). Therefore there is no doubt that it is the hope of researchers that an effective vaccine will be available by 2010. However, by that time, WHO predicts that half the world's population will be living in areas of high exposure to the disease compared to 41% now (Alonso *et al.*, 2005). Meanwhile, in the absence of an effective vaccine, investigation of alternative methods of protection such as chemoprophylaxis and chemotherapy should be sought.

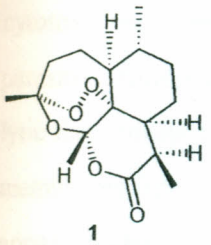
1.7.2 Chemotherapy and drug resistance

There are a limited number of drugs for the effective treatment of malaria today. Some give little protection against infection, but are effective in treatment, while others serve as both prophylactic and curative drugs (Service, 1986). The disease has become so difficult to treat due to increasing drug resistance in many parts of the world (Fairly, 1947; Trigg *et al.*, 1997). Resistance *in vivo* has been reported to all anti-malarial drugs except artemisinin (**1**) and its derivatives (Zucker & Campbell, 1992). To date, there is no report of *in vivo* resistance of malaria parasites to artemisinin based drugs (Adjuik *et al.*, 2004).

Resistance development forces many malaria patients to use drugs, which are more expensive. This may lead to undesirable side effects. In some parts of the world, artemisinin-based drugs are the first line of treatment of suspected uncomplicated malaria (http://www.frontlineonnet.com/fl1913/19130870.htm). The implication of this is that malaria forms resistant to artemisinin are expected to appear soon (WHO, 1987). The areas affected most with drug resistance are the Indo-Chinese peninsula and the Amazon region of South America (WHO, 1995a).

The problem of drug resistance may be attributed mainly to increased selection pressures on *P. falciparum*, due to indiscriminate and incomplete doses for self treatment (Zucker & Campbell, 1992). In Thailand and Vietnam, *An. dirus* and *An. minimus* spread the drug resistant parasites (Kevin, 1994). These mosquitoes adapt their biting activity to human behaviour patterns and therefore maintain intense transmission cycles (Terrenato *et al.*, 1998).

Drug resistant *P. falciparum* was first reported in Thailand in 1961 (Kevin, 1994). Various *P. falciparum* 'strains', have now attained resistance to all commonly used and generally available anti-malarial drugs (Kevin, 1994). In man, the problem of resistance to the common anti-malarial drugs such as chloroquine (**2**) and pyrimethamine (**3**) as well as the decreasing efficacy of quinine (**4**) are mainly limited to *P. falciparum* infection (WHO, 1995b; Spencer, 1985). However, chloroquine still remains the treatment of choice for *P. vivax* (Peters, 1987). Several mechanisms can account for changes in drug sensitivity of the malaria parasites: physiological adaptations due to non-genetic changes, selection of previously existing drug resistant parasites from a mixed population under drug pressure, spontaneous mutation, mutation of extra-nuclear genes, or the existence of plasmid-like factors (Spencer, 1985).



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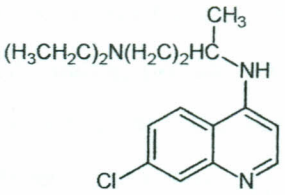

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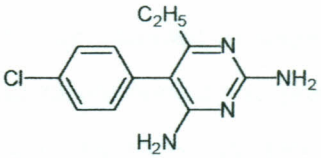

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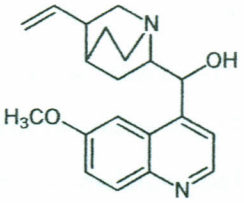


4

Selection of mutants by the drugs themselves appears to be an important mechanism (WHO, 1987). In an environment with sub-therapeutic levels of the anti-malarial drugs, the parasites which have resistance through their natural variation or through mutations have an important biological advantage. This means that even though the drug-resistant forms were initially in the minority, the continued drug mediated elimination of intra-specific competition from the non-resistant forms, has allowed the resistant ones to attain numerical superiority - to the point that drugs like chloroquine are considered ineffective (Peters, 1987). Majority of studies indicate that drug-created selection pressure is to blame for the emergence of resistant malaria (WHO, 1987). Sub-curative plasma levels of drugs found in many areas with uncontrolled and irresponsible prophylactic and treatment regimes will kill the most drug sensitive forms of the parasite, but select the less sensitive ones. Besides, spontaneous mutations in these forms tend to reduce the sensitivity of the parasite to the drug (Yamanda & Sherman, 1979). Fortunately, the problem of irresponsible prophylaxis has been recognized and precautions are being taken. For instance, in Zimbabwe and Kenya it is illegal to sell chloroquine other than full courses (Bradley & Behrens, 1994).

The rapid spread of drug-resistant malaria may be due to an increasing efficiency of vector. This phenomenon may be explained by the increased efficiency of oocyst formation that has been observed with drug-resistant species (WHO, 1987).

In order to appreciate the physiological nature of resistance, it is necessary to understand the metabolism of the parasites and their mode of action on anti-malarial drugs. Intra-erythrocytic stages of malaria ingest the haemoglobin into food vacuoles. Here exo-peptidases break down haemoglobin into haemozoin (malaria pigment), of which the

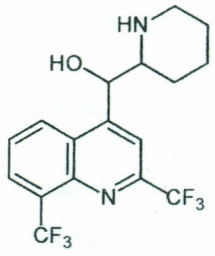
cytotoxic ferriprotoporphyrin IX is a major component (Wernsdorfer & Trigg, 1984). A parasite synthesized binding protein, 'haembinder', seemed to sequester the membrane-lytic ferriprotoporphyrin IX into the inert haemozoin complex to protect the parasite cell membranes from damage. It is now appropriate to discuss a number of anti-malarials and apparent adaptations seen in resistance.

Quinolines: This class of antimalarial drugs includes quinine (**4**) and mefloquine (**5**). These cause blabbing of the parasite membranes and aggregations of haemozoin. Parasite-resistance to this class of compounds occurs by uncertain mechanisms, but is stable and transmissible (WHO, 1987; White, 1999). Quinine is still drug of choice in severe and complicated cases like cerebral malaria (Hien *et al.*, 1999; Birku *et al.*, 1999; D'Alessandro, 2004; Aceng *et al.*, 2005).

Aminoquinolines: This class of compounds includes the 4-aminoquinolines and the 8-aminoquinolines. The 4-aminoquinolines constitutes the highest number of anti-malarial drugs and includes chloroquine (**2**), amodiaquine (**6**) and related compounds. It is known that chloroquine mediates its effects on the haemoglobin metabolism in malaria parasites, perhaps preventing the neutralization of the toxic ferriprotoporphyrin IX. Chloroquine resistant parasites are unable to produce haemozoin, but are still able to digest haemoglobin. In non-resistant forms, most of the ferriprotoporphyrin IX is sequestered in haemozoin, but in the resistant forms, this toxic metabolite becomes available to the host cell haemoxygenase system for elimination (Fitch, 1983). In chloroquine-sensitive parasites, the drug is taken up into food vacuoles, and it is proposed that it competes with the haembinder for the ferriprotoporphyrin IX, to form a destructive compound (Bradley, 1995).

A representative of the 8-aminoquinolines is primaquine (**7**). This drug has been used mainly against gametocytes and hypnozoites (Brueckner *et al.*, 1998; Lell *et al.*, 2000). The drug is thought to work by inhibiting the ion transport chain of the parasite, though the precise metabolic interaction is not known. Neither is it certain as to whether it is the drug itself or derived metabolites, which have the desired effects (Merhil & Peters, 1976).

There is no evidence that gametocyte resistance exists, but if the drug is used against schizonts, then resistance is rapidly attained (Ferone, 1970). The surviving resistant parasites exhibited increased numbers of mitochondria suggesting that the resistance mechanism involves the production of extra organelles to compensate for the damage caused by the drug (WHO, 1987).



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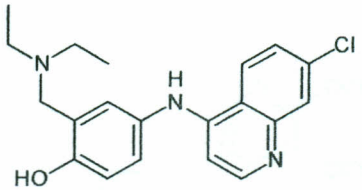

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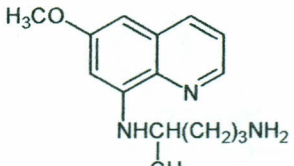
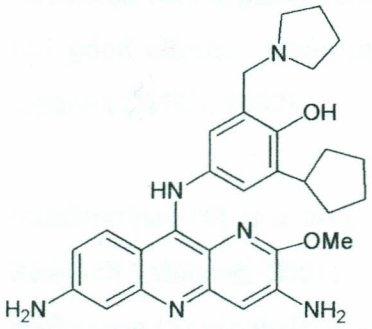


7

Acridines: 9-Anilinoacridines are potent inhibitors of parasite DNA topoisomerase II both *in vitro* and *in situ* (Chavalitshewinkoon *et al.*, 1993; Auparakkitanon & Wilairat, 2000). 3,6-Diamino substitution on the acridine ring greatly improves anti-parasitic activity against *P. falciparum*. A number of 9-anilinoacridines such as pyronaridine (**8**), 3,6-diamino-9-anilinoacridine (**9**) and 3,6-dichloro-9-anilinoacridine (**10**) have the ability to inhibit ß-hematin formation, to form drug-hematin complexes, and to enhance hematin-induced lysis of red blood cells (Egan *et al.*, 2001).



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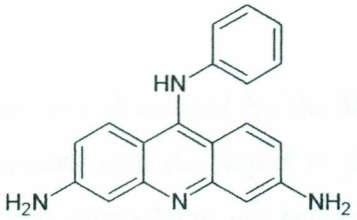

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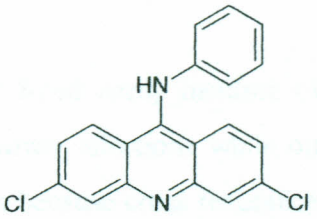
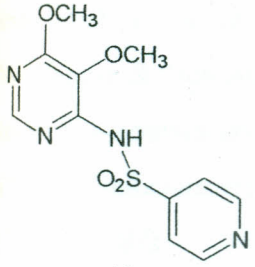


10

Sulfonamides: Parasites, which become resistant to sulfonamides like pyridine-4-sulfonic acid (5,6-dimethoxy-pyrimidin-4-yl)-amide (**11**), sulfadoxine (**12**) and dapsone (**13**), must bypass the metabolic step at which *p*-aminobenzoic acid (*p*ABA) is incorporated into

dihydropterate. Sulfonamides work by inhibiting *p*ABA synthesis, which is required to synthesise the dihydropterate - an intermediate compound in the synthesis of tetrahydrofolate. They are dihydropterate synthase (DHPS) inhibitors. Tetrahydrofolate derivatives serve as donors of C_1 groups in a number of essential biosynthetic pathways. Little is known about this aspect of parasite metabolism or the exact mechanism of resistance - though resistance is clearly stable, transmissible and prolific (WHO, 1987).



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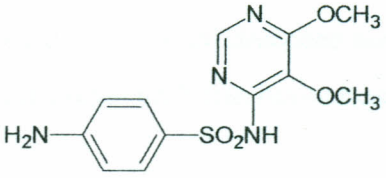

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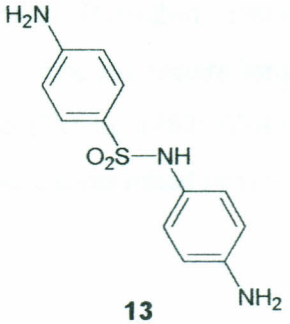

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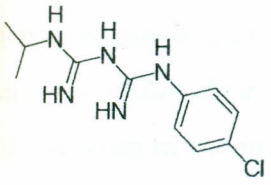
Antifolics: Compounds like proguanil (**14**) and pyrimethamine (**3**) inhibit the action of dihydrofolate reductase. As with the sulfonamides, resistance occurs in all stages of the life cycle. Dihydrofolate reductase (DHFR) enzymes of resistant strains bind to pyrimethamine (**3**) 400-800 times less readily than the enzymes of drug sensitive strains (Eckman *et al.*, 1977). Interestingly, high levels of resistance to sulfonamides are associated with hypersensitivity to antifols and vice versa, so combination treatments have had good effects. Unfortunately, resistance to these drug cocktails has now become apparent (WHO, 1987).

Halofantrine (**15**) is a drug that was developed by the Walter Reed Army Institute of Research (Milhous, 2001). Patients who developed *P. falciparum* infections while on mefloquine (**5**) prophylaxis are not supposed to use halofantrine because cross resistance to both drugs is common (Bangchang *et al.*, 1992). In addition, fatalities have occurred when halofantrine is used for treatment in patients who received mefloquine prophylaxis (Bangchang *et al.*, 1992; Baune *et al.*, 1999). Cardiac complications have also been reported in patients under halofantrine treatment (Nosten *et al.*, 1993).

Artemisinins: Are among the newest and most effective of all anti-malarial drugs, and seem to affect protein synthesis. Artemisinins must be protected and used rationally to prevent the emergence of inevitably resistant *P. falciparum* for as long as possible. Schizontocidal activity of artemisinin (**1**) and several of its derivatives and analogues like artemether (**16**), arteether (**17**) and sodium artesunate (**18**) against *P. falciparum* strains resistant to all known anti-malarial drugs, with virtually no toxicity, have been well evaluated in clinical tests in China (Klayman, 1993; Kinghorn & Balandrin, 1993; Wolfender & Hostettmann, 1995). However, the derivatives and analogues require long treatment courses and, when used alone, re-crudescence may occur (Peters, 1987; WHO, 1998). Unfortunately, artemisinin resistant forms have already been demonstrated *in vitro* (WHO, 1987).



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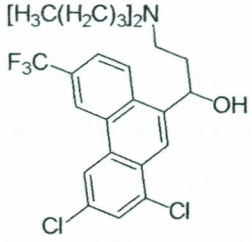

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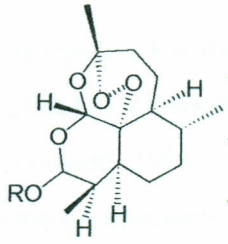
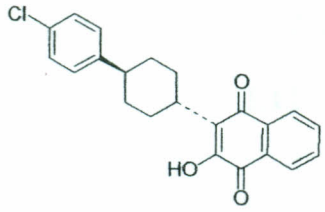


	R
16	CH_2CH_3
17	CH_3
18	$CO(CH_2)_2CO_2Na$

The use of a combination of two or more anti-malarial drugs, which have different mechanisms of action and different bio-chemical targets in the parasite, to manage resistant strains of *P. falciparum* is now a common practice (Sowumni, 2002). An ideal combination is one that is potentiating, well matched, has reduced toxicity and delays the emergence of resistance to the individual components (Kremsner *et al.*, 1999). The combination of sulfadoxine (**12**) and pyrimethamine (**3**) (Fansidar®) represents one of the most important chemotherapeutic agent that is used in managing chloroquine resistant malaria (Sowumni, 2002). Malarone®, is a combination of proguanil (**14**) and atovaquone (**19**), which is highly effective for treatment of acute uncomplicated malaria caused by *P. falciparum* resistant to first line treatment anti-malarials (Kremsner *et al.*, 1999). Maloprim®, a combination of daspone (**13**) and pyrimethamine (**3**) has also been developed, though, resistance to this drug is now widespread and its use is no longer recommended (Canfield *et al.*, 1995).

Antibiotics are often used in conjunction with other drugs to combat chloroquine resistant *falciparum* malaria (Anderson *et al.*, 1995). Tetracycline (**20**) and chlorotetracycline (**21**) have been used successfully for this (WHO, 1987). *Plasmodium* protein synthesis appears to be eukaryotic and insensitive to chloramphenicol, but affected by cycloheximide. It has been suggested that antibiotics such as tetracyclines act on the mitochondrial ribosomes of the parasite, thus inhibiting protein synthesis. Macrolides such as erythromycin seem to inhibit autophagic vacuole formation, thus potentiating the action of chloroquine (WHO, 1987). Resistance to these compounds is not a current problem. Resistance to combination therapy has been reported. A typical example is resistance of *P. falciparum* to Fansidar® in north-eastern Tanzania (Mutabingwa *et al.*, 2001). This has led to multiple therapy.

Triple therapy is currently the most viable approach to fighting the multi-drug resistant strains of *Plasmodium falciparum* (Mutabingwa *et al.*, 2001). It slows down development of resistance to the individual drugs (McIntosh & Greenwood, 1998). Chloroquine plus sulfadoxine-pyrimethamine (SP), amodiaquine® (**6**) alone or in combination with SP and chloroguanil-dapsone (Winstanley *et al.*, 2002) have been used successfully and are also affordable. However, it is obvious, that resistance to SP drugs is an ongoing problem. By 1973, sulfadoxine-pyrimethamine cocktails replaced chloroquine, but by 1985, this too was ineffective (WHO, 1987). Though quinine remains effective, there is a 50% failure rate unless it is supplemented by tetracyclines (WHO, 1987). Its compliance with the 7-day regimen is poor. Between 1985 and 1990, the recommended treatment for malaria in Thailand was mefloquine, combined with sulfadoxine-pyrimethamine at a dose of 15/30/1.5 mg/kg body weight. However, 1990 the cure rate had fallen to 71% in adults and 50% in children. This treatment is no longer used due to resistance (Kevin, 1994). The future of chloroquine is not clear, although it has been suggested that, due to the current withdrawal of drug pressure, chloroquine-sensitivity may be regained (IDRC, 1995).



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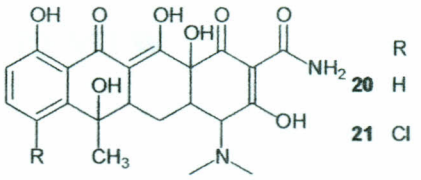


The common prophylactic drugs are, for many areas, obsolete (IDRC, 1995), and the use of advanced drugs such as artemisinin derivatives for uncontrolled prophylaxis, would be downright irresponsible, given the obvious ability of *P. falciparum* to attain a high degree of resistance in a short period. It has been suggested that strains resistant to artemisinins will eventually appear (Zucker & Campbell, 1992), and this seems inevitable. Vector manipulation for malaria control, therefore, has a pivotal role in the future.

CHAPTER TWO

LITERATURE REVIEW

2.1 Vector control

Vector control includes non-chemical as well as chemical control methods (pesticides). Presently, vector control is thought of as one of the best approaches in the war against malaria, especially in the absence of a malaria vaccine and effective chemotherapeutic agents. Control of the malaria vector can be achieved through environmental management (Githeko, 2000; Martens *et al.*, 1997), biological control adjuncts (Lu, 1996; Neri-Barbosa *et al.*, 1997), insect growth regulators (IGRs) (Katinka, 1999), genetically modified mosquitoes (Cutkomp, 1967), larvicides (WHO, 1996; Collins & Paskewitz, 1995), insecticides (Curtis, 1990; Marbiah *et al.*, 1998), traps baited with semiochemicals (attractants and pheromones) and the use of repellents (WHO, 1984; Trigg, 1996) among other methods. In the search for novel techniques in vector control methods, the protection of human targets from infective mosquito bites has been found to be the most effective.

2.1.1 Environmental management

Environmental management includes modification and manipulation of breeding sites. The former is permanent removal or prevention of larval habitat formation, such as drainage, land leveling and filling, tree planting, design of water impoundment/irrigation structures. The later is temporary removal or disturbance of larval habitat, such as intermittent irrigation, stream flushing, manipulating salinity, vegetation removal/shading, expanded polystyrene beads (Patz *et al.*, 2000; Mouchet *et al.*, 1998). Other forms of habitat reduction include open marsh water management, in which mosquito-producing areas in the marsh are connected by shallow ditches to deep water habitats to allow drainage or introduction of fish. Rotational impoundment management, in which the marsh is minimally flooded during summer but is flap-gated to reintegrate impoundment to the estuary, for the rest of the year, can also be employed. All these strategies could eliminate

larval habitats or render such habitats unsuitable for larval development. However, some of the developmental activities have interfered with the environment, leading to global warming and climatic changes that may be responsible for most malaria epidemics (Githeko, 2000; Patz *et al.*, 2000; Mouchet *et al.*, 1998; Bouma *et al.*, 1997; Lindsay & Birley, 1996). Moreover, some malaria vectors have undergone some adaptations that make their larvae survive in temporary water collections in holes of tree trunks, animal hoof prints, old disused tins, tyres and marshy grounds making environmental management inadequate on its own.

2.1.2 Biological control

This involves the use of biological organisms or their by-products to control the mosquitoes. Biological control is not a panacea for mosquito control in the 21st century. However, it should be considered as a tool that mosquito control programs have at their disposal and use whenever feasible. Entomo-pathogenic larvicidal organisms such as the bacteria *Bacillus thuringiensis* var. *israelensis*, *B. sphaericus* and the fungi *Tolypocladium cylindrosproum* (Deuteromycotina: Hyphomycetes), which have proved effective, are not yet on an operational scale (WHO, 1996; Nadeau & Boisvert, 1994; Collins & Paskewitz, 1995). Baumgartner (1987) experimented with aquatic carnivorous plants like, *Urticularia vulgaris*, for larval control. In Florida, other invertebrates like *Toxorhynchites* species, *Dugesia dorotocephala* (Planarian), *Triops* spp. (Crustacean), *Mesocyclops* spp. (Copepod), and some insects (in the orders Odonata, Notonectidae and Dytiscidae) have been under trial for various malaria vectors (Schrieber & Jones, 2000). Corbat (1986) used dragonfly nymphs to eliminate the larvae of *Aedes aegypti*. *Azollo fulcoides*, a floating water fern was also tested by Lu (1996) on *Anopheles* and *Aedes* larvae. Third-stage juvenile mermithid nematodes have also been used to suppress mosquito population. The nematodes usually enter the insect through its breathing holes, mouth, anus, or penetrate thin areas of the insect's cuticle (Lacey, 1998). Once inside the body of the insect, they release special bacteria, which produce toxins that kill the host after a few days (Skovmand *et al.*, 2000).

The most commonly used biological control adjunct is introduction of mosquito fish (or larvivorous fish) in larval habitats. These include *Gambusia affinis*, *G. holbrooki*, *Cyprinus carpeo*, *Ctenopharyngodon idella*, *Macropodus opercularis* and *M. chinensis*. Naturally occurring *Fundulus* spp. and possibly *Rivulus* spp. predate on mosquitoes; also play an important role in larval control in open marsh water management and rotational impoundment management (Bay, 1967).

Most of the biological control agents such as the *B. thuringiensis*, *Romanomeris jindeensis* and *R. culicivorax* are not easy to culture. The high cost of culturing bio-control agents makes the control program expensive. The larvivorus fishes can only thrive in specific habitats and cannot be employed in managing some larval habitats especially temporary ones like holes in tree trunks, hoof prints, tyres prints, wells, old tyres and tins.

2.1.3 Genetic modification

One of the methods of controlling malaria is to come up with genetically modified vectors that can be used to limit reproduction and survival of their own species in natural populations. Such insects are mass produced in the laboratory and released among the wild populations so that, mating with normal insects will either not result in an offspring or lead to reduced fitness (sterility or failure to adapt properly to the environment) of the progeny (Cutkomp, 1967; Coleman & Alphey, 2004). Genetic approaches include the chromosomal translocations, hybrid sterility and cytoplasmic incompatibility. None of these has been proven successful. Otherwise, they are largely theoretical in nature (Popiel & Olkowski, 1990; Schrieber & Jones, 2000).

GM mosquito work has attracted the attention of many research groups since the insect control measures are conducted over large areas under professional supervision thus avoiding mistakes or omissions by individuals. Secondly, such insects will have the potential to increase efficiency as densities of target populations decline (Spielman *et al.*, 2002). Insecticidal control measures normally become less cost-effective as the target population decreases towards the economic threshold, below which it becomes

uneconomical to employ insecticides. Thirdly, the genetic control is specific and avoids undesirable effects on other organisms (Coleman & Alphey, 2004). No residues are involved and other adverse effects associated with the use of insecticides are avoided. Finally, once established, it may cause dramatic savings by completely eliminating the insect (Cutkomp, 1967; Mshinda *et al.*, 2004). As long as the idea of GM mosquitoes is not operational, control of malaria transmission can be achieved using other methods that target adult insects.

2.1.4 Sterile insect techniques

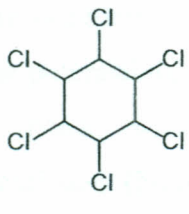
One possibility for the control of *Anopheles* mosquitoes is the Sterile Insect Technique (SIT) (Benedict & Robinson, 2003). SIT relies on mass rearing and release of male insects sterilized by gamma radiation or chemosterilants (Knipling, 1955; Hendrichs, 2000; Cayol *et al.*, 2002). The released males mate with wild fertile female mosquitoes, preventing production of viable offspring (Alphey, 2000). Repeated releases lead to population control and eventual eradication (Thomas *et al.*, 2000; Alphey & Andreasen, 2002). Highly successful, area-wide SIT programs have been conducted against the screwworm *Cochliomyia hominivorax* in the USA, Mexico and Central America; also in Libya, where SIT was used in the successful control of a serious outbreak (Knipling, 1982). Other targets of area-wide SIT programs include the Mediterranean fruit fly (medfly), *Ceratitis capitata*, in various parts of Latin America and the codling moth *Cydia pomonella* in Canada (Knipling, 1982; Robinson, 2002; Hendrichs *et al.*, 2002). The technique has also achieved some suppression of the boll weevil *Anthonomus grandis* Boh, Codling moth *Laspeyresia pomonella* (L.), tsetse fly *Glossina mortisan morsitan* and certain mosquitoes (*An. quadrimaculatus*, *An. albimanus*, *An. culicifacies*, *Culex tritaeniorhynchus* and *Cx. tarsalis*) (Knipling, 1982; Weidhaas & Patterson, 1982).

Even though SIT is a highly effective, species-specific and environmentally-friendly area-wide method of insect control, it has some limitations (Lindquist, 2001). In order for SIT to work, billions of sterile mosquitoes need to be released into the environment over a period of time. One application of sterile males would not be enough to reduce the natural

population of mosquitoes. Multiple applications to treat newly emerging mosquitoes would need to be used (Benedict & Robinson, 2003). As a consequence of breeding large numbers of males, females inevitably become mixed with the males. Females bite and are thus able to transmit diseases. A good SIT program should therefore reduce the number of released females in order to reduce risk of disease. Thus the methods of reducing the number of females mixed with the sterile males need to be researched. It is also important to note that large production of the sterile males is not easy; and even if successful, would lead to unavoidable costs in terms of the fitness of the irradiated insects and the financial costs of constructing and operating the radiation facility. In addition methods of release need to be researched, as mosquitoes are fragile and can be damaged during release (Lindquist, 2001). Methods of sterilization also need to be addressed since current sterilization techniques harm the male and make them less competitive for females.

2.1.5 Larvicides

Many chemicals as well as biological larvicides have been used to control mosquito larvae. In the USA, oil sprays on water have been used to control mosquito larvae (Wigglesworth, 1976). Synthetic inorganic compounds like paris green $\{Cu(C_2H_3O)_2.3Cu(AsO_2)_2\}$ and metarsenite $\{Cu(AsO_2)_2\}$ have also been used as larvicides (Metcalf & Flint, 1962). Paris green is suitable for controlling larvae in water under much vegetation cover (Wigglesworth, 1976). Chlorinated synthetic organic compounds like lindane **(22)**, chlordane **(23)** and DDT **(24)** have also been used as larvicides (Kirk & Othmer, 1992). However, these compounds are non-biodegradable and as a result accumulate in nature.



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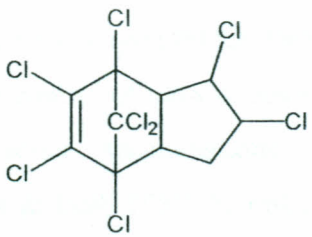

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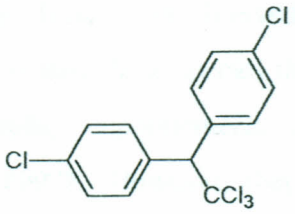

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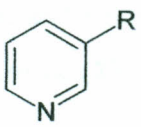
One of the earliest reports of the use of plant extracts against mosquito larvae demonstrated that plant alkaloids (Katinka, 1999) like, nicotine (**25**), anabasine (**26**) and methylanabasine (**27**) extracted from the Russian weed, *Anabasis aphylla*, killed the larvae of *Culex pipiens* Linn, *Cx. territans* Walker and *Cx. quinquefasciatus* Say (Wigglesworth, 1976).



R

25 1-methyl-2-pyrrolidinyl

26 2-piperidinyl

27 1-methyl-2-piperidinyl

Even though chemical and bio-larvicides are effective on the larvae of mosquitoes, they are toxic to most non-target aquatic organisms (WHO, 1996). Moreover, some larvae may develop resistance to the lethal effect of the larvicides and emerge into adults. There is no doubt that total control of mosquitoes requires the management of larval and adult population.

2.1.6 Insect growth regulators (IGRs)

Chemicals exhibiting insect growth inhibiting properties have been used to control the growth of most insects at the larval stage (Kirk & Othmer, 1992). Most of the IGRs so far discovered, can be classified as juvenile hormone mimics or chitin synthesis inhibitors (Kirk & Othmer, 1992; WHO, 1996). These compounds have mild or no toxicity to most non-target organisms (WHO, 1996). Their activity is against immature stages of mosquitoes, flies and other insects. However, some IGRs induce sterility, among other reproductive disorders in the adult stage (WHO, 1996). It has been observed that IGRs have a great potential for the control of mosquitoes especially those species that breed in containers, pools, waste water accumulations, wells, impoundments and other developmental sites (Manson & Bell, 1987; Katinka, 1999). However, they cannot be applied on drinking water sources due to their mild toxicity to man. Consequently, new and safer methods of vector management are necessary.

2.1.7 Insecticides

Control of the adult mosquito population or bites is possible by the use of insecticides or repellents (Kirk & Othmer, 1992). Several classes of insecticides have been used to control insect pests. These include chlorinated hydrocarbons, carbamates, organophosphates, synthetic pyrethroids and organic compounds of plant origin. Chlorinated hydrocarbons with insecticidal activity are so much persistent in nature. They accumulate in food webs and chains with long term effects (Kumar, 1984). Examples include lindane (**22**), chlordane (**23**), toxapane ($C_{10}H_{10}C_{18}$) and DDT (**24**).

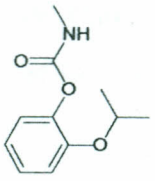
Global eradication of malaria seemed possible with the discovery of DDT in 1942 and its first use in Italy in 1944 (Wigglesworth, 1976). Despite the initial successes, there was a complete failure to eradicate malaria in most countries (Curtis & Lines, 1985; Service, 1986). This was mainly due to resistance developed by mosquitoes to DDT and other insecticides among many other factors (Kumar, 1984; Kirk & Othmer, 1992).

Carbamates are generally stable, easily affordable, broad spectrum and bio-degradable insecticides (Kirk & Othmer, 1992). They are however, known for their rapid yet transient poisoning effect in mammals (Dethier, 1947). Resistance to these insecticides has also been reported (Brown, 1986). Examples include propoxur (**28**), carbaryl (**29**) and carbofuran (**30**).

Organophosphates are esters or organic salts of phosphoric acid or its derivatives. They are less stable than both the carbamates and organochlorines. They are degradable through water hydrolysis, reaction with oxygen, heat and bacterial action (Palchick, 1996). Examples include methylparathion (**31**), parathion (**32**) and malathion (**33**) (Kumar, 1984).

Natural pyrethrins from the flowers of *Chrysanthemum cinerariaefolium* are powerful insecticides with low mammalian toxicity. The powder from the flower is rich in a mixture of six insecticidal esters. These are pyrethrin **I** (**34**) and **II** (**35**), cinerin **I** (**36**) and **II** (**37**) and jasmolin **I** (**38**) and **II** (**39**) (Elliott & Janes, 1979). Even though, the use of natural

pyrethrins depends on the rapid knock down effect, lack of prolonged residual action and cost have restricted their application (Kumar, 1984).



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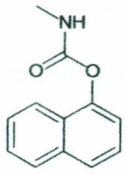

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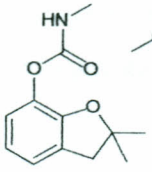

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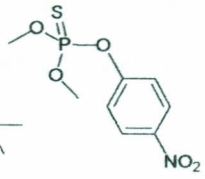

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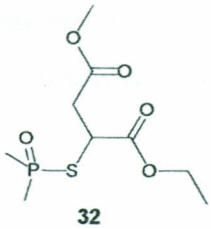

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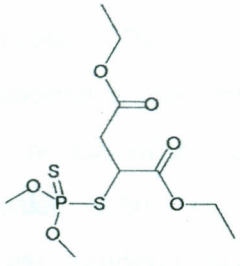

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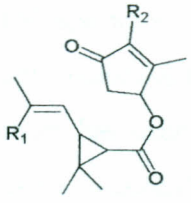
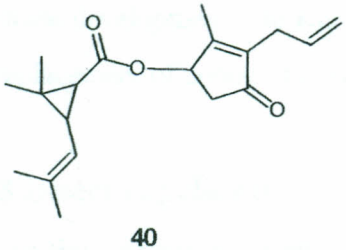


	R_1	R_2
34	CH_3	$CH_2CH{=}CHCH{=}CH_2$
35	$COOCH_3$	$CH_2CH{=}CHCH{=}CH_2$
36	CH_3	$CH_2CH{=}CHCH_3$
37	$COOCH_3$	$CH_2CH{=}CHCH$
38	CH_3	$CH_2CH{=}CHCH_2CH_3$
39	$COOCH_3$	$CH_2CH{=}CHCH_2CH_3$

This has led to the development of synthetic pyrethroids, such as allethrin (**40**), permethrin (NRDC 143) (**41**), cyhalothrin (**42**), and decamethrin (NRDC 161) (**43**) (Elliott *et al.*, 1973; 1974; Beach *et al.*, 1993), that have improved photo-stability and diminished mammalian toxicity.



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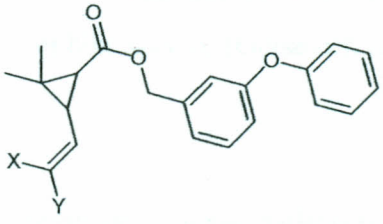


	X	Y
41	Cl	Cl
42	Cl	CF_3
43	Br	Br

There has been increasing interest in the use of insecticide treated bed nets for malaria control. They were first used in Russia in the 1930's and by American and German forces during World War II (Curtis & Lines, 1985). Bed nets have been widely used against nuisance mosquitoes in China, Thailand, Latin America, Papua New Guinea and Africa (Katinka, 1999). Since most *Anopheles* species bite at night, it has been assumed that nets should reduce the chances of contracting malaria (Lindsay & Gibson, 1988; Mathenge *et*

al., 2002). In the Gambia (Snow *et al.*, 1988; Curtis, 1990; Alonso *et al.*, 1991; 1993), Guinea Bissau (Joenson *et al.*, 1994), Kenya (Beach *et al.*, 1993) and elsewhere (Curtis & Lines, 1985; Curtis *et al.*, 1987), introduction of insecticide-impregnated nets in the communities has remarkably reduced parasite prevalence and malaria cases (Marbiah *et al.*, 1998).

Insecticide resistance has been observed in almost every major group of arthropod vectors of major diseases. By 1991, resistance to all major classes of insecticides in public health use was reported in more than 150 species of vectors and nuisance pests (WHO, 1996). Today, the number of the resistant vectors is much greater than it was in 1991 (WHO, 1996; Katinka, 1999). This number has been increasing steadily. Furthermore, cross-resistance has occurred between older insecticides such as DDT and synthetic pyrethroids in different species of mosquitoes and among different synthetic pyrethroids themselves (Metcalf & Flint, 1962). Taking *An. gambiae* as an example, it has been reported in several West African countries that this vector is resistant to permethrin (**41**) with decreased susceptibility to decamethrin (**43**) (Chadwick *et al.*, 1977; WHO, 1996). The resistance is believed to be mostly due to miss-use of insecticides. For instance, spraying insecticides without washing off the trace chemicals left after applications contributes to resistance development (De Kumar, 1995). This has led to the emergence of new species and replacement of endophilic with exophilic species (Gutsevich *et al.*, 1974).

2.1.8 Insect repellents

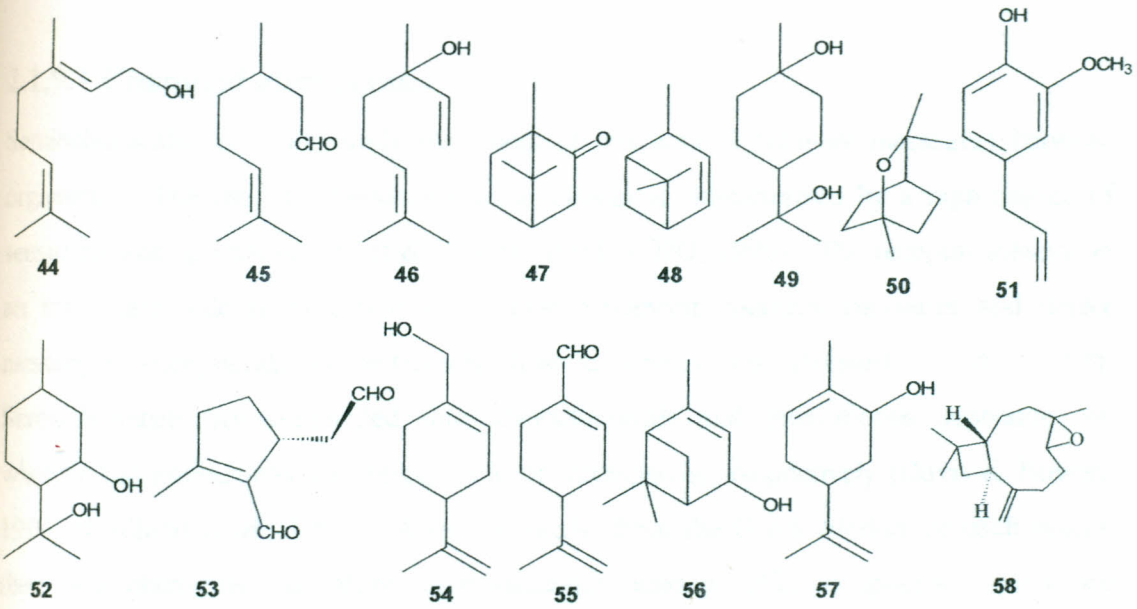
Perhaps the best and most effective protection against malaria is avoiding being bitten by mosquitoes. Personal protection can be achieved by the use of bed nets, suitable clothing, and repellents (WHO, 1995). Repellents are chemical substances that protect animals, plants or materials such as fabrics, grain and timber from insect attack by rendering them unattractive, unpalatable or offensive (Metcalf & Flint, 1962). During world war II there was need to search for new repellents for use by the military (Wigglesworth, 1976). Almost 7000 organic synthetic compounds were screened for repellency against mosquitoes on human skin and clothing in USA (Service, 1986). Dimethyl phthalate

(DMP), 2-ethyl-1,3-hexanediol (Rutgers 6/2) and *n*-butylmesityloxide oxalate (indalene) are some of the earliest repellents synthesized in the laboratory (Kirk & Othmer, 1992). The search for new and longer acting repellents resulted in the discovery of DEET (*N, N*-diethyltoluamide), which provides relatively long protection against blood-feeding insects (Fradin, 1998; Goodyer & Behrens, 1998). On the other hand, rapid skin penetration and bio-distribution of DEET in both humans and animals have raised concerns on its toxic side effects (Miller, 1982; Roland *et al.*, 1985; Qui *et al.*, 1998). Recently, a related repellent, 1-piperidinecarboxylic acid, 2-(2-hydroxyethyl)-1-methylpropylester (KBR 3023 or Picaridin, or Bayrepel®) was developed by Bayer. On the basis of available evidence, KBR 3023 represents a promising alternative to DEET (Badolo *et al.*, 2004; Debboun *et al.*, 2000).

DEET, Dimethyl phthalate (DMP), 2-ethyl-1,3-hexanediol and 2-phenylcyclohexanol have been used as general purpose insect repellents, whereas *n*-butyl-6,6-dimethyl-5,6-dihydro-1,4-pyran-2-carboxylate, *cis*-dimethylbicyclo[2.2.1]-5-heptane-2,3-dicarboxylate, 2-ethyl-2-butyl-1,3-propanediol and *n*-propyl-*N,N*-diethyl succinate have been used specifically as mosquito repellents (Kirk & Othmer, 1992). The disadvantages of synthetic repellents include; resistance developed by insects, toxicity to other animals, high cost and environmental pollution (De Kumar, 1995).

Natural repellents have also been used to control mosquitoes. These include the pyrethrin (an excito-repellent/insecticide) (WHO, 1984), essential oils of some plants like lemon grass, thyme, geranium, bergamot, baylaurel, pine, wintergreen, pennyroyal and eucalyptus (Curtis *et al.*, 1991). Examples of natural repellents which have been isolated from a wide range of plant species include, geraniol (**44**), citronellal (**45**), linalool (**46**), camphor (**47**), δ-pinene (**48**), *p*-menthane-1,8-diol (**49**), 1,8-cineole (**50**), eugenol (**51**) (Dethier, 1947), *p*-menthane-3,8-diol (**52**) (Trigg, 1996; Barassa *et al.*, 2002), rotundial (**53**) (Grayson, 2000), perilly alcohol (**54**), perillaldehyde (**55**), *cis*-verbenol (**56**), *cis*-carveol (**57**) and caryophyllene oxide (**58**) (Omolo *et al.*, 2004). Plant derived repellents are generally volatile compounds and as a result do not protect for long when applied on skin or

clothing, thus requiring repetitive treatments. Consequently, they are costly and therefore unaffordable to resource-limited rural farming and pastoral communities.



2.1.9 Mosquito attractants

Host-attractant baited traps and targets are promising technologies currently being considered for mosquito control (Mathenge *et al.*, 2002). A widely used trap for the surveillance of mosquitoes is the Centers for Disease Control (CDC) trap. The trap uses carbon dioxide and a light source to attract mosquitoes (Bellamy & Reeves, 1952; Sudia & Chamberlain, 1962; Newhouse *et al.*, 1966; Kline, 1994). However, the CDC miniature light traps are unsatisfactory for field surveys because they consistently produce too small catches for evaluation (Miller *et al.*, 1969).

Mosquitoes use attractants to locate their mates and find hosts for blood meals (Takken *et al.*, 2001). Host attractants therefore appear to offer potential application for use in traps and targets in the effort to minimize the threat of human diseases transmitted by mosquitoes. However, attractants of disease vectors and human pests are not adequately studied (WHO, 1996) and therefore not well understood. It is possible that chemical attractants from the insects themselves (pheromones), the host (kairomones) or habitat (apneumones) may offer new avenues for the management and control of vectors and

pests. The combination of baited traps with entomopathogens may facilitate bio-control tools for adult mosquito control.

2.1.9.1 Chemical attractants

Semiochemicals are compounds that mediate interactions (convey messages) between organisms. The use of semiochemicals in insects is characterized by a high degree of sensitivity and specificity (Davis & Bowen, 1994; WHO, 1996). The receptor systems in an insect are able to screen out countless irrelevant chemical messages and detect messenger compounds at extremely low concentrations (Benard *et al.*, 1994). Semiochemicals are subdivided into allelochemicals and pheromones depending on whether the interactions are interspecific or intraspecific, respectively (Davis & Bowen, 1994; Nordlund *et al.*, 1981). Allelochemicals (from the Greek *allelon*, of each other), then, are chemicals that allow communication among different species. They are disseminated by an individual of one species and received by an individual of another species (Davis & Bowen, 1994; Bracks *et al.*, 1999). The semiochemicals are grouped according to which individual benefits from the interaction:

- Kairomones benefit the recipient. For instance, rabbits give off a scent that attracts predators.
- Allomones benefit the disseminator. For example, protective substances are scattered to repel an attacker.
- Synomones benefit both the disseminator and the recipient, like the scent produced by a flower attracts a pollinating insect. Symbiotic organisms use these substances widely.
- Apneumones are emitted by non-living material evoking behavioral or physiological reactions that are adaptively favourable to receiving organisms (Nordlund *et al.*, 1981).

Within both allelochemicals and pheromones it is sometimes useful to refer to chemicals as arrestants, attractants, repellents, deterrents, stimulants or other descriptive terms (Nordlund *et al.*, 1981).

Attractants can be classified as pheromones, kairomones, allomones and synomones (Benard *et al.*, 1994; WHO, 1996). Pheromones are semiochemicals that are produced and received by members of the same species (Benard *et al.*, 1994). A range of behaviours and biological processes are influenced by pheromones. Pest management programs use compounds that attract a mate (sex pheromones), call others to a suitable food or nesting site (aggregation pheromones) or encourage deposition of eggs (oviposition pheromones or apneumones) (Sastry *et al.*, 1994; Bracks *et al.*, 1999). Other pheromones regulate reproductive development in social insects (honey bees and termites), alarm signal (in honey bees, ants and aphids), trail marks (ants) (Sastry *et al.*, 1994), among other functions.

Of known allelochemicals, volatile compounds similar to those given off by food sources (plants or animals) are important in pest management. Feeding attractants are examples of kairomones (Sastry *et al.*, 1994; Bracks *et al.*, 1999). For example, carbon dioxide given off by humans and other animals is used as a kairomone by female mosquitoes seeking a blood meal (Reisen *et al.*, 2000; Rueda *et al.*, 2001). In contrast, allomones are allelochemicals that favour the producer (Davis & Bowen, 1994). For example, secretions that deter predators are allomones (Sastry *et al.*, 1994). However, it should be noted that a single chemical signal may act as both a pheromone and kairomone. For instance, the compounds emitted by a bark beetle colonizing a host tree, attract other bark beetles (aggregation pheromone for the beetle), but the same compounds also attract certain predators and parasites that attack these bark beetles (feeding attractant or kairomone) (http://edis.infas.ufl.edu/Body-IN080).

Insect parasitoids are faced with a reliability-deductibility difficulty as far as the location of their hosts is concerned (Steinberg *et al.*, 1993). To cover this problem, parasitoids

use herbivores-induced synomones which are chemicals that are produced by plants in response to feeding damage by herbivores, and which attract the parasitoids (Udayagiri & Jones, 1992ab; Steinberg *et al.*, 1993; Tumlinson *et al.*, 1993). Many parasitoids are known to discriminate, and respond to volatile chemicals produced by un-infested plants and plants infested with a particular herbivores species (Steinberg *et al.*, 1993; Tumlinson *et al.*, 1993). Once the herbivore feeds on a plant, the plant starts producing large amount of volatile chemicals which are used as cues leading females of parasitoid to the microhabitat of its hosts (Turlings & Tumlinson, 1991; Tumlinson *et al.*, 1993). For instance, beet armyworm-damaged corn seedlings are the primary source of volatile allelochemicals that attract females of *Cotesia marginiventris* (Turlings *et al.*, 1991ab). It has been shown that *C. marginiventris* females fly to and land on undamaged plants, but any minor damage would increase responsiveness and the parasitoids would fly directly to the damaged sites (Turlings *et al.*, 1991ab). Thus, plants are the principal source of volatile synomones that not only direct insect parasitoids to the host patch, but also to get them into the direct location of the host. The end result is that the chemicals produced by the plant under damage by herbivores help the plant to get protection from further attack by attracting the female parasitoids which will then attack the herbivores. Both the plant and the insect parasitoids become the beneficiaries of the plant produced synomones.

Practical use of pheromones or host-produced attractants (kairomones) for pest management requires that specific active chemicals be isolated, identified and produced systematically (Eiras & Jepson, 1994; Bracks *et al.*, 1999). The systematic attractants, usually comprise of sex or aggregation pheromones or feeding attractants, and are used in one of the following ways: (i) as lures in traps used to monitor pest populations; (ii) as lures in traps designed to reduce a pest population; (iii) as broadcast signals intended to disrupt insect mating; or (iv) as attractants in baits containing insecticides or bio-insecticides (Bracks *et al.*, 1999; Mathenge *et al.*, 2002).

Mosquitoes have evolved a wide range of host-oriented responses. The four main sources of attractants are: (i) human body odour; (ii) long-range olfactory responses; (iii) short-range olfactory, thermal and visual responses; (iv) human skin micro-flora (Bracks *et al.*, 1999).

2.1.9.2 Human body odour

Why do mosquitoes bite people who like camping in the summertime in the evening? The answer may lie in body odour. The suggestion is supported by a study done with three volunteers sleeping for nine nights in separate tents outfitted with one mosquito entrance and two exit traps (Knols *et al.*, 1995). Analysis of the mosquito catches showed that one volunteer was significantly less attractive to two *Anopheles* and one *Culex* species than the other two volunteers. It was concluded that three of the mosquito species must have selected their host based on a blend of human odours. Carbon dioxide (CO_2) was ruled out as a selection factor since all volunteers were of about the same weight, size and age. Van den Hurk *et al.* (1997) showed that it is possible to sample an appropriate fraction of the mosquito population with a non-suction trap, baited with human odours.

In a separate study, extracts of whole mouse odour excluding CO_2 or other highly volatile chemicals, elicited highly significant responses in *Aedes aegypti* (McCall *et al.*, 1996). Knols & De Jong (1996) and Knols (1997) demonstrated that *An. gambiae s.s.* is attracted by volatile odours from limburger cheese (used as a mimic of human foot odour). Andreasen (1997) found that membrane blood-feeding was enhanced when recently worn socks were placed next to the device. The fecundity of laboratory colonies of *An. gambiae* and *An. stephensi* were also enhanced. It has been shown that *An. gambiae s.s.* prefers feeding on the feet to other parts of the human body (De Jong & Knols, 1995a). This may be due to bacterial foot odour (Bracks *et al.*, 1999). Preference for landing was lost when the feet were washed (Knols *et al.*, 1995).

A field study done to determine if mosquitoes were more attracted to tents baited with human odour or CO_2, found that significantly fewer mosquitoes were caught in the tent with CO_2 than the human odour-baited tent (Mboera & Takken, 1997a). Even when the CO_2 concentration was increased, mosquitoes were still more attracted to the human odour-baited tent. In this study mosquitoes had a choice of entering the two tents.

Lactic acid is a component of the body odour that was isolated from human arms through thin layer chromatography and identified as a mosquito attractant (Acree *et al.*, 1968). It was found that the *L*-isomer of lactic acid (**59**) was 5 times as attractive as the *D*-isomer. It was demonstrated that CO_2 was essential for the acid to act as a mosquito attractant (Acree *et al.*, 1968). In the search for components that are attractive to *Ae. aegypti*, it was demonstrated that *L*-lactic acid was only slightly effective in attracting these mosquitoes (Geier, 1996). Earlier on, Smith *et al.*, (1970), had found that lactic acid alone, or in combination with convection currents (heat), failed to elicit response in *Ae. aegypti* at close range. The observation was also confirmed by Eiras & Jepson (1994).

Previous researchers in this field have focused on the role of individual components as attractants. Moreover, wind tunnel has been employed to bioassay these compounds. However, wind tunnel bioassay does not mimic the host feeding conditions. In this study, a new bioassay set up that mimics the host feeding conditions was developed to test blends of compounds as well as the individual components in human foot odour under laboratory conditions.

2.1.9.3 Long-range olfactory cues

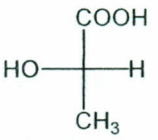
Chemicals that act as long-range attractants (or carry messages across distances) are volatile compounds (Reisen *et al.*, 2000). When released into the air, certain insects, a few inches to hundreds of yards away, can detect them at very low concentrations. Reeves (1953), was among the first to demonstrate that CO_2 is a mosquito attractant and that this chemical was dose dependent in its action. Sensitivity to a variety of chemicals exists, but none is more important in long-distance orientation than carbon dioxide (Van

den Hurk *et al.*, 1997; Mboera *et* al., 1997b; Reisen *et al.*, 2000). Mosquitoes are not only attracted to the host CO_2, but also to CO_2 when offered alone in a wind tunnel. In the absence of all other host odours, mosquitoes still become activated towards the CO_2 (Healy & Copland, 1995). CO_2 when used as bait enhances the catch size of any type of trap (Reisen *et al.*, 2000). It enhances the attractiveness of whole host odour including, some components of host odour and induces attraction to some chemicals, which are non-attractive (Van den Hurk *et al.*, 1997). The range of attraction increases with dose and removal of CO_2 from human breath decreases attraction to the host (Constantini *et al.*, 1998).

Comparative studies on the landing-site preferences of a range of mosquito species, from generalists to specialists in their host preference, have shown that olfactory cues emanating from particular areas of the human body enhances landing on those areas for the specialist species, whereas generalist feeders land at random (De Jong & Knols, 1996). It was established that out of the eight species of mosquitoes studied, five of them preferred getting blood meals at the head region (De Jong & Knols, 1996). Of the five, three preferred biting the face directly (De Jong & Knols, 1996). This observation suggested that CO_2 is universally attractive to mosquitoes (Gibson & Torr, 1999) and tend to direct the attraction to the part of the body that emits the highest amount. In fact, the universal attractancy of CO_2 as the main olfactory cue used for trapping mosquitoes has been demonstrated for several malaria vector species: *An. atroparvus* (Van Thiel, 1947; Laarman, 1955), *An. arabiensis* (Omer, 1979), *An. gambiae* s.s (Knols *et al.*, 1994a; Knols, 1996a). However, some schools of thought suggest that CO_2 is an activator that induces flight activity only, after which other orientation factors promote host location (Khan & Maibach, 1966; Snow, 1970).

CO_2 is generally considered as a kairomone of mosquitoes (Reisen *et al.*, 2000). Other kairomones or long-range olfactory cues are; lactic acid (**59**), 1-octen-3-ol (**60**), acetone and fatty carboxylic acids (Takken & Knols, 1999). However, lactic acid may not be important in long-range distance orientation, but its role in close-range host seeking is

extremely important (Acree *et al.*, 1968; Kline *et al.*, 1990). Other studies have also clarified the role of ammonia and phenols (Davis & Bowen, 1994) in host seeking behaviour. Under laboratory conditions, a combination of CO_2 and 1-octen-3-ol has been shown to increase responsiveness in *Ae. taeniorhynchus*, *Ae. crucians*, *Ae. quadrimaculatus* and *Wyeomyia mitchellii* (Takken & Kline, 1989). A combination of octenol, CO_2 and light as bait in CDC trap caught significantly more *An. farauti s.l.* than CO_2 and light alone (Van den Hurk *et al.*, 1997; Rueda *et al.*, 2001). Only small numbers of *An. farauti* complex were captured when CDC traps were baited with octenol alone in the absence of light or CO_2 (Van den Hurk *et al.*, 1997). Most vertebrates produce octenol (Gikonyo *et al.*, 2002). Vertebrates, such as oxen that produce high amounts of octenol are extremely attractive to host-seeking mosquitoes (Van den Hurk *et al.*, 1997). In fact, in some places, 1-octen-3-ol is routinely used for collection of mosquitoes in the field (Kline, 1994).



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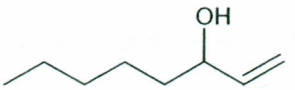

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2.1.9.4 Short-range olfactory, thermal and visual cues

Mosquito eyesight is poor, but its sensitivity to light is high. This has been demonstrated with *Ae. aegypti* (Muir *et al.*, 1992a). Anemotaxis is a movement in response to a learned behaviour (Gibson, 1995; Gibson & Torr, 1999). The enhanced sensitivity allows mosquitoes to follow host-odour plumes even at low light intensities (Bidlingmayer, 1994; Gibson, 1995), by using optomotor anemotaxis (Gillies, 1980). Optomotor anemotaxis was first demonstrated in *Ae. aegypti* (Kennedy, 1940).

Diurnal species respond to visual characteristics of hosts, such as colour/brightness, pattern and movement (Allen, 1987). For example, in *Ae. aegypti* the attractiveness of coloured targets is related to spectral sensitivity by the mosquito (Muir *et al.*, 1992b). Nocturnally active mosquitoes have specific responses to conspicuous objects and

barriers. The females of some species are attracted to conspicuous objects from as far as 15-20 m away even in the absence of hosts (Prokopy, 1986).

From earlier discussions, CO_2 is the best-understood long range mosquito-host attractant. The least understood cues are those used by mosquitoes to land on or near hosts at short-range distances. Early studies showed that mosquitoes respond to humidity and temperature gradients associated with convection currents (Takken, 1991). It has been confirmed that convection currents alone are attractive to *Ae. aegypti*. Lactic acid and sweat enhance the number of landings in the presence of CO_2. However, the human hand is significantly more attractive than the best combination of the tested components (Eiras & Jepson, 1994).

Although Eiras & Jepson (1994) confirmed that convection currents alone were attractive, no laboratory bioassay method, which exploits this research finding, has been developed. In this work, convection currents have been incorporated in the bioassay set up to help in mimicking the host-feeding conditions.

2.1.9.5 Human skin micro-flora

Researchers are just beginning to trap emanations (volatiles) that may act as host attractants. Human skin is inhospitable to most micro-organisms, and only a few species can survive on it (Bracks *et al.*, 1999). The composition and growth of skin micro-flora depends on skin temperature, humidity, pH, the concentration of inhibitors and the availability of nutrients (James *et al.*, 1981; Bracks *et al.*, 1999). Human skin micro-flora may be divided into three groups: (i) gram-positive cocci, (ii) diphtheroids-like species, and (iii) fungi. Gram-positive cocci can be obtained from nearly all body sites, with *Staphylococcus epidermidis* being the most dominant species. Three genera of diphtheroids are common. *Brevibacterium* spp. have a limited distribution and appear mainly on the toewebs. *Corynebacterium* spp. are spread over the entire skin surface, and together with various micro-coccal species, are found in high densities in the armpit. The anaerobic *Propionibacterium* spp., together with the fungus *Malassezia furfur*, are

abundant in the areas with high densities of sebaceous glands, such as the face and scalp (Bracks *et al.*, 1999). *Propionibacterium* spp., *M. furfur* and, to a lesser extent *Corynebacterium* spp. are lipophilic (Korting *et al.*, 1988). Due to metabolic processes, volatiles arise from the skin flora and fauna (James *et al.*, 1981). When oxidation of nutrients by skin micro-flora occurs, it results in the production of water and CO_2 (James *et al.*, 1981; Bracks *et al.*, 1999). Incomplete oxidation produces other small volatile breakdown products and excretory molecules. Humans are unique in having a high level of triglycerides (Stoddart, 1990), which are broken down mainly by *Propionibacterium* spp and give rise to a large number of long and short chain free fatty acids (James *et al.*, 1981; Bracks *et al.*, 1999). *Corynebacterium* spp are responsible for modification of initially odourless apocrine secretions including androsterone sulphate and dehydroepiandosterone, into the typical axillary smell of 5-androst-16-en-3-one and short chain fatty acids (Bracks *et al.*, 1999). *Brevibacterium epidermidis* is responsible for production of methanethiol, isovaleric acid and components of pungent foot odour (Sastry *et al.*, 1994; Bracks *et al.*, 1999). *Anopheles gambiae s.l.* responds positively to the volatiles produced by a related species, *Brevibacterium linens* (Bracks *et al.*, 1999). Meijerink & Van Loon (1999), found that limburger cheese, the odour of which is similar to that produced by feet, get its flavour by microbial action of *B. linens* and raises the number of mosquitoes in olfactometer traps by 2-3 folds. Similar responses to the acid fraction of the cheese and artificial mixture of short-chain fatty acids occurring in the cheese and foot odour were also observed (Knols, 1996b). Furthermore, significant electrophysiological responses of *An. gambiae s.s* were observed towards short chain fatty acids (Meijerink & Van Loon, 1999). Similarly, it was found that *Ae. aegypti* were attracted by air led through broth cultures of transient skin bacteria, *Bacillus cereus* (Schreck & James, 1986).

In attempts to identify human-produced kairomones, that are used for host location by *Ae. aegypti*, 346 peaks were detected by GC-MS analysis (Bernier *et al.*, 2000). Of these, 43 remain unidentified while 303 were characterized and 26 confirmed to be of background origin, leaving 277 as candidate attractants for *Ae. aegypti*. The number of compounds

identified in human-produced kairomones makes their bioassay a difficult and tedious process. Even with such a large number, there is no guarantee that all of the important compounds have been detected, and it is strongly believed that they have not been (Bernier *et al.*, 2000). Furthermore, the mosquitoes may be responding to volatile blends and not the individual compounds.

In 1992, S.C. Johnson and Sons Inc. sponsored Wageningen Agricultural University to identify the semiochemicals that mosquitoes use to locate their host (Van de Meent, 1994). It was shown that, depending on the species, mosquitoes show preferences for distinct parts of the human body. *Anopheles gambiae s.s* was attracted to the human foot whereas *Ae. aegypti* preferred the head region (De Jong *et al.*, 1993, Van de Meent, 1994). A pilot study on the analysis of the headspace volatiles of human feet, of which nothing was known, both from chemical as well as methodological point of view was undertaken (Van de Meent, 1994). The mosquito landing tests showed that once the feet had been washed, the preference disappeared (Knols *et al.*, 1995). In this work, 3-methylbutanal, butanal, heptanal, 2-methyl-1-propanol, limonene, octanal, 3-hydroxy-2-butanone, 6-methyl-5-hepten-2-one, menthol, phytane, geranylacetone, benzothiazole, phenol, 1-tetradecanol, 1-decanol, pristine, 1-dodecanol, 1-tridecanol, octanoic, nonanoic and acetic acids were listed as possible candidate attractants from unwashed feet (Van de Meent, 1994). However, the identification of these components was not confirmed (Van de Meent, 1994) by co-injection with authentic standards. Similarly, bioassays of the above compounds were not done (Van de Meent, 1994). Effectively, the mosquito attractants from the human feet have not been identified, even though most of the piloting work was completed.

The use of host attractants together with the known mosquito repellents may help in controlling the adult population through the 'push and pull mechanism'. In this case, the repellents can be used to form a perimeter fence in malaria prone area, thereby pushing the insects away from the fence, while the attractants in a trap, will pull them towards the

center. The biological or chemical agents can then be used to kill them. This method has been found to be effective in controlling the tsetse flies (Gikonyo *et al.*, 2002).

2.2 Statement of the problem

Attractive components and blends of compounds from human feet remain unidentified despite their potential as odour baits in *An. gambiae s.s.* traps for mosquito population monitoring and control.

2.3 Hypotheses

Human foot odour is attractive to *An. gambiae s.s.* mosquitoes, and contains short-range kairomones that can be trapped, extracted, characterized and reconstituted into attractive blends for population monitoring and control.

2.4 Objectives

The general objective was to characterize the mosquito attractive semiochemicals from the human foot odour and to evaluate their use under semi-field conditions. Specific objectives included:

- Ranking human volunteers in the order of the attractiveness of their feet odour to *An. gambiae s.s.*
- Collecting volatiles from the most and least attractive individuals with various adsorbents and determining their chemical compositions
- Identification of the active principles/blend(s) of the odour through electro-physiological and behavioural bio-assays.
- Development of synthetic blends for trapping *An. gambiae s.s.* in the field.

2.5 Justification

Mosquito behaviours are mediated by a wide range of semiochemicals that remain largely unknown or poorly understood. With the improved knowledge of the chemical cues influencing mosquito behaviours, new methods of malaria control could be developed.

Previous studies have demonstrated the importance of human foot odour in the selection of biting sites (Knols *et al.*, 1994b; De Jong & Knols, 1995a; 1996). Subsequently, the role of human skin micro-flora in the production of mosquito kairomones has been demonstrated (De Jong & Knols 1995b; 1996; Knols, 1996b; Bracks *et al.*, 1999). Synthetic mixtures of fatty acids (Knols, 1997), incubated human sweat (Bracks *et al.*, 1999), ammonia and several other compounds, like indole, geranylacetone, 3-methylbutanone, 6-methyl-5-hepten-2-ol, 1-dodecanol and hexadecanoic acid (Kline *et al.*, 1990; Meijerink *et al.*, 2001), have yielded varying behavioural or electrophysiological responses in *An. gambiae s.s.* Apart from whole human odour, none of the afore-mentioned compounds or blends thereof has resulted in increased trap catches under field conditions. This is indicative of incomplete characterization of the bioactive blends and/or flaws in experimental laboratory studies. It is therefore necessary that comprehensive identification of the components and attractive blends together with thorough evaluation of the field-based trapping systems be undertaken. This may lead to alternative mosquito population monitoring and control tools. Due to concerns for environmental pollution and pesticide safety, Integrated Vector Management (IVM) approaches are recommended for mosquito control. Pesticides should only be applied selectively, especially in emergency situations. It therefore follows that reduction of the human-mosquito contact through behavioural manipulation with infochemicals would be more acceptable than the widely applied chemical control methods.

CHAPTER THREE

DIFFERENTIAL ATTRACTIVENESS OF HUMAN FEET ODOURS TO MOSQUITOES

3.1 Mean mosquito catches using socks worn by human volunteers as baits

In a screen house trapping experiment using socks previously worn by male human volunteers as baits, the mean *An. gambiae s.s.* catches based on 32 replicates done at each of the traps at 4 different sites and classified by student LSD t-test (SAS® Institute, 2000) was 17.68 ± 2.61^{a}, 20.00 ± 2.25^{a}, 22.75 ± 3.06^{a} and 22.56 ± 2.92^{a}, for traps 1, 2, 3 and 4, respectively. Thus the means were not significantly different ($p=0.512$), implying that there was no biasness of catches in any of the traps at the 4 different sites. On the other hand, the mean mosquito catches of each of the 4 different groups of human subjects I, II, III and IV based on 32 replicates and student t-test was 25.53 ± 3.12^{a}, 24.68 ± 2.52^{a}, 18.31 ± 2.57^{b} and 14.46 ± 2.24^{b}, for the groups III, II, IV and I respectively. This shows that the mean mosquito catches obtained from the socks baits worn by human subjects that constituted groups III and II differed significantly ($p=0.009$) from those that constituted groups I and IV.

3.2 Attractiveness of human feet odours to mosquitoes

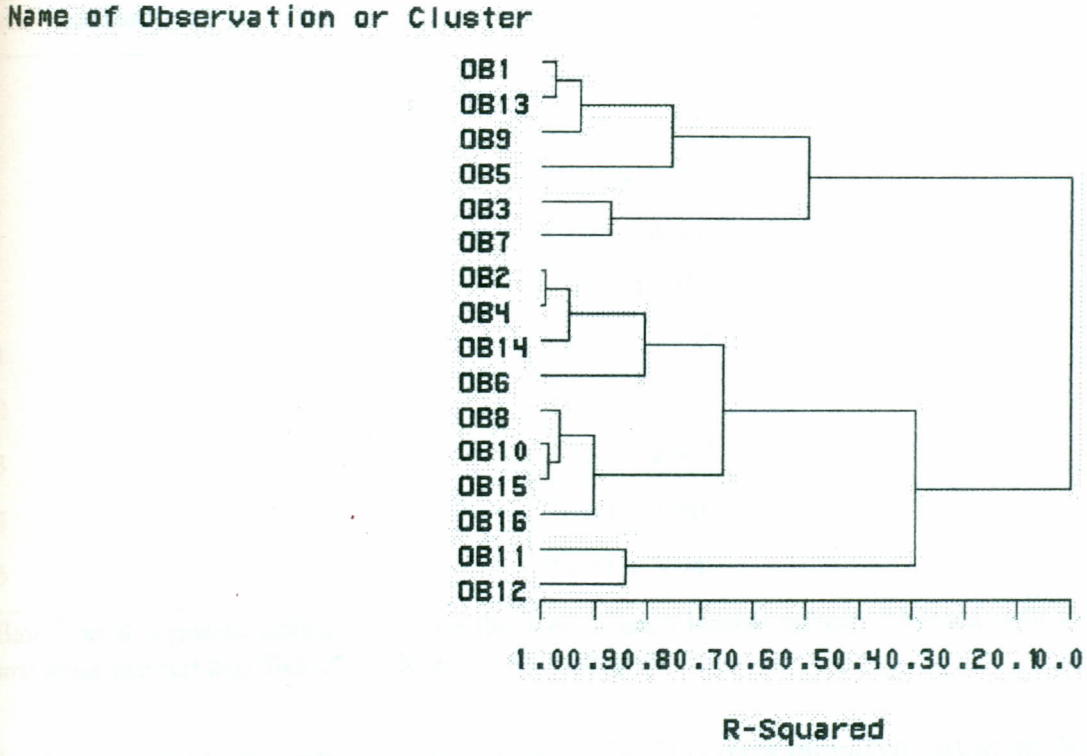
The mean number of *An. gambiae* mosquitoes caught using feet odour adsorbed on polyester and cotton socks worn by each of the 16 male human subjects are summarised in table 1. Feet odours of persons number 11 and 12, caught the highest number of *An. gambiae* with no significant difference between them. These were significantly higher ($p=0.001$) than those caught by feet odours of persons number 9 and 10 (SAS® Institute, 2000), which were the least attractive in group III with no significant difference between them. Mean catches of mosquitoes from other subjects in the other three groups I, II and IV (SAS® Institute, 2000) were not significantly different ($p=0.527$, $p=0.458$, $p=0.679$ for groups I, II and IV, respectively). The dendrogram (Fig. 3) obtained from the

multivariate cluster analysis (SAS® Institute, 2000) of the mean mosquito catches revealed 3 distinct clusters of the subjects ($R^2 = 0.35$). The three clusters constitute the most, moderate and least attractive groups. Persons number 11 and 12 comprise the most attractive group, while persons 1, 3, 5, 7, 9 and 13 belong to the least attractive cluster. The rest belong to the moderately attractive cluster.

Table 1: Mean mosquito catches* in the CFG traps baited with human foot odour

Group	Person number	Mean catches ± SE	Group	Person number	Mean catches ± SE
I	1	12.50 ± 8.92[a]	III	9	6.75 ± 2.19[a]
	2	12.63 ± 14.08[a]		10	15.88 ± 5.28[a]
	3	20.38 ± 14.96[a]		11	42.50 ± 7.9[b]
	4	12.38 ± 12.38[a]		12	37.00 ± 17.13[b]
	(LSD test p=0.527)			(LSD test P=0.001)	
II	5	18.63 ± 15.17[a]	IV	13	14.88 ± 7.14[a]
	6	28.88 ± 14.41[a]		14	21.50 ± 19.85[a]
	7	28.13 ± 18.39[a]		15	21.63 ± 17.96[a]
	8	23.13 ± 6.64[a]		16	15.25 ± 10.98[a]
	(LSD test p=0.458)			(LSD test p=0.679)	

*Based on 8 replicate collections from the feet of each human subject. Means with the same letter are not significantly different (LSD test, at stated p values)



3.3 Order of attractiveness of the human feet odour to *An. gambiae*

In a follow up experiment involving 10 selected subjects (4 least, 3 moderate and 3 most attractive) together with the control, mosquito catches from socks worn by persons number 11 and 12 (most attractive) were significantly higher ($p=0.001$) than those of feet odours from persons number 1, 4 and 9 (least attractive) (Table 2).

Table 2: Transformed mean mosquito catches* in CFG traps baited with human foot odour

Person number	Mean catches ± SE

Table 2: Transformed mean mosquito catches* in CFG traps baited with human foot odour

Person number	Mean catches ± SE
1	26.63 ± 2.93^{e}
3	28.75 ± 2.58^{c}
4	22.63 ± 2.72^{e}
6	35.25 ± 4.86^{b}
9	7.75 ± 1.85^{f}
11	58.88 ± 3.55^{a}
12	51.25 ± 4.83^{a}
13	33.63 ± 4.89^{b}
14	26.63 ± 3.80^{c}
15	31.63 ± 3.94^{c}

*Based on 8 replicate collections from the feet of each human subject. Means with the same letter are not significantly different (LSD test, p=0.001).

The cluster analyses of mean mosquito catches in the CFG traps prompted further studies on the chemical variations in the feet odours of the most and least attractive persons (number 9 and 11, respectively). The results of this are detailed in the next chapter.

CHAPTER FOUR

THE CHEMISTRY OF HUMAN FOOT ODOUR

4.1 Feet odour collections from most and least attractive subjects

The GC profiles of the feet odour of the least attractive human subject (person number 9) (Fig. 4) and the most attractive subject (person number 11) (Fig. 5) collected on porapak Q, activated charcoal, reverse phase octadecyl (C_{18}) bonded silica and reverse phase octyl (C_8) bonded silica revealed different chemical compositions. Porapak Q and activated charcoal on one hand and C_8 and C_{18} on the other hand had very similar chemical profiles. Porapak Q was the best adsorbent for the feet odour volatiles based on the number and quantity of compounds adsorbed by it compared to the other adsorbents.

The odour profiles from the feet of most (person number 11) (Fig. 6a) and least (person number 9) (Fig. 6b) attractive human subjects trapped on porapak Q were different qualitatively and quantitatively. The difference may be attributed to the variation in the microbial composition on the feet of the subjects. It is likely, that the type and population of the skin micro-flora and fauna on human foot determines the quality and quantity of the volatile odour composition emanating from the foot. Consequently, the difference in the chemical profiles of the feet odours of most and least attractive persons might be due to the differences in the feet micro-flora and fauna (James *et al.*, 1981; Bracks *et al.*, 1999).

Figure 4: GC profiles for the foot odour of least attractive person on different adsorbents

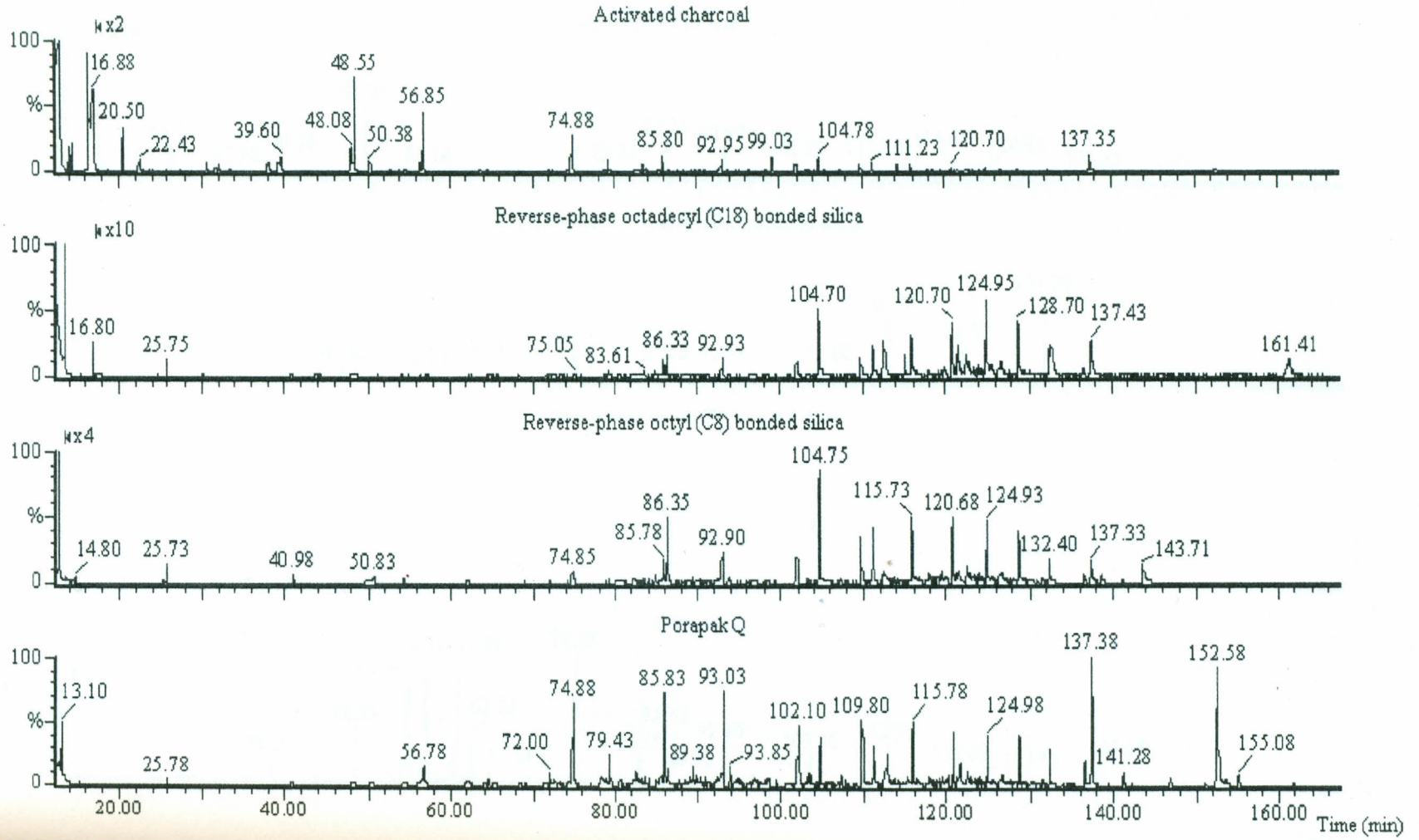


Figure 5: GC profiles for the foot odour of most attractive person on different adsorbents

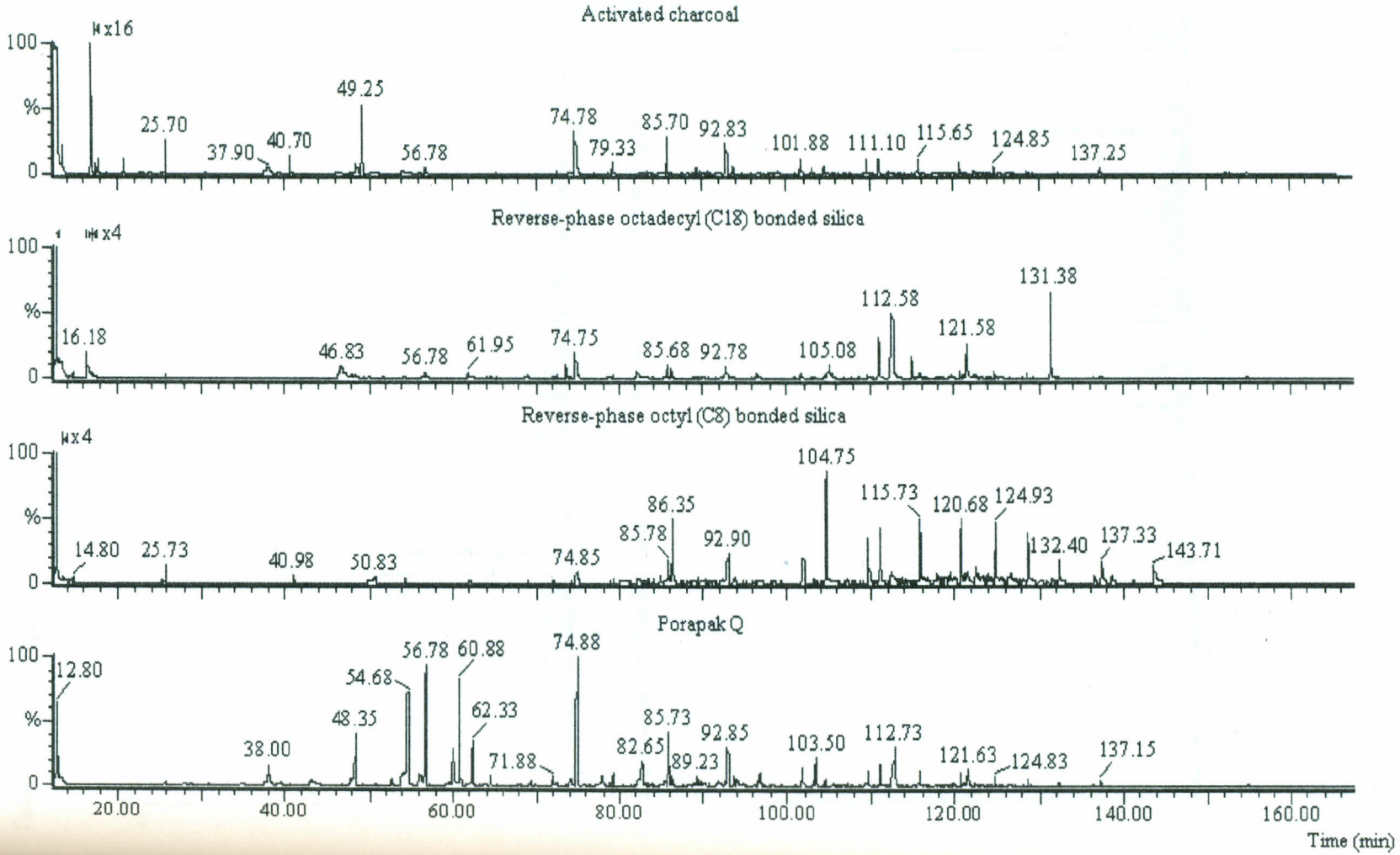


Figure 6a: GC profile for foot odour of most attractive person trapped on porapak Q

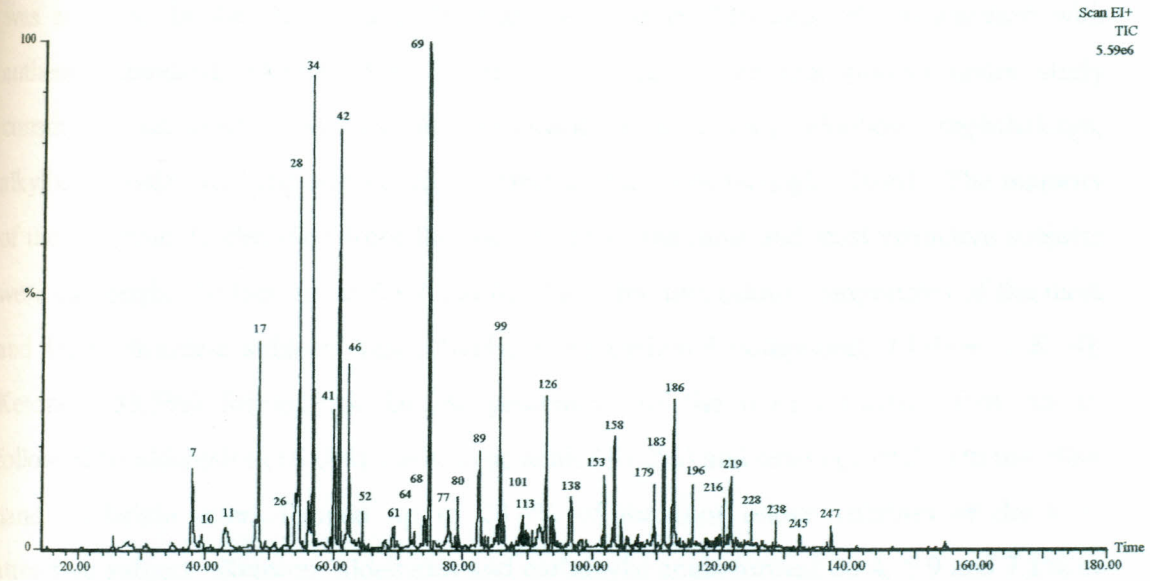
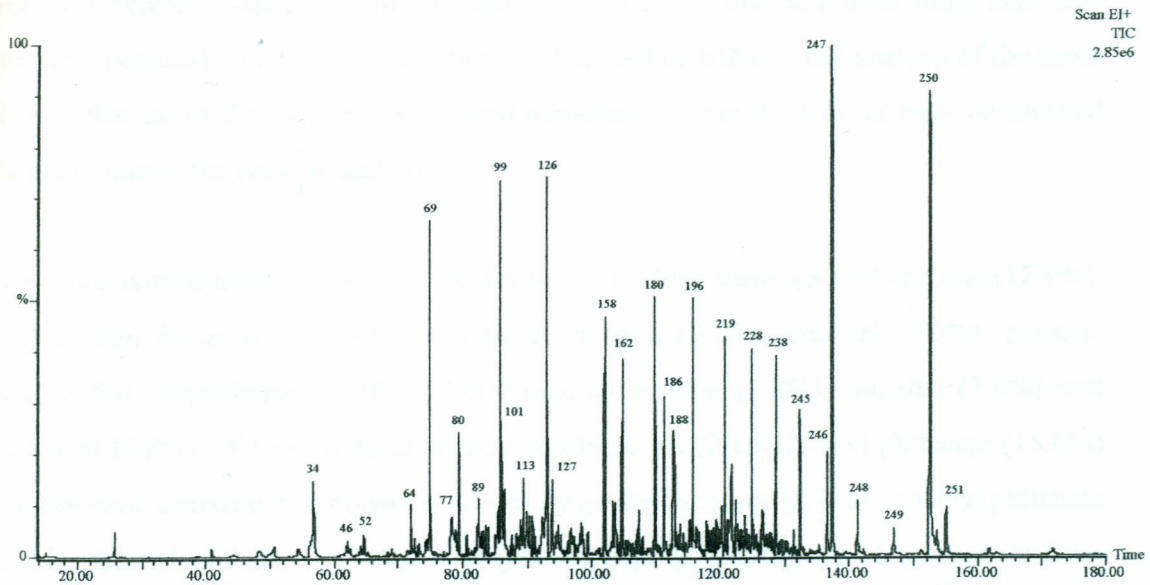


Figure 6b: GC profile for foot odour of least attractive person trapped on porapak Q



4.2 Identification of feet odour components

The composition of the feet odours of the most and least attractive male human subjects was analyzed by GC (Fig. 6a & b) and identified by MS and GC co-injection with authentic standards (Tables 3-8). The compounds in the feet odours under study constituted carbonyls, halo-organics (aliphatic & aromatic), alcohols, naphthalenes, alkylbenzenoids, aliphatic hydrocarbons, amines and thiols (straight chain). The majority of the compounds identified from the feet odour of the most and least attractive subjects were carbonyls. In fact, up to 65.7 and 61.8% of the feet odour components of the most and least attractive subjects respectively, were carbonyl compounds (Tables 3 & 4). Ketones (33.5%) formed the largest percentage of the most attractive foot odour, followed by aldehydes (18.0%), carboxylic acids (13.2%) and esters (1.0%). On the other hand, phthalate esters formed up to 28.4% of the foot odour volatiles of the least attractive subject. Ketones, aldehydes and carboxylic acids formed 20.4, 5.9 and 7.1% of the odour components, respectively. Alcohols (Table 5) and aliphatic hydrocarbons (Tables 7 & 8) formed up to 8.9 and 12.5% in most and 5.5 and 25.0% in least attractive feet, respectively. About 65 of the detected peaks (13 and 8% from most and least attractive persons) could not be classified or identified by EIMS. The analysis of the blank showed that up to 8 peaks that got eluted immediately after the solvent peak constituted the peaks due to the background noise.

The major components of the most attractive foot odour were geranyl acetone (12.9%), 1,3,5-trichlorobenzene (10.9%), 4-ethylacetophenone (9.2%), decanal (9.0%), palmitic acid (6.9%), heptadecane (5.0%), 4-ethoxyacetophenone (4.8%), nonanal (3.4%) and oleic acid (2.8%). From the least attractive subject *bis*-(2-ethyl)hexyl phthalate (15.6%) was the most abundant component followed by geranyl acetone (8.2%), isobutyl phthalate (6.3%), dibutyl phthalate (5.8%), 4-ethylacetophenone (4.5%), neryl acetone (3.6%), among others (Tables 3-8). Except in a few cases, most of these results are qualitatively in agreement with what was previously reported on the analysis of human skin emanations (Bernier *et al.*, 2000). The difference could be as a result of the different approaches

taken in trapping the foot odour volatiles. Whereas we employed static method, Bernier *et al.* (2000) trapped the odours through head space.

Table 3: Carboxylic acids and esters in human feet odour

Peak No.	Compound	Retention time (min)	% Composition MA subject	LA subject
	Carboxylic acids			
1	Isobutyric acid	20.7	0.142	0.213
2	isovaleric acid	28.1	0.328	0.480
3	2-methylbutyric acid	28.8	0.233	0.422
9	pentanoic acid	38.5	0.070	0.178
14	hexanoic acid	46.8	0.045	0.050
21	Heptanoic acid			
25	2-ethylhexanoic acid	50.8	0.605	1.284
30	octanoic acid	55.1	0.175	0.397
44	nonanoic acid	58.3	0.158	0.314
47	decanoic acid	62.2	0.406	0.684
61	undecanoic acid	69.3	0.924	0.229
92	hexadecanoic (palmitic) acid	96.8	6.937	0.546
138	9-octadecenoic (oleic) acid	112.7	2.755	2.264
147	octadecanoic acid*	116.2	0.126	0
164	docosanoic acid*	105.4	0.268	0
	Esters			
143	*cis*-3-hexenyl acetate*	98.2	0	0.432
161	isobutyl phthalate	104.5	0.744	6.346
171	diisobutyl phthalate	107.3	0	0.106
174	butyl phthalate	108.2	0	0.184
183	dibutyl phthalate	111.3	0	5.798
195	methyl-2-oxooctadecanoate*	115.4	0.036	0
215	octyl laurate*	120.2	0.222	0
250	*bis*-(2-ethyl)hexyl phthalate	137.4	0	15.625

MA = most attractive, LA = least attractive *Compounds not confirmed through GC co-injection.

Table 4: Aldehydes and ketones in human feet odour

Peak No.	Compound	Retention time (min)	% Composition MA subject	LA subject
	Aldehydes			
5	2-ethylbutanal	30.8	0.121	0.029
9	2-octenal	39.3	0.152	0
10	octanal	39.5	0.199	0.968
16	2-nonenal	47.8	0.602	0
17	nonanal	48.4	3.439	2.116
33	2-decenal	56.1	1.784	0
34	decanal	56.8	9.014	0.709
39	4-(1-hydroxyethyl)benzaldehyde*	59.5	0.610	0
52	undecanal	64.5	0.624	0.051
77	dodecanal	70.5	0.550	0.554
84	tridecanal	78.8	0.239	1.506
98	tetradecanal	85.4	0.665	0
110	6-nonenal*	88.4	0.041	0
	Ketones			
7	6-methyl-5-hepten-2-one	38.1	3.683	0
18	3,5,5-trimethylcyclohex-2-en-1-one	49.3	0.140	0
41	2 -ethylacetophenone	58.2	0.345	0.067
42	4-ethylacetophenone	60.8	9.225	4.540
43	3,4-dimethylacetophenone	62.3	0.673	0
46	4-ethoxyacetophenone	63.2	4.785	3.864
67	4-phenyl-2-butanone	73.6	0.136	0
68	nerylacetone	74.2	1.747	3.604
69	geranylacetone	74.8	12.912	8.200
73	2,6-dibutyl-2,5-cyclohexadiene-1,4-dienone*	76.3	0.095	0
94	2-undecanone	84.5	0.079	0
121	2-nonadecanone*	91.5	0	0.221

MA = most attractive, LA = least attractive *Compounds not confirmed through GC co-injection.

Table 5: Alkyl halides, alcohols, amines and thiols in human feet odour

Peak No.	Compound	Retention time (min)	% Composition MA subject	LA subject
	Alkyl halides			
78	1-chlorododecane	78.6	0.015	0
91	1-fluorododecane*	86.7	0	0.059
106	1-bromohexadecane	87.3	0.060	0
114	1-chlorohexadecane	97.8	0	0.079
149	1-iodo-2-methylundecane	100.6	0.065	0.183
213	1,2-dibromodecane	119.8	0.233	0
	Alcohols			
6	1-acetoxy-2-propanol	34.9	0.685	0
11	2-ethyl-1-hexanol	43.1	3.041	0.395
12	*trans*-2-hexenol	45.3	0.054	0
31	1-nonanol	55.5	0.112	0
37	2-phenoxyethanol	58.1	0.053	0
38	4-*t*-butylcyclohexanol	58.4	0.030	0
70	2-phenyl-2-propanol	75.5	0.509	0
79	dodecanol	77.7	1.877	0
105	1-tetradecanol	91.8	0.051	0
120	3,7,11-trimethyl-3-dodecanol*	92.3	0	0.890
123	2-hexadecyloxyethanol*	94.1	0	0.389
130	2-octadecyloxyethanol*	94.5	1.068	0
179	6,10,14-trimethylpentadecan-2-ol*	115.3	0	1.483
200	1-hexadecanol	116.7	0.026	0.093
211	1-octadecanol	119.1	0.057	0.256
237	farnesol	128.2	0.162	1.907
	Amines			
4	3-methyl-1-butamine	27.8	0.035	0
90	O-decylhydroxylamine*	83.1	0.251	0.367
	Thiols			
155	2-methyl-2-undecanethiol*	102.8	0.052	0.037
231	1-octadecanethiol*	126.5	0.184	0

MA = most attractive, LA = least attractive *Compounds not confirmed through GC co-injection.

Table 6: Aromatic compounds in human feet odour

Peak No.	Compound	Retention time (min)	% Composition MA subject	LA subject
	Alkylbenzenoids			
53	1-ethyl-4-isopropylbenzene*	63.4	0	0.046
96	1'-decylundecylbenzene	87.4	0	0.242
97	1'-octyldodecylbenzene	87.6	0	0.688
111	1'-propyloctylbenzene	88.4	0	0.416
112	2'-decyldodecylbenzene	89.8	0.041	1.399
124	1'-methylundecylbenzene	94.3	0.000	0.577
127	1'-pentylheptylbenzene	94.8	0.160	0.410
128	1'-butyloctylbenzene	95.2	0.124	0.518
131	1'-pentyloctylbenzene	95.7	0	0.211
132	1'-propylheptadecylbenzene	96.3	0.032	0.351
136	1'-propylnonylbenzene	96.1	0.062	0
159	1'-butylnonylbenzene	104.2	0.083	0.458
163	1'-isopropylheptadecylbenzene	105.4	0	0.438
	Halo aromatics			
27	1,2,3-trichlorobenzene	53.6	0.020	0
28	1,3,5-trichlorobenzene	54.7	10.997	0
35	1,2,4-trichlorobenzene	57.3	0.159	0
55	1,2,3,4-tetrachlorobenzene	66.5	0.047	0
62	1,2,4,5-tetrachlorobenzene	69.8	0.226	0
	Phenols			
13	2-methylphenol	45.9	0.036	0.069
48	*o-t*-butylphenol	63.2	0.100	0
50	2-ethyl-4,5-dimethylphenol	63.8	0.409	0
	Naphthlenes and azulene			
23	naphthalene*	52.3	0.138	0
56	2-methylnaphthalene*	65.3	0	0.204
57	2,6-dimethylnaphthalene*	67.8	0	0.118
65	2-ethylnaphthalene*	73.2	0	0.348
66	1,3-dimethylnaphthalene*	73.5	0	0.106
71	1,4-dimethylnaphthalene*	74.1	0	0.047
81	isopropylnaphthalene*	81.3	0	0.130
85	1,4,6-trimethylnaphthalene	81.5	0	0.051
54	azulene	60.7	0	0.166
74	2,4,6-trimethylazulene	80.9	0	0.240

MA = most attractive, LA = least attractive *Compounds confirmed through GC co-injection.

Table 7: Straight aliphatic hydrocarbons in human feet odour

Peak No.	Compound	Retention time (min)	% Composition MA subject	LA subject
19	decane	52.6	0.066	0.347
20	undecane	55.3	0.094	0.131
36	dodecane	59.7	0.022	0.048
58	tridecane	65.8	0.087	0.208
72	1-undecene	76.9	0	2.015
75	tetradecane	78.3	0.047	0.140
76	isotetradecane	79.3	0.846	0
83	1-tetradecene	79.4	0.022	0.117
86	pentadecane	80.1	0	2.604
87	isohexadecane*	80.3	0.040	0
88	hexadecane	83.4	0.231	0.056
89	heptadecane	85.7	5.011	0.034
107	eicosane	90.1	0.287	9.376
117	7-tetradecene*	90.9	1.779	0
122	1-hexadecene	91.2	0	0.468
125	heneicosane	91.7	0.018	0.263
126	docosane	93.4	0.210	0.572
128	pentacosane*	100.9	0.173	0.411
134	heptacosane*	105.6	0	0.039
137	octacosane*	107.1	0.357	0.153
198	5-eicosene*	120.8	0.050	0
246	hexatriacontane*	138.2	0	0.136

MA = most attractive, LA = least attractive *Compounds not confirmed through GC co-injection.

Table 8: Branched aliphatic hydrocarbons in human feet odour

Peak No.	Compound	Retention time (min)	% Composition MA subject	LA subject
118	2-methyldecane	90.7	0	0.772
128	2,6,10,14-tetramethylheptadecane*	95.3	0.018	2.018
129	3-methyltridecane	95.8	0.159	0.606
142	5,8-diethyldodecane*	98.7	0.056	0.208
143	3-methylhexadecane	100.4	0.351	0.869
145	4-ethyltetradecane	102.6	0.083	0.323
147	2,6-dimethylheptadecane	103.2	0.662	1.007
166	3-ethyltetracosane	107.8	0.085	0.079
169	2,6,8-trimethyldecane	108.1	0.043	0
173	2,6,7-trimethyldecane	108.9	0.028	0
175	4-methyloctadecane*	109.5	0.118	0
178	isooctacosane*	110.1	0.056	0
189	5-butyl-4-nonene*	114.7	0	0.054
194	2,4,6-trimethyldecane	116.8	0.023	0
197	5-methyl-2-undecene	117.9	0.019	0
205	3-ethyl-5-(2-ethylbutyl)octadecane*	118.3	0.111	0
209	9-cyclohexyleicosane*	119.4	0.188	0
224	6-ethyl-2-methyldecane	122.3	0.356	0.109
225	3,5,24-trimethyltetracontane*	123.8	0.156	1.144
227	9-(2-cyclohexylethyl)heptadecane*	127.5	0.038	0
243	7-hexyleicosane*	132.9	0	0.120

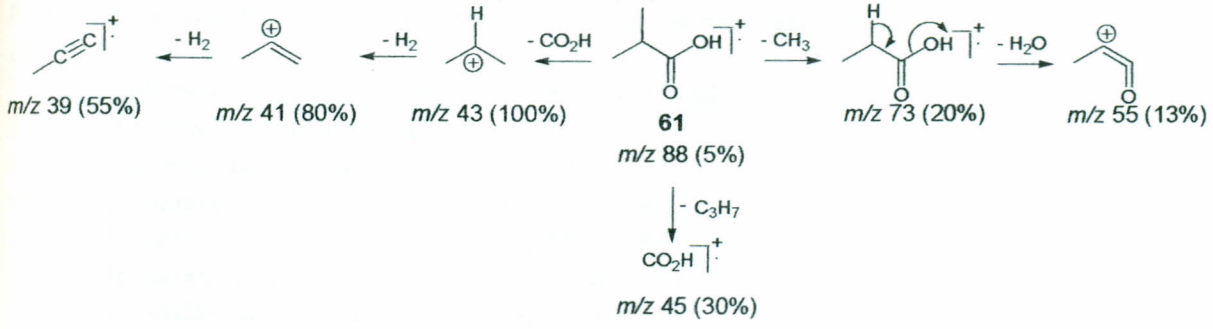
MA = most attractive, LA = least attractive *Compounds not confirmed through GC co-injection.

4.3 Mass fragmentation patterns

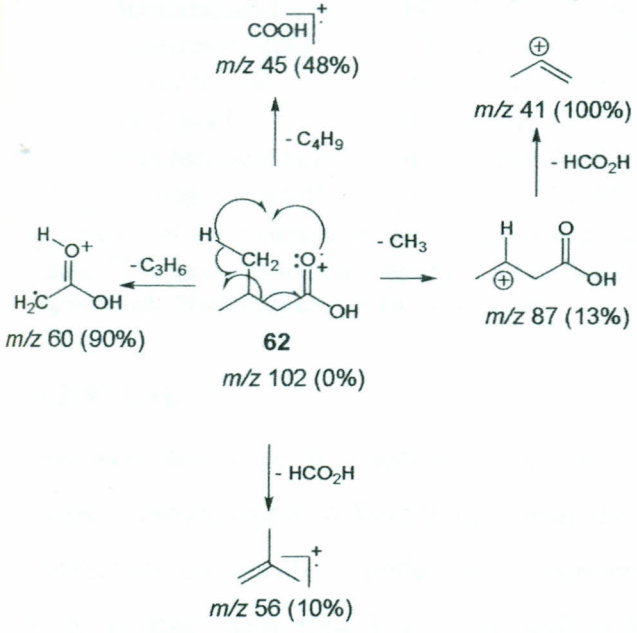
4.3.1 Carboxylic acids

Most of these acids were easily recognized through the fragment ion CO_2H of *m/z* 45, which was as a result of the cleavage of C-C bond that is α to the CO_2H functional group (Scheme 1 & 2). In addition to this ion, all the aliphatic acids with γ-protons and no branching at C-2 showed a diagnostic peak of *m/z* 60 due to β-cleavage that resulted from McLafferty re-arrangement (Kemp, 1991). The other fragment ions varied from one acid to the other depending on the chain length, branching and complexity of the structure as indicated in schemes 1 & 2.

Scheme 1: Mass fragment ions of isobutyric acid **(61)**



Scheme 2: Mass fragment ions of 3-methylbutyric acid **(62)**



Another related compound was 2-methylbutyric acid. All its fragment ions were the same as those of 3-methylbutyric acid except the ion that resulted from β-cleavage followed by McLafferty re-arrangement {*m/z* 74 (70%)}. This could be due to the methyl substituent at C-2. The characteristic mass spectral ions for the carboxylic acids are summarized in table 9 below.

Table 9: Mass spectral ions of carboxylic acids

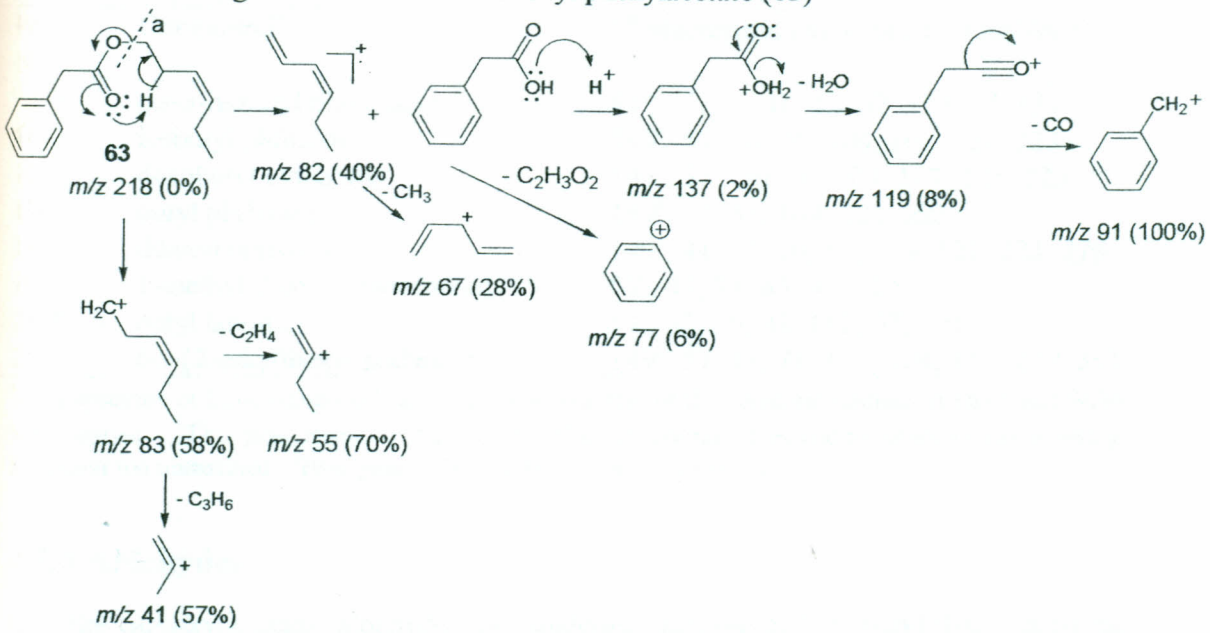
Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
1	isobutyric acid	43, 41, 39, 45, 73, 55, 88
2	3-methylbutyric acid	41, 60, 87, 56
3	2-methylbutyric acid	41, 74, 57, 39, 45
9	pentanoic acid	60, 41, 39, 55, 73, 87
14	hexanoic acid	60, 73, 57, 45, 55, 87
21	heptanoic acid	60, 73, 55, 45, 87, 101
25	2-ethylhexanoic acid	73, 41, 88, 57, 101, 116
30	octanoic acid	60, 73, 45, 85, 101, 115
44	nonanic acid	60, 73, 45, 115, 129, 158
47	decanoic acid	60, 73, 57, 129, 87, 172
61	undecanoic acid	73, 60, 55, 87, 129, 143, 157 186
92	palmitic acid	43, 57, 60, 73, 85, 97, 115, 129, 157, 171, 185, 213, 256
138	oleic acid	43, 60, 41, 73, 55, 85, 99, 129, 157, 171, 185, 199
147	octadecanoic acid*	43, 57, 69, 83, 85, 97, 111, 129, 285
164	docosanoic acid*	43, 57, 60, 73, 85, 97, 171, 185, 241, 297

[a]mass spectra of listed compounds are consistent with those of the standards in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. *Not confirmed by GC co-injection

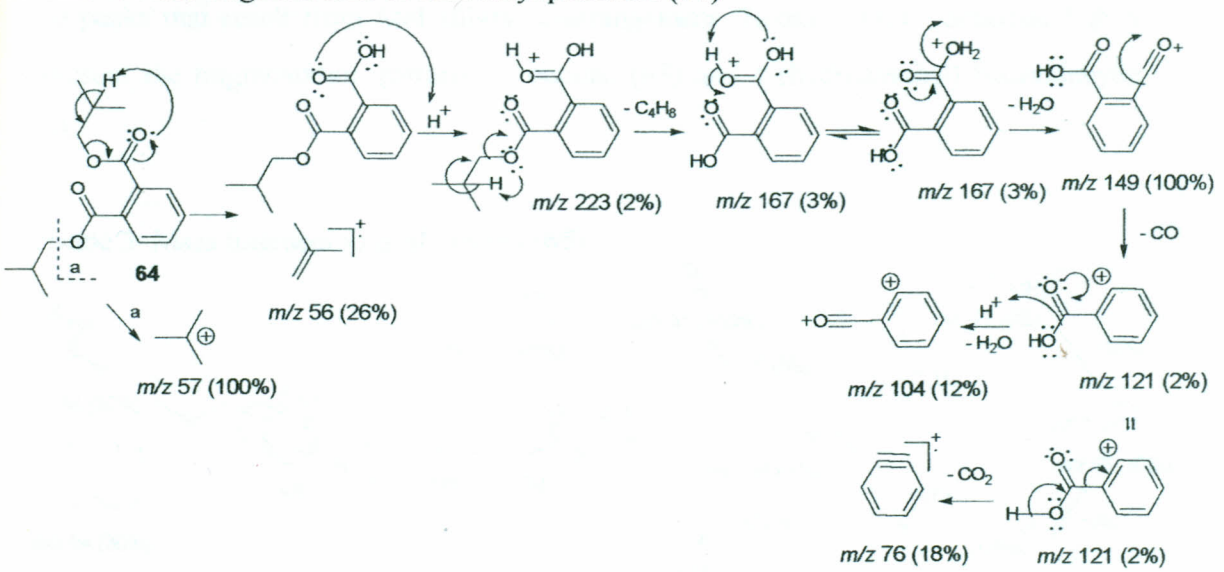
4.3.2 Esters

There were two main fragmentations common to the esters. The first was double hydrogen rearrangement to form the protonated acid of *m/z* 137 and 223 for *cis*-hexenyl phenylacetate and diisobutyl phthalate, respectively as illustrated in the schemes 3 and 4. The protonated acids then lost water and carbon monoxide successively. Another characteristic fragmentation was due to acid elimination from the molecular ions, which yielded the alkenes CH_2=CHCH=$CHCH_2CH_3$ and CH_2=$C(CH_3)_2$ ions at *m/z* 82 and 56, repectively (Scheme 3 & 4). The other major features of the spectra of *cis*-3-hexenyl phenylacetate (**63**) and isobutyl phthalate (**64**) could be accounted for by formation of hexenyl and *tert*-butyl ions (*m/z* 83 and 57). Loss of ethylene from the hexenyl ion gave *m/z* 55, which then lost a methylene yielding *m/z* 41.

Scheme 3: Mass fragment ions of *cis*-3-hexenyl phenylacetate **(63)**



Scheme 4: Mass fragment ions of diisobutyl phthalate **(64)**



The mass fragment ions of the esters in the foot odour are summarized in table 10.

Table 10: Mass spectral ions of esters

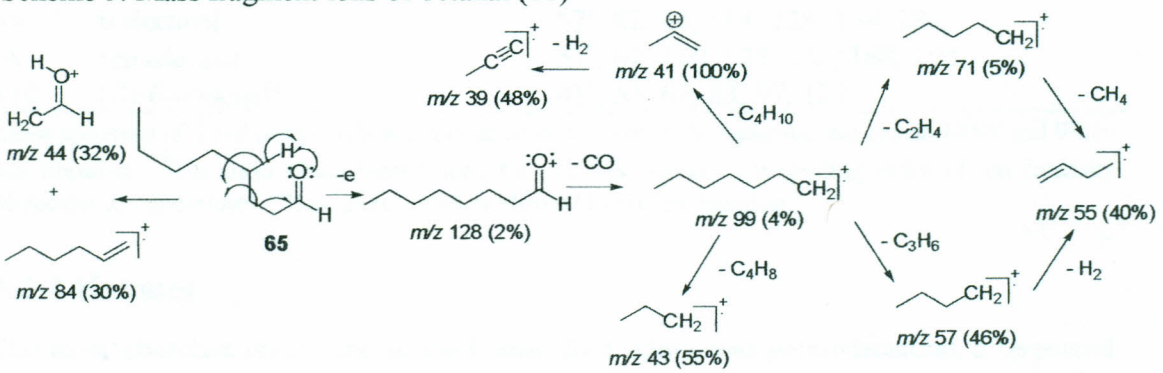
Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
143*	*cis*-3-hexenyl phenylacetate	91[c], 55, 83, 41, 82, 67, 119, 77, 137
161	isobutyl phthalate	149[c], 57, 56, 76, 104, 167, 121, 223
171	diisobutyl phthalate	149[c], 57[c], 56, 76, 104, 167, 121, 223
174	butyl phthalate	149[c], 41, 55, 104, 121, 223
183	dibutyl phthalate	149[c], 41, 57, 76, 65, 104, 121, 223, 278
195*	1-methyl-2-oxooctadecanoate	57[c], 43, 71, 85, 97, 125
215*	octyl laurate	57[c], 43, 70, 83, 112, 97, 201,
250	*bis*-(2-ethyl)hexyl phthalate	149[c], 57, 43, 71, 167, 104, 113, 279, 391

[a]mass spectrum of listed compounds are consistent with that of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]Base peak. *Not confirmed by GC co-injection.

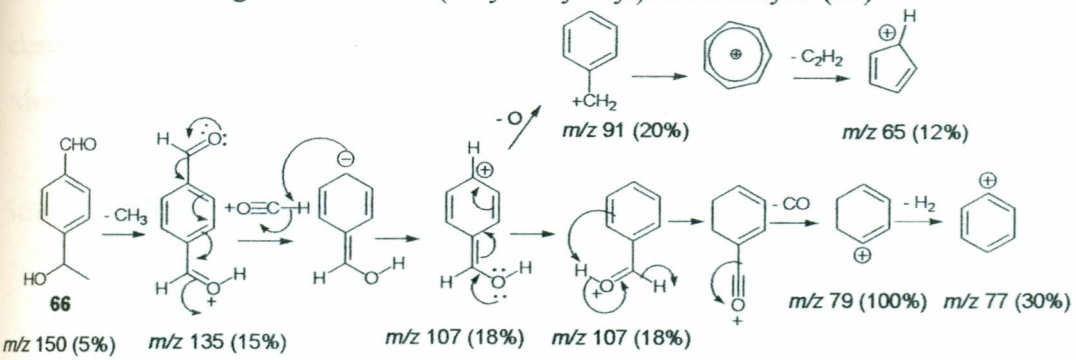
4.3.3 Aldehydes

Like the carboxylic acids, aldehydes also undergo cleavage of C-C bond that is α to the CHO functional group. In addition to this, straight chain aldehydes with γ-protons also give peaks that result from McLafferty re-arrangement (Kemp, 1991). Schemes 5 & 6 represent the fragmentation patterns of octanal (**65**) and 4-(hydroxyethyl) benzaldehyde (**66**).

Scheme 5: Mass fragment ions of octanal (**65**)



Scheme 6: Mass fragment ions of 4-(1-hydroxyethyl)benzaldehyde (**66**)



The characteristic fragment ions of the aldehydes are summarized in table 11.

Table 11: Mass spectral ions of aldehydes

Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
5	2-ethylbutanal	43[c], 72, 100
9	(*E*)-2-octenal	41[c], 55, 70, 83, 97, 126
10	octanal	41[c], 43, 39, 57, 55, 44, 84, 71, 100, 128
16	(*E*)-2-nonenal	41[c], 70, 83, 96, 111, 140
17	nonanal	57[c], 44, 98, 82, 114, 142
33	(*E*)-2-decenal	43[c], 70, 83, 97, 121, 168
34	decanal	57[c], 44, 82, 112, 128, 156
39	4-(1-hydroxyethyl)benzaldehyde*	79[c], 77, 91, 107, 135, 65, 150
52	undecanal	57[c], 44, 82, 110, 126, 170
77	dodecanal	57[c], 82, 44, 110, 140, 156, 184
84	tridecanal	57[c], 82, 44, 111, 124, 154, 180,
98	tetradecanal	57[c], 82, 110, 124, 138, 168, 194
110	(*Z*)-6-nonenal*	41[c], 55, 67, 83, 97, 122

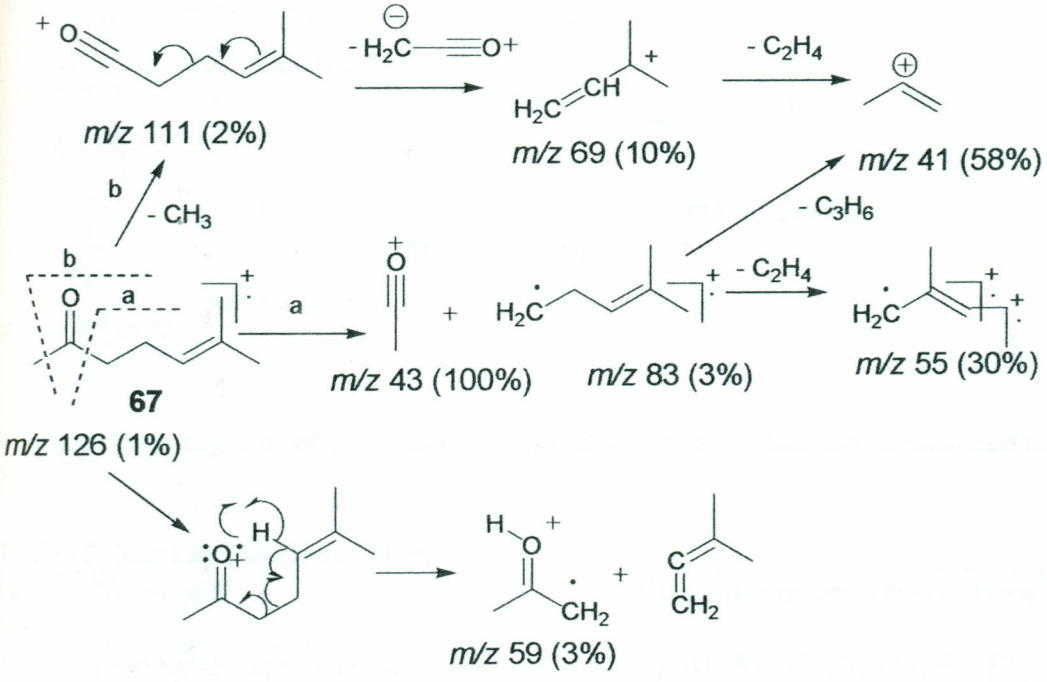
[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity Molecular ion underlined. [c]Base peak. *Not confirmed by GC co-injection.

4.3.4 Ketones

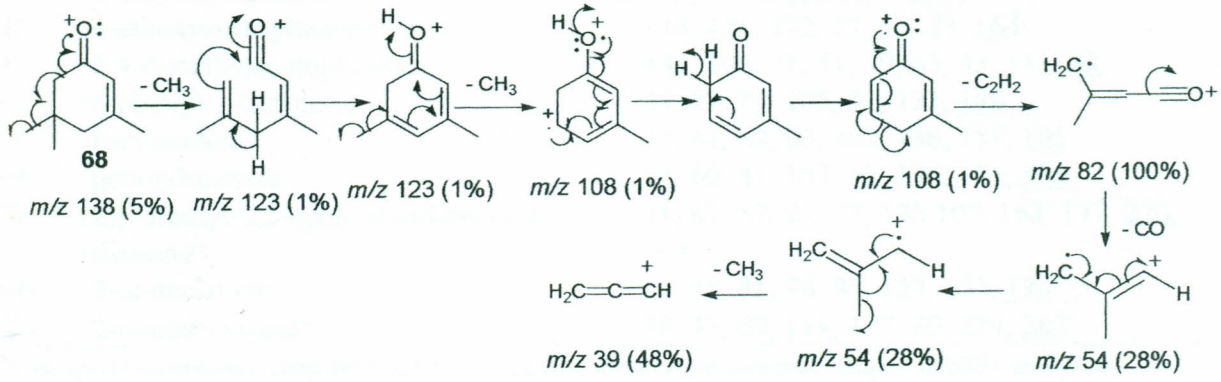
The most abundant compound in the human foot odour was geranylacetone, a terpenoid ketone. Whereas foot odour was found to be rich in straight short chain aliphatic acids, and aldehydes; branched aliphatic and aromatic ketones dominated its composition. The mass spectral fragmentation patterns of 6-methyl-5-hepten-2-one (**67**), 3,5,5-trimethylcyclohex-2-ene-1-one (**68**), and 4-ethylacetophenone (**69**) are presented in schemes 7, 8 & 9, respectively; as representatives of the group. Apart from the cleavage

at α position from the carbonyl group, ketones with protons at γ position are expected to cleave through McLafferty rearrangement. These are also illustrated by the following scheme 7 & 8.

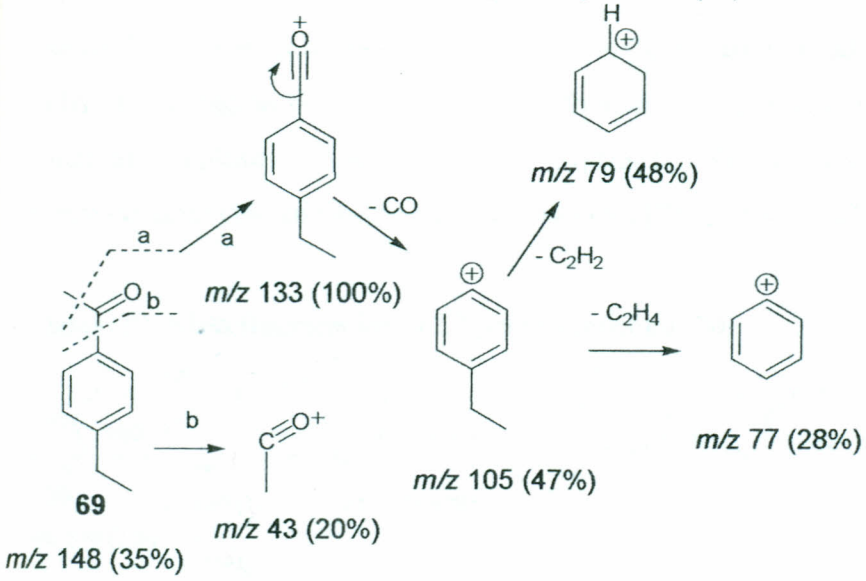
Scheme 7: Mass fragment ions of 6-methyl-5-hepten-2-one (**67**)



Scheme 8: Mass fragment ions of 3,5,5-trimethylcyclohex-2-ene-1-one (**68**)



Scheme 9: Mass fragment ions of 4-ethylacetophenone (**69**)



The mass fragment ions of the ketones identified in the foot odour are summarized in table 12.

Table 12: Mass spectral ions of ketones

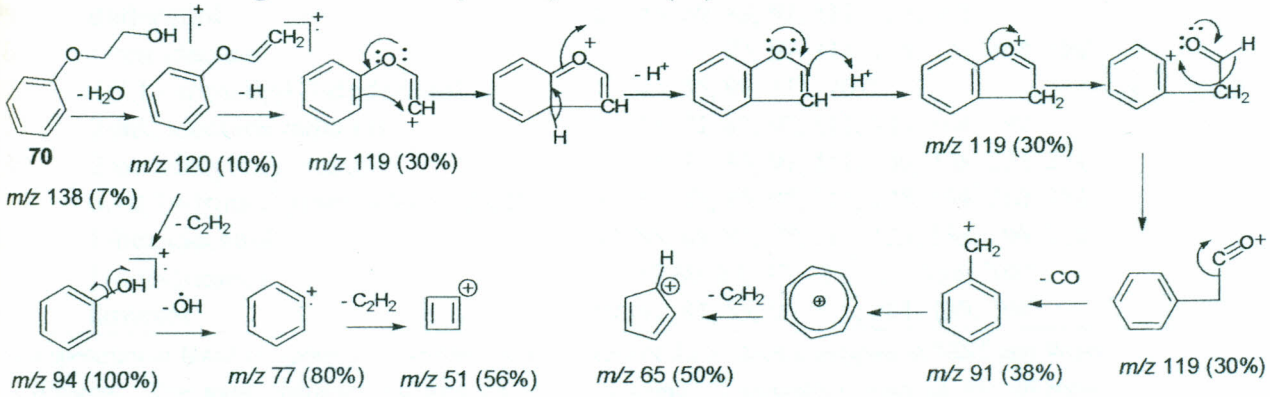
Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
7	6-methyl-5-hepten-2-one	43, 41, 55, 69, 83, 111, 97, 126
18	3,5,5-trimethylcyclohex-2-ene-1-one	82, 39, 54, 138, 108, 123
41	2-ethylacetophenone	133, 105, 148, 79, 77, 51, 43, 65,
42	4-ethylacetophenone	133, 79, 105, 148, 77, 43, 65
43	4-ethoxyacetophenone	138, 121, 122, 77, 65, 43, 164
46	3,4-dimethylacetophenone	133, 105, 77, 51, 79, 65, 43, 51, 148,
67	4-phenyl-2-butanone	43, 77, 79, 105, 91, 133, 148
68	nerylacetone	43, 41, 69, 93, 107, 136, 151, 195
69	geranylacetone	43, 69, 41, 107, 93, 136, 151, 195
73	2,6-dibutyl-2,5-cyclohexadiene-1,4-dienone*	41, 67, 57, 91, 77, 135,107, 163, 177, 220, 205
94	2-undecanone	43, 58, 71, 98, 85, 127, 155, 170
121	2-nonadecanone*	58, 43, 69, 111, 127, 80, 224, 282

[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. *Not confirmed by GC co-injection.

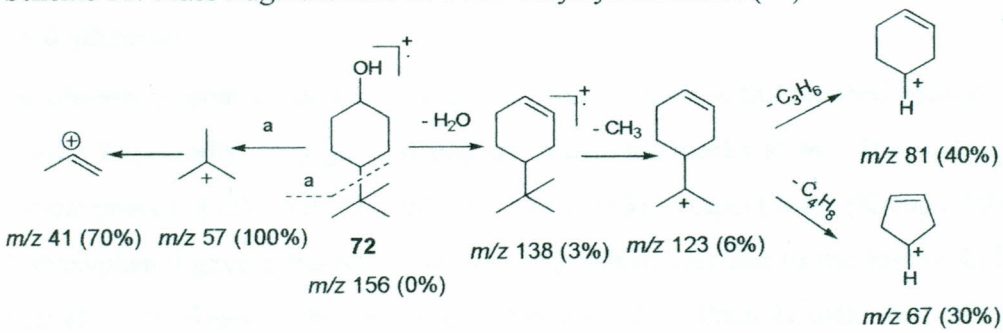
4.3.5 Alcohols

Like the ketones most of the alcohols present in the human foot odour were branched and cyclic. Their fragmentation patterns varied from one compound to the other depending on structural complexity. Schemes 10 & 11 explain the observed mass fragment ions of 2-phenoxyethanol (**70**) and 4-*tert*-butylcyclohexanol (**71**) (Crews *et al.*, 1985).

Scheme 10: Mass fragment ions of 2-phenoxyethanol (**70**)



Scheme 11: Mass fragment ions of 4-*tert*-butylcyclohexanol (**71**)



The characteristic fragment ions of the alcohols are summarized in table 13.

Table 13: Mass spectral ions of alcohols

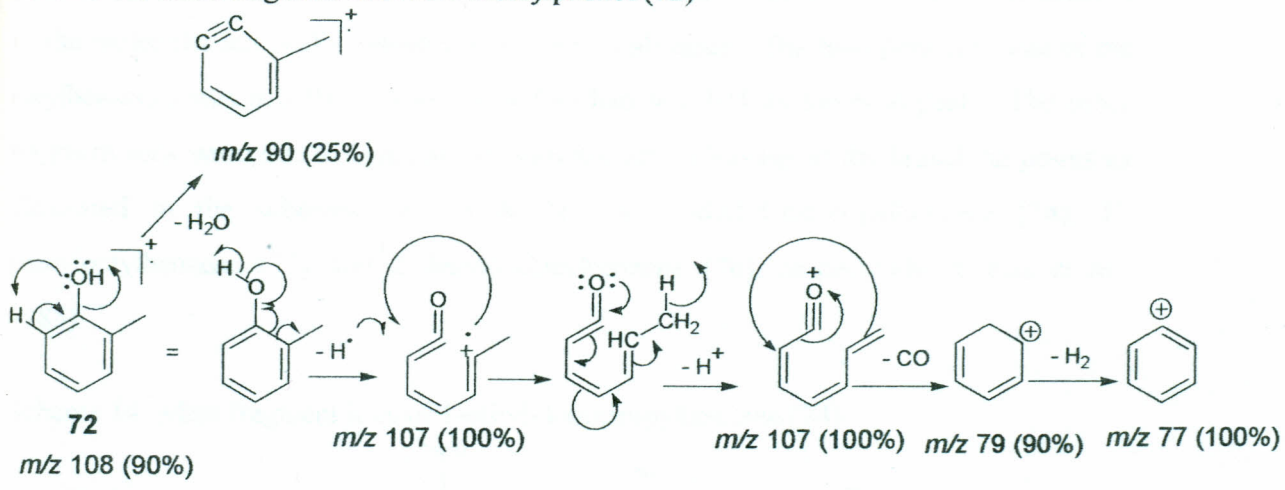
Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
6	1-acetoxy-2-propanol	43, 74, 45, 58, 74, 87
11	2-ethyl-1-hexanol	57, 41, 43, 70, 83, 98, 112[c]
12	*trans*-2-hexenol	57, 41, 43, 82[c], 67, 100
31	1-nonanol	41, 43, 55, 56, 70, 83, 97, 126[c]
37	2-phenoxyethanol	94, 77, 51, 65, 91, 119, 120[c], 138
38	4-*tert*-butylcyclohexanol	57, 41, 81, 67, 109, 123, 138[c]
70	2-phenyl-2-propanol	43, 121, 77, 103, 91, 65, 136
79	dodecanol	55, 43, 69, 83, 97, 111, 125, 168[c]
105	1-tetradecanol	43, 55, 69, 83, 97, 111, 125, 139, 168, 196[c]
120	3,7,11-trimethyl-3-dodecanol*	73, 43, 55, 97, 111, 207[c]
123	2-hexadecyloxyethanol	57, 43, 71, 85, 97, 111, 145, 168, 199
130	2-octadecyloxyethanol*	57, 43, 71, 85, 97, 111, 140, 168, 224, 254
179	6,10,14-trimethylpentadecan-2-ol*	43, 57, 71, 85, 97, 111, 125, 154, 210, 252[c]
200	1-hexadecanol	43, 55, 69, 83, 97, 111, 125, 154, 196, 224, 280[c]
211	1-octadecanol	43, 57,69, 83, 97, 111, 125, 154, 308[c]
237	farnesol	69, 41, 81, 55, 93, 136, 161, 189, 204[c]

[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]M-H_2O ions. *Not confirmed by GC co-injection.

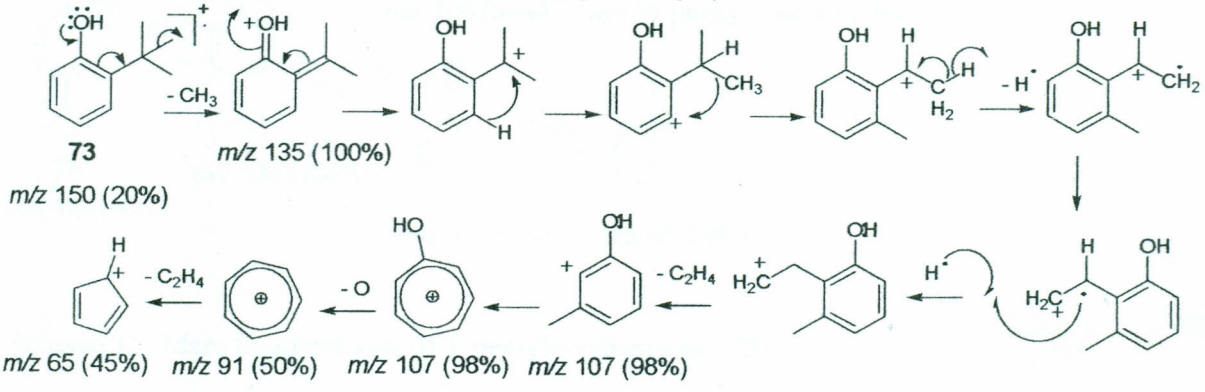
4.3.6 phenols

The phenols present in the foot odour were simple ones with branched hydrocarbon side chains. Expectedly, they gave strong molecular ion peaks at *m/z* 108 and 150 for 2-methoxyphenol (**72**) and *O-tert*-butylphenol (**73**), respectively (Kemp, 1991). 2-Methoxyphenol gave a fragment ion (M - 29), which was due to the loss of CHO, in the order (M – H) followed by loss of CO (Scheme 12). Phenols with alkyl side chain are known to undergo benzylic fission, leaving variants of the hydroxytropylium ion (Kemp, 1991). This is illustrated in the fragmentation pattern of *o-tert*-butylphenol (Scheme 13).

Scheme 12: Mass fragment ions of 2-methylphenol (**72**)



Scheme 13: Mass fragment ions of *o-tert*-butylphenol (**73**)



The characteristic fragment ions of the phenols are summarized in table 14.

Table 14: Mass spectral ions of phenols

Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
13	2-methylphenol	77, 107, 79, <u>108</u>, 90[c]
48	*o-tert*-butylphenol	135, 107, 77, 91, 65, 51, <u>150</u>
50	2-ethyl-4,5-dimethylphenol	135, 91, 77, 107, <u>150</u>, 115, 65

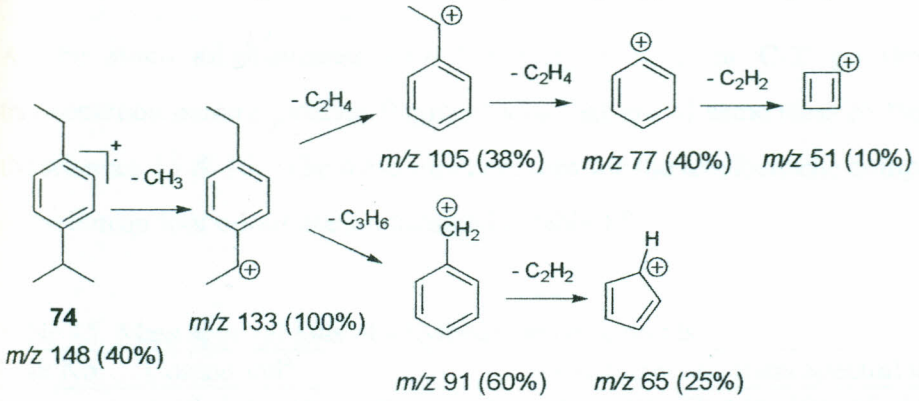
[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]M-H_2O ions.

4.3.7 Alkylbenzenoids

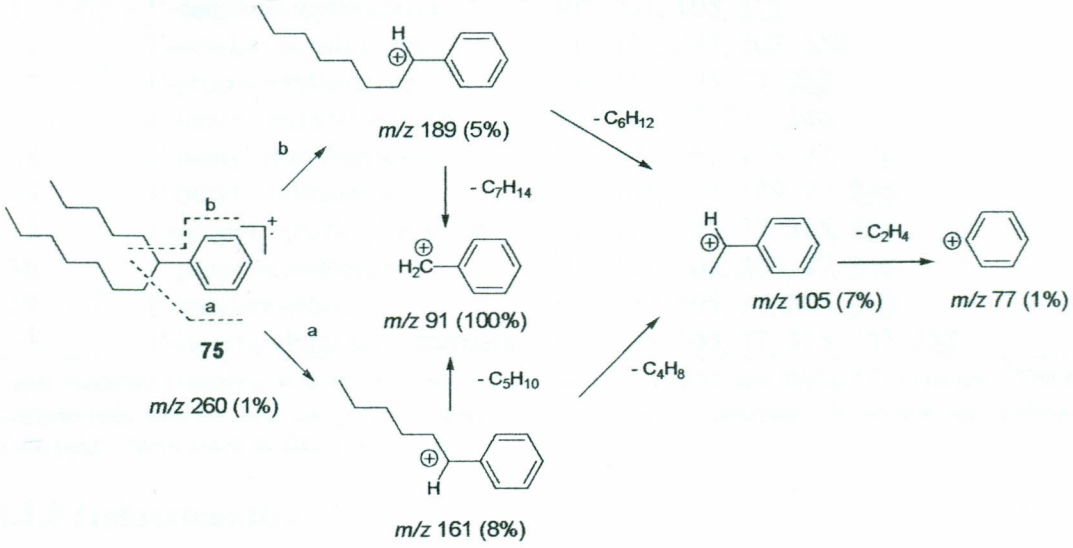
All the alkylbenzenes in the human foot odour were branched at C-1' or C-2' of the aliphatic section. The fragmentation pattern of the branched alkylbenzenes was

characterized by the presence of the mass fragment ions at *m/z* 105, 91 and 77 in addition to the molecular ion peaks, which was present in all cases. The base peak for most of the alkylbenzenes was *m/z* 91. However, a few had *m/z* 105 as the base peak. The other fragment ions were due to benzene residues left after cleavage at the branching points as illustrated in the schemes 14, 15 & 16 for 1-ethyl-4-isopropylbenzene (**74**), 1'-pentyloctylbenzene (**75**) and 2'-decyldodecylbenzene (**76**), respectively (Crews *et al.*, 1985).

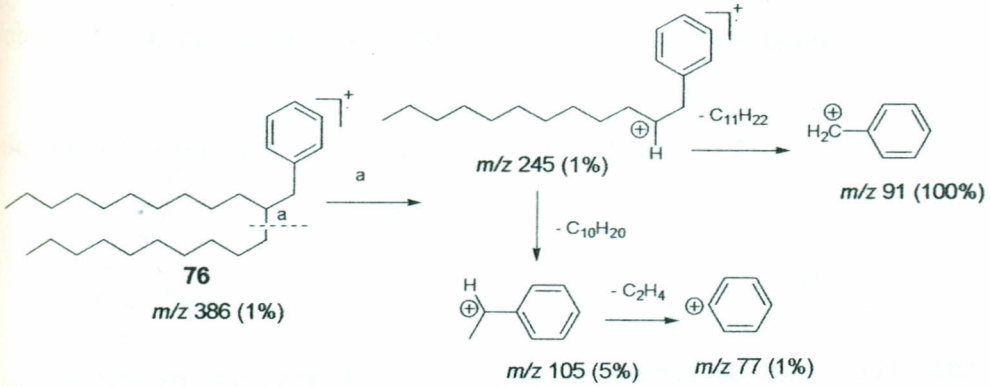
Scheme 14: Mass fragment ions of 1-ethyl-4-isopropylbenzene (**74**)



Scheme 15: Mass fragment ions of 1-pentyloctylbenzene (**75**)



Scheme 16: Mass fragment ions of 2-decyldodecylbenzene (**76**)



All the *mono* alkylbenzenes with branching at C-1' or C-2' go through the same fragmentation pattern yielding fragment ions that are of same mass to those illustrated in the schemes 15 & 16. The mass fragment ions for the alkylbenzene compounds identified in the human foot odour are summarized in table 15.

Table 15: Mass spectral ions of alkylbenzene compounds

Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
53	1-ethyl-4-isopropylbenzene*	133[c], 91, 77, 148, 105, 65, 51
96	1'-pentyloctylbenzene	91[c], 161, 105, 189, 77, 260
97	2'-decyldodecylbenzene	91[c], 105, 77, 245, 386
111	1'-decylundecylbenzene	91[c], 231, 105, 372
112	1'-octyldodecylbenzene	91[c], 105, 245, 203, 358
124	1'-propyloctylbenzene	91[c], 133, 105, 77, 232
127	1'-methylundecylbenzene	105[c], 91, 77, 231, 246
128	1'-pentylheptylbenzene	91[c], 105, 161, 175, 77, 246
131	1'-butyloctylbenzene	91[c], 105, 147, 189, 77, 246
132	1'-propylheptadecylbenzene	91[c], 133, 105, 77, 315, 358
136	1'-propylnonylbenzene	91[c], 133, 105, 203, 77, 246
159	1'-butylnonylbenzene	91[c], 147, 105, 77, 203, 260
163	1'-isopropylheptadecylbenzene	91[c], 133, 105, 77, 315, 343, 358

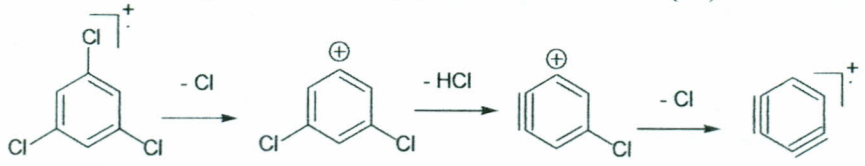
[a]mass spectrum consistent with those of authentic standards in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]Base peak. *confirmed by GC co-injection.

4.3.8 Haloaromatics

These were amongst the most abundant compounds in the most attractive foot odour. The diagnostic mass fragment ions for 1,3,5-trichlorobenzene (**77**) are shown in scheme 17. Characteristic group of peaks observed at *m/z* 180, 182; 145, 147; and 109, 111 were

indicative of isotopic effects of Cl atom (Crews *et al.*, 1985; Kemp, 1991). This was observed in all the chlorobenzene compounds identified in the odour.

Scheme 17: Mass fragment ions of 1,3,5-trichlorobenzene (77)



77

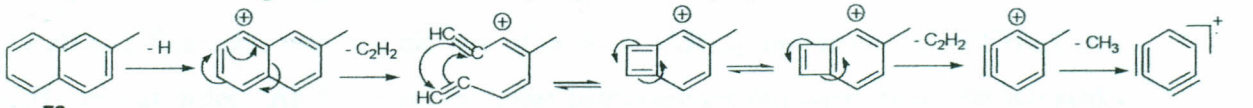
m/z 180, 182 (100, 95%) *m/z* 145, 147 (40, 35%) *m/z* 109, 111 (40, 15%) *m/z* 74 (38%)

The fragmentation pattern and mass fragment ions of 1,2,3-trichlorobenzene and 1,2,4-trichlorobenzene were very close to that of 1,3,5-trichloro isomer except for their base peaks and relative abundance. For 1,2,3-trichlorobenzene the peak intensities: *m/z* 180 (90%), 145 (40%), 109 (58%), 74 (100%) and 50 (40%), were observed for the above mass fragment ions. 1,2,4-Trichlorobenzene showed mass fragment ions of the following peak intensities: *m/z* 180 (30%), 145 (50%), 109 (30%), 84 (40%) and 74 (100%). 1,2,3,4-Tetrachlorobenzene and its 1,2,4,5-tetrachloro isomer also had similar pattern of fragmention but with the molecular fragment ions at *m/z* 216 (100%) as the base peaks.

4.3.9 Naphthalenes

Naphthalene and all alkylated naphthalene derivatives went through a fragmentation pattern in which the base peak was always either the molecular ion peak M^+ or $[M+CH_3]$. The other diagnostic peaks were *m/z* 51, 63, 77, 115, 128 and 141. Schemes 18 and 19 represent the fragmentation pattern of 2-methylnaphthalene (**78**) and 1-isopropylnaphthalene (**79**), respectively (Crews *et al.*, 1985).

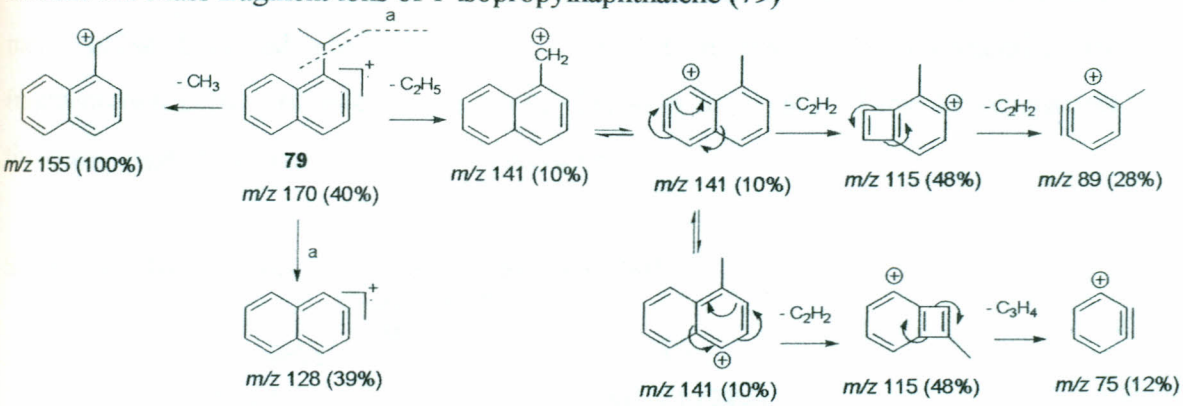
Scheme 18: Mass fragment ions of 2-methylnaphthalene (**78**)



78

m/z 142 (100%) *m/z* 141 (80%) *m/z* 115 (100%) *m/z* 115 (100%) *m/z* 89 (28%) *m/z* 74 (26%)

Scheme 19: Mass fragment ions of 1-isopropylnaphthalene (79)



The mass fragment ions of the naphthalene derivatives are summarized in table 16.

Table 16: Mass spectral ions of naphthalenes

Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
23	naphthalene	128[c], 102, 63, 74, 63, 51
56	2-methylnaphthalene	142[c], 115[c], 141, 89, 74
57	2,6-dimethylnaphthalene	156[c], 141, 115, 128, 63, 77, 51
65	2-ethylnaphthalene	141[c], 156, 115, 128, 63, 77, 51
66	1,3-dimethylnaphthalene	141[c], 156, 115, 128, 63, 51, 77
71	1,4-dimethylnaphthalene	141[c], 156, 115, 128, 63, 77, 51
81	1-isopropylnaphthalene	155[c], 170, 128, 115, 63, 51, 77
85	1,4,6-trimethylnaphthalene	155[c], 170, 128, 115, 63, 51, 77

[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]base peak.

4.3.10 Long chain hydrocarbons

These formed the largest number of compounds in the human foot odour. Their fragmentation patterns in most cases did not reveal the molecular ion peaks. That was usual for higher *n*-alkanes as the intensity of their molecular ion peaks are expected to fall off rapidly with increasing chain length (Kemp, 1991). The most characteristic feature of the alkanes fragments were the clusters of peaks at C_nH_{2n+1} amu from loss of CH_3, C_2H_5, C_3H_7, in that order. All the saturated linear hydrocarbons displayed molecular ion peaks accompanied by a set of fragment ion peaks spaced 14 amu apart. The fragment ions of all the saturated linear hydrocarbons at m/z 43 or 57 were often intense. The alkyl fragment ions from $C_4H_9^+$ up of these linear saturated hydrocarbons are known to undergo

Wagner-Meerwein rearrangement in the ionization chamber of mass spectrometer to form more stable branched chain forms as illustrated in scheme 20 representing the fragmentation pattern of *n*-tetradecane (**80**) (Crews *et al.*, 1985). Table 17 summarizes the mass fragment ions of the linear hydrocarbons.

Scheme 20: Mass fragment ions of *n*-tetradecane (**80**)

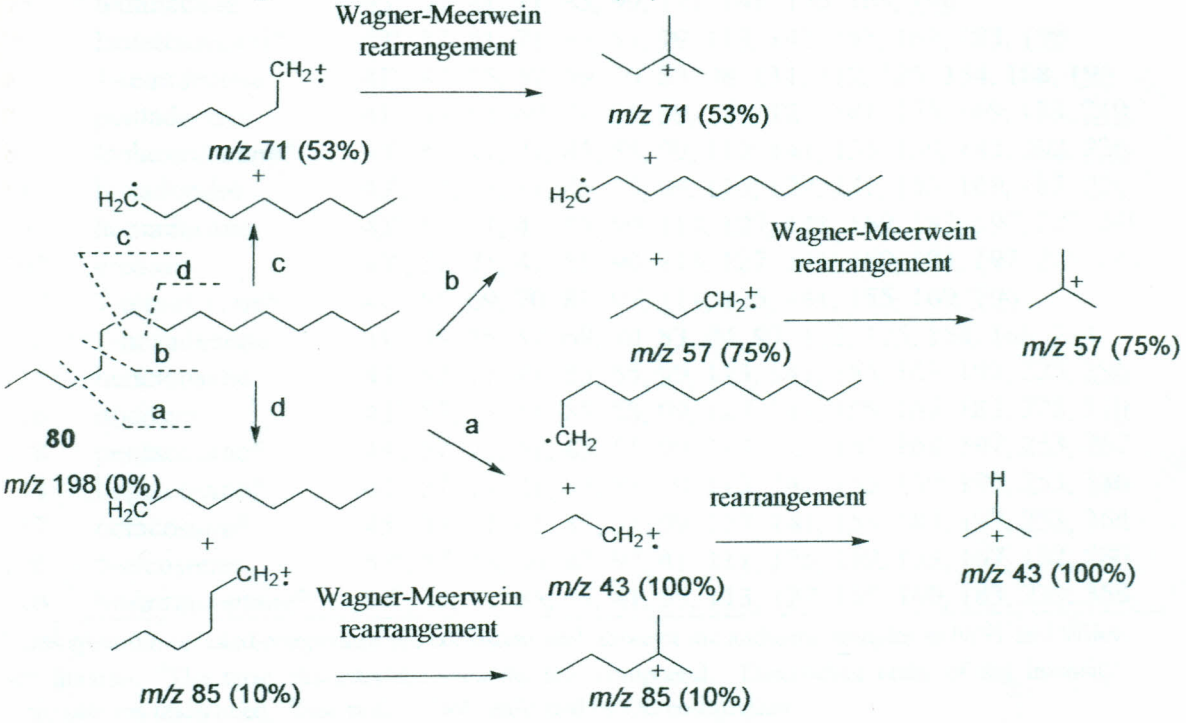


Table 17: Mass spectral ions of linear hydrocarbons

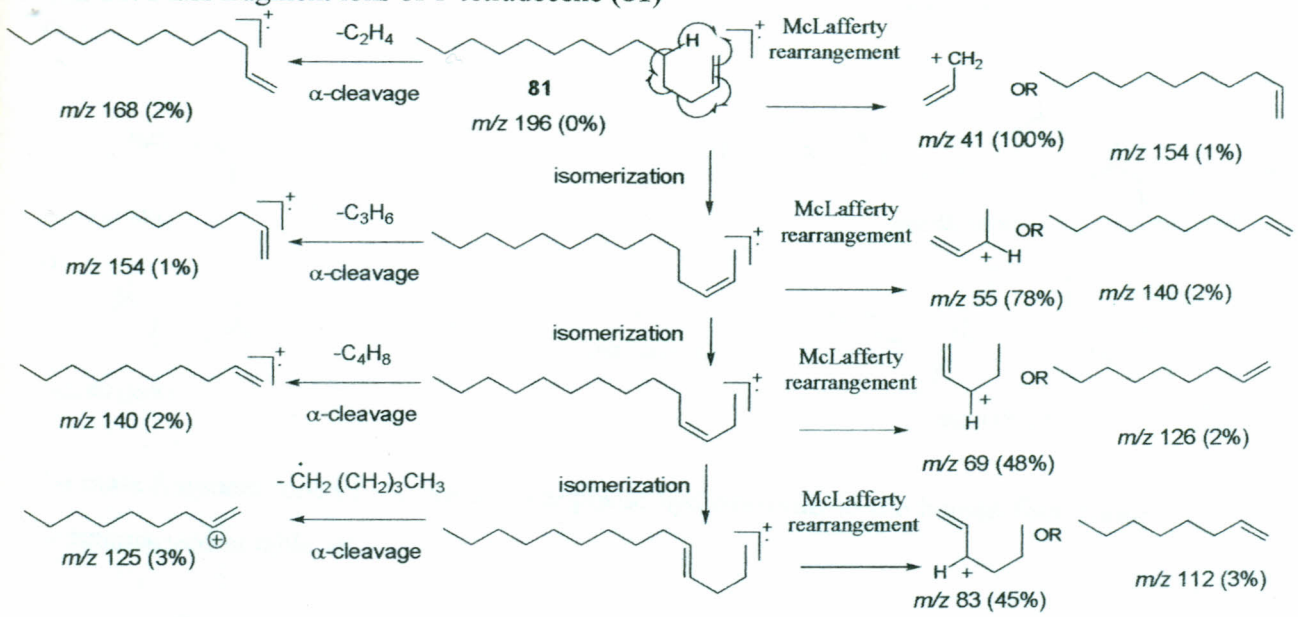
Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
19	decane	43[c], 57, 41, 71, 55, 85, 99, 126, 142
20	undecane	43[c], 57, 41, 71, 55, 85, 99, 156
36	dodecane	43[c], 57, 41, 71, 85, 99, 127, 141, 170
58	tridecane	43[c], 57, 41, 71, 85, 99, 127, 141, 155, 169, 184
72	1-undecene	41[c], 43, 55, 56, 57, 69, 83, 97, 111, 112, 126, 140, 154
75	tetradecane	43[c], 57, 41, 71, 85, 99, 127, 141, 155, 169, 198
76	Isotetradecane*	43[c], 57, 41, 71, 85, 55, 99, 113, 141, 155, 169, 183, 198
83	1-tetradecene	41[c], 43, 55, 57, 69, 70, 83, 98, 111, 112, 125, 154, 168, 196
86	pentadecane	41[c], 43, 55, 69, 71, 85, 99, 111, 127, 141, 155, 169, 183, 210
87	Isohexadecane*	43[c], 57, 41, 71, 85, 55, 99, 113, 141, 155, 169, 183, 202, 226
88	hexadecane	43[c], 57, 41, 71, 85, 55, 99, 113, 127, 141, 155, 169, 183, 226
89	heptadecane	43[c], 57, 71, 41, 55, 99, 113, 127, 141, 169, 183, 197, 227, 240
107	eicosane	43[c], 57, 71, 41, 55, 99, 113, 127, 141, 169, 183, 197, 225, 282
117	7-tetradecene*	41[c], 55, 69, 70, 83, 97, 111, 125, 141, 155, 169, 196
122	1-hexadecene	41[c], 43, 55, 57, 69, 70, 83, 84, 97, 112, 125, 154, 168, 224
125	heneicosane	43[c], 57, 71, 41, 85, 55, 99, 113, 141, 155, 169, 197, 225, 296
126	docosane	43[c], 57, 71, 41, 85, 55, 99, 127, 141, 155, 169, 183, 225, 310
128	pentacosane*	43[c], 57, 71, 41, 85, 55, 99, 113, 127, 141, 169, 197, 253, 267
134	heptacosane*	43[c], 57, 71, 41, 85, 55, 99, 113, 141, 155, 169, 197, 253, 336
137	octacosane*	43[c], 57, 71, 41, 85, 55, 99, 127, 141, 155, 183, 197, 253, 364
198	5-eicosene*	55[c], 57, 43, 69, 83, 97, 41, 111, 125, 139, 153, 167, 182, 280
246	hexatriacontane*	57[c], 43, 71, 85, 55, 41, 99, 113, 127, 155, 169, 183, 239, 358

[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]base peak. *Not confirmed by GC co-injection.

Linear unsaturated hydrocarbons showed prominent fragment ion peaks at C_nH_{2n-1} amu. The most intense peaks were frequently at *m/z* 41, 55, or 69, in that order as shown in scheme 21 for 1-tetradecene (**81**) and table 16. Such fragmentation patterns occurred by simple heterolysis of the parent ion producing species of general structure $[C{=}C\text{-}C]^+$ (Crews *et al.*, 1985), with an intense peak at *m/z* 41, $[H_2C{=}CH\text{-}CH_2]^+$ observed for most of the alkenes except a few, which showed a base peak at *m/z* 55, $[H_2C{=}C(CH_3)\text{-}CH_2]^+$ (Table 16). McLafferty rearrangements were observed for the alkenes with a sp^3 H at a γ-carbon in relation to the vinyl carbon (Scheme 21). The linear alkene ions also went through allylic fragmentation by α-cleavage. In order to rationalize some of the fragment ions observed, there was a possibility of the parent alkenes undergoing isomerization

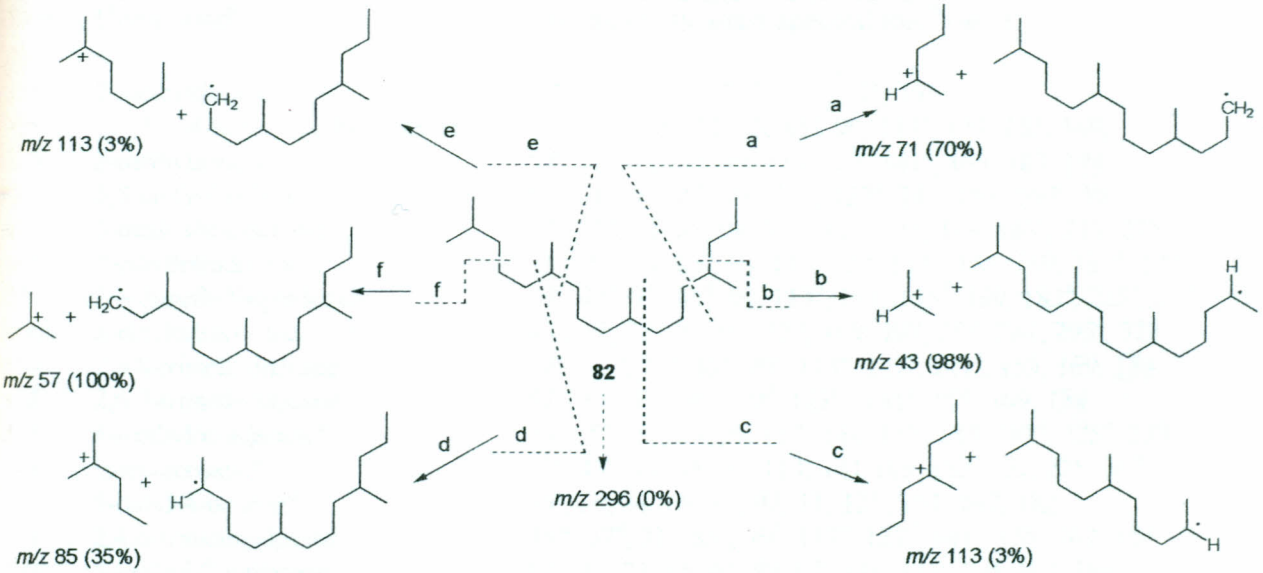
through migration of the double bond followed by either α-cleavage and/ or McLafferty rearrangement (Scheme 21).

Scheme 21: Mass fragment ions of 1-tetradecene (**81**)



Like the linear alkanes, branched ones fragmented by statistically controlled loss of C_nH_{2n+1} arrays, which could be seen in the intensities of the low molecular weight ions at *m/z* 29, 43, 57, 71 and 85. In addition to these peaks, branched alkanes showed enhanced fragmentation at the branching points, where the largest group attached to the branching point got preferentially expelled as a radical. Scheme 22 illustrates the fragmentation pattern of 2,6,10,14-tetramethylheptadecane (**82**).

Scheme 22: Mass fragment ions of 2,6,10,14-tetramethylheptadecane **(82)**



The mass fragment ions of the branched aliphatic hydrocarbons in the human foot odour are summarized in table 18.

Table 18: Mass spectral ions of branched aliphatic hydrocarbons

Peak No.	Compound[a]	Characteristic mass spectral ions[b] (*m/z*)
118	2-methyldecane	57[cd], 71, 85, 55, 99[d], 113[d], 141, 156,
128	2,6,10,14-tetramethylheptadecane*	57[cd], 43[d], 71[d], 41, 55, 85[d], 99, 113[d], 127, 155, 169,
129	3-methyltridecane	57[cd], 43, 71, 85, 99, 113, 127, 141[d], 169, 183, 198
142	5,8-diethyldodecane*	57[cd], 43, 71, 85, 99[d], 113, 127[d], 141, 155, 169[d], 197
143	3-methylhexadecane	57[cd], 43, 71, 85, 99, 113, 127, 141, 169, 183[d], 211, 225
145	4-ethyltetradecane	43[cd], 57, 71, 85[d], 99, 113, 127, 141[d], 155, 169, 183[d], 197
147	2,6-dimethylheptadecane	57[c], 43[d], 71, 85[d], 99, 113[d], 141, 155[d], 169, 183[d], 225[d], 253
166	3-ethyltetracosane	43[c], 57, 71[d], 85, 99, 127, 169, 197, 253, 281, 295[d], 323
169	2,6,8-trimethyldecane	57[cd], 43[d], 71[d], 85[d], 99[d], 113[d], 127[d], 141[d], 155, 169, 184
173	2,6,7-trimethyldecane	57[c], 43[d], 71[d], 85[d], 99[d], 113[d], 141[d], 155, 169, 184
175	4-methyloctadecane*	43[cd], 57, 71, 85, 99, 113, 141, 155, 183, 197[d], 225[d], 239
178	Isooctacosane*	57[c], 43[d], 71, 85, 99, 113, 141,169, 183, 197, 225, 267
189	5-butyl-4-nonene*	55[c], 69, 41, 69, 83, 97, 11, 125, 153, 167, 182
194	2,4,6-trimethyldecane	43[cd], 57[d], 71[d], 85[d], 99[d], 113[d], 127[d], 141[d], 155, 169, 184
197	5-methyl-2-undecene	57[c], 43, 71, 55, 41, 83, 97, 111, 125, 139, 168, 169
205	3-ethyl-5-(2-ethylbutyl)octadecane*	43[c], 57, 71[d], 85[d], 99, 141, 169, 183[d], 225, 281[d], 295[d]
209	9-cyclohexyleicosane*	57[c], 55[c], 43, 83, 71, 97[d], 111, 139, 153, 209, 251[d], 281
224	6-ethyl-2-methyldecane	57[cd], 43[d], 71, 85[d], 99[d], 113, 127[d], 141[d], 155
225	3,5,24-trimethyltetracontane*	57[cd], 43, 71[d], 85, 99[d], 113, 141, 155, 206, 238
227	9-(2-cyclohexylethyl)heptadecane*	43[c], 57, 71, 97, 111, 125[d], 139, 153, 167, 181, 237[d]
243	7-hexyleicosane*	43[c], 57, 85[d], 97, 111, 125, 139, 181, 281[d]

[a]mass spectrum of listed compounds are consistent with those of the authentic samples in NIST and Wiley MS libraries. [b]The most characteristic ions for that compound. Descending order of ion intensity. Molecular ion underlined. [c]base peak. *Not confirmed by GC co-injection. [d]Fragment ion at branching point.

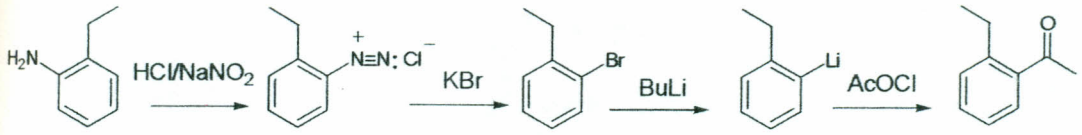
4.4 Synthesis of some compounds in human feet odour

Some compounds present in the human feet odour were not commercially available and had to be synthesized to confirm the structures deduced from GC-MS.

4.4.1 2-Ethylacetophenone (85)

From GC-EAG and GC-MS of the foot odour, this compound was one of the suspected candidate attractants of *An. gambiae* but could not be obtained commercially. It was therefore necessary to avail it through synthesis using the commercially available precursors; 2-ethylaniline **(83)** and 1-bromo-2-ethylbenzene **(84)** (Scheme 23).

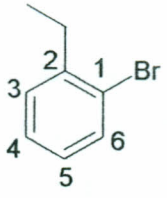
Scheme 23: The synthetic route to 2-ethylacetophenone (**85**)



83 **84** **85**

2-Ethylaniline (**83**) was converted to 1-bromo-2-ethylbenzene (**84**), in 58% yield through diazotization (Dadley & Dadley, 1996; Solomons, 1990; Vogel, 1987) and Sandmeyer reactions (Dadley & Dadley, 1996; Solomons, 1990; Vogel, 1987). 1-Bromo-2-ethylbenzene (**84**) was obtained as a colourless liquid after purification by column chromatography and identified by NMR and EIMS analyses.

The ^{1}H NMR revealed three distinct signal regions: δ 7.45 – 7.5 (m, 4H), 2.80 (q, 2H) and 1.27 (t, 3H). The signal at δ 7.045 – 7.55 was due to aromatic protons. The quartet, at δ 2.80 and triplet at 1.27 were assigned to methylene and methyl groups of side chain, respectively. The broad peak at δ 3.4 – 3.7, which is conspicuous in the ^{1}H NMR of 2-ethylaniline (Appendices) was not observed in 1-bromo-2-ethylbenzene. The ^{13}C NMR spectrum revealed 8 signals at δ 124.3, 127.3, 127.5, 129.5, 132.7, 143.2, 29.4 and 14.2. The six signals down field were due to the benzene ring skeleton and the two up field due to the alkyl side chain. The protonated carbons resonated at δ 129.5, 127.3, 127.5 and 132.7 and were assigned to C-3, C-4, C-5 and C-6, respectively (Table 19).



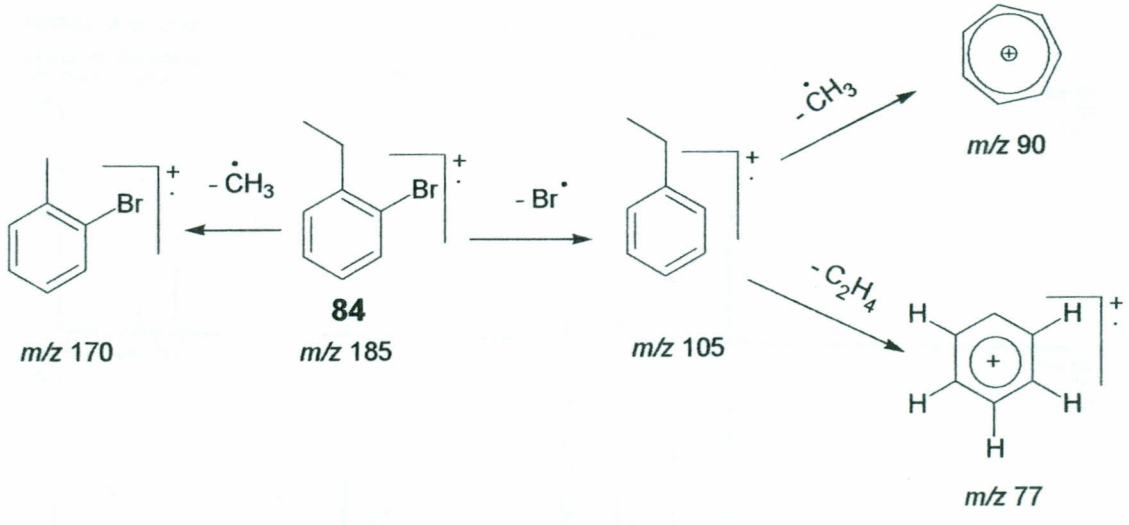
84

Table 19: Observed and predicted ^{13}C NMR data for1-bromo-2-ethylbenzne

Position	Base value	substituent		$\delta_{predicted}$	$\delta_{observed}$
		Br	C_2H_5		
1	128	-5.8	-0.5	121.7	124.28
2	128	+3.2	+15.6	146.8	143.24
3	128	+1.6	-0.5	129.1	129.49
4	128	-1.6	0.0	126.4	127.33
5	128	+1.6	-2.7	126.9	127.48
6	128	+3.2	0.0	131.2	132.65

The observed δ values are very close to the predicted values (Kemp, 1991; Crews *et al.*, 1985) as summarized in table 19. The EIMS further confirmed the identity of the product, which gave molecular ion peaks at *m/z* 183 and185 in the ratio intensity 1:1 and a base peak at *m/z* 105 [M-Br] from the molecular ion. The other fragment ions are summarized in scheme 24.

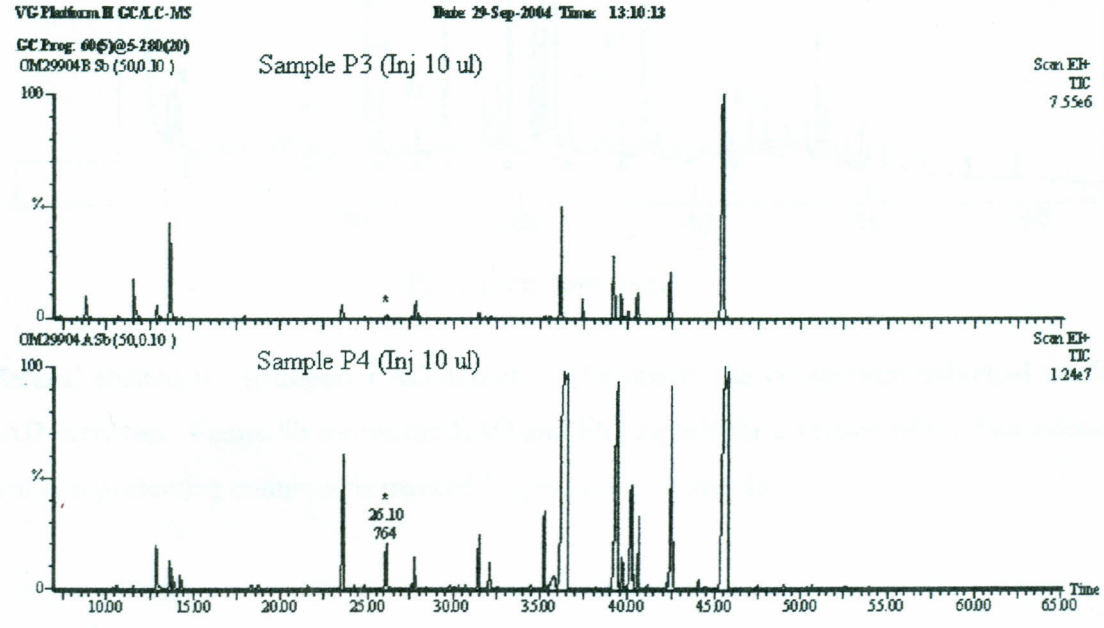
Scheme 24: Mass fragment ions of 1-bromo-2-ethylbenzene (**84**)



1-Bromo-2-ethylbenzene was converted into the final product in a single pot two step reaction, which was done in two experimental procedures. The first attempt was made without molecular sieves in the reaction flask leading to product labeled P3; while the 2nd one had molecular sieves included in the reaction flask before adding the solvent and reagents and was labeled P4. The products of the two experimental procedures were analyzed by GC and GC-MS. GC and GC-MS analysis confirmed that all the starting material had reacted and revealed the presence of the 2-ethylacetophenone (**85**) peak, at R_t 26.10 min (Fig. 7).

From the GC profiles of the two reactions (Fig. 7), the one with molecular sieves yielded more 2-ethylacetophenone than the other one without. The inclusion of molecular sieves helped to mop up moisture in the reaction flask and therefore improve the dry conditions necessary for the lithiation and acetylation reactions. However, from the R_t, the product was not the same as the electro-physiologically active one in the foot odour as confirmed by GC-coinjection of the product with foot odour sample. Moreover it showed no EAG activity when assayed with mosquito antennae. Consequently, there was no need of further purification of the 2-ethylacetophenone.

Figure 7: Comparison of the GC profiles of the conversion products of 1-bromo-2-ethylbenzene to 2-ethylacetophenone by 2 experimental procedures



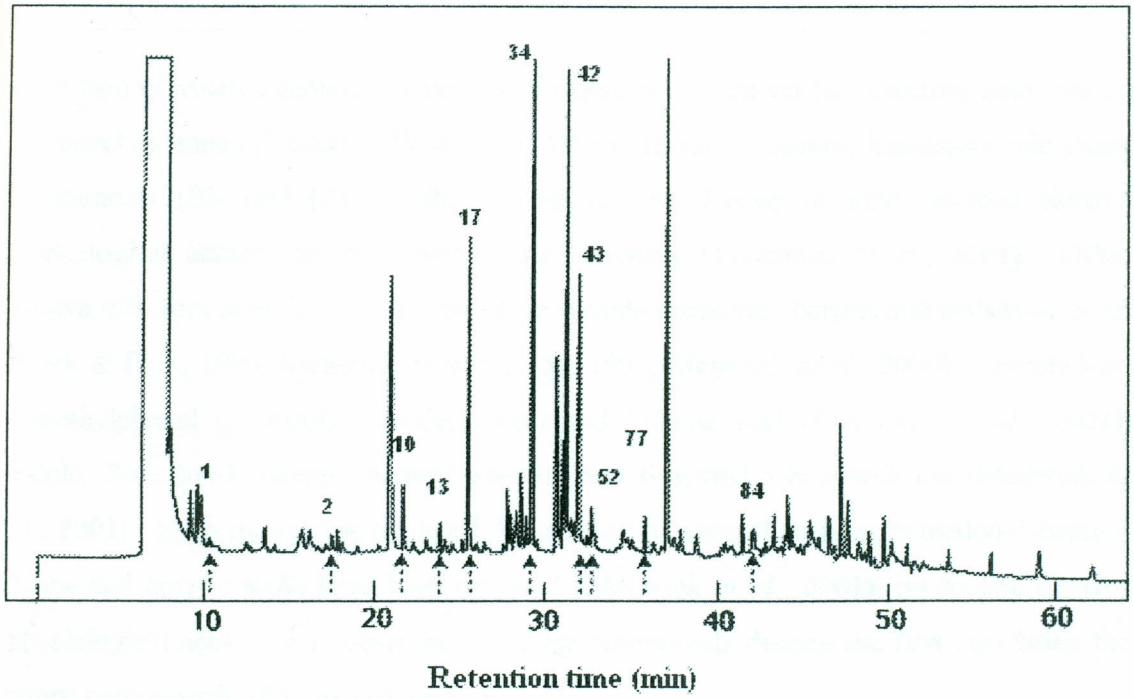
CHAPTER FIVE

ELECTRO-PHYSIOLOGICAL AND BEHAVIOURAL BIO-ASSAYS

5.1 Electro-physiologically active components of foot odour

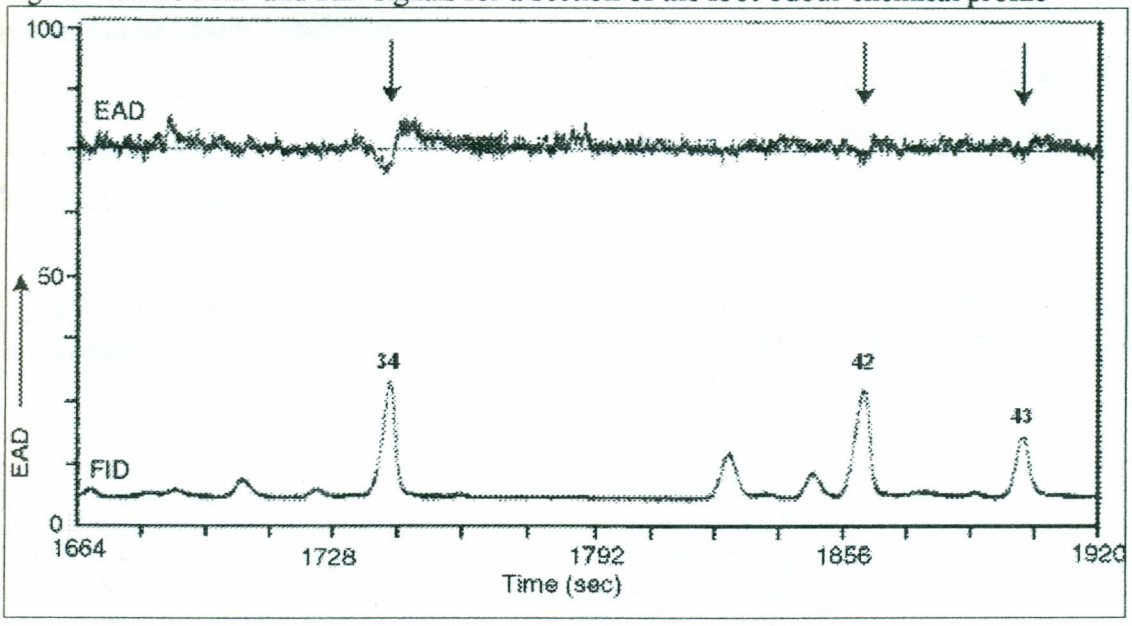
The on line GC-EAD analysis of the human foot volatiles revealed 11 EAD active components (Fig. 8a). These included 2 carboxylic acids (isobutyric peak (1) and isovaleric acids (2)); 6 aliphatic aldehydes (*n*-octanal (10), nonanal (17), decanal (34), undecanal (52), dodecanal (77) and tridecanal (84); 2 ketones (4-ethylacetophenone (42) and 4-ethoxyacetophenone (43) and 1 phenol (2-methylphenol (13).

Figure 8a: GC profile of human foot odour showing EAD-active components of the odour



Decanal elicited the strongest EAD activity. The rest of the compounds exhibited weak EAD activities. Figure 8b shows the EAD and FID signals for a section of the foot odour profile representing compounds marked by peaks 34, 42 and 43.

Figure 8b: The EAD and FID signals for a section of the foot odour chemical profile



Detection of volatile semiochemicals by mosquitoes occurs via the olfactory neurones on the insect antennae (Pickett & Woodcock, 1996). In earlier studies, human-specific sweat components (E)- and (Z)-3-methyl-2-hexenoic and 7-octenoic acids elicited electro-physiological activity on *An. gambiae s.s.* antennae (Costantini *et al.*, 2001). Other known olfactory stimulants of *An. gambiae* include propionic, butyric and isobutyric acids (Cork & Park, 1996; Meijerink & Van Loon, 1999; Meijerink *et al.*, 2000); 1-octen-3-ol, 4-methylphenol (*p*-cresol), isovaleric acid and *L*-lactic acid (Costantini *et al.*, 2001); indole, 3-methyl-1-butanol, geranyl acetone and 6-methyl-5-hepten-2-one (Meijerink *et al.*, 2001). Even though the off line EAD activity of geranyl acetone, 6-methyl-5-hepten-2-one and butyric acids have been reported (Meijerink *et al.*, 2001), no on line electro-physiological activity was observed for these compounds despite the first two being the major components of human foot odour.

Of the 11 EAD active components of the human foot odour, two (isovaleric and isobutyric acids) have been previously reported to exhibit electro-physiological activity (Costantini *et al.*, 2001; Meijerink & Van Loon, 1999). The electro-physiological activity of the remaining nine compounds is being reported for the first time in this work.

5.2 Assay of behaviourally active components of the human foot odour under laboratory conditions

The 11 EAG active components were assayed both individually and as blends in the convective bioassay chamber under laboratory conditions. The results of the assay of individual components are presented in table 20.

Table 20: Transformed mean number* of mosquitoes responding to test compound

Compound	Mean number responding ± SE
1	-2.5 ± 0.681^{b}
2	-1.875 ± 0.895^{b}
10	0.125 ± 0.895^{a}
13	0.500 ± 0.732^{a}
17	-6.50 ± 0.627^{c}
34	0.375 ± 0.652^{a}
42	-5.50 ± 0.598^{c}
43	-4.875 ± 0.743^{c}
52	1.250 ± 0.453^{a}
77	1.875 ± 0.479^{a}
84	1.875 ± 0.479^{a}

*Based on 8 replicates of number of mosquitoes responding to the test chemical. Means with the same letter are not significantly different (LSD test, $p<0.0001$).

Generally, the landing or resting response of the mosquitoes near to or on the test source was very low. The low mosquito landing or resting response indicated that the EAG active components, when assayed individually, elicited very low behavioural response in the laboratory reared *An. gambiae s.s.* The negative mean values for landing or resting mosquitoes due to the components 1, 2, 17, 42 and 43 showed that their respective controls had relatively higher responses. However, it is interesting to note that the landing or resting responses due to these components were significantly lower ($p=0.0001$) than those from the components 10, 13, 34, 52, 77 and 84 (Table 20). From these results, the role of each of these compounds in the attraction of *An. gambiae* mosquitoes to human foot was not very clear due to the low mosquito landing or resting responses recorded with the tested samples. However, when these samples were subjected to subtraction assays in which one component was omitted and the remaining 10 assayed as a blend,

higher mosquito landing or resting responses were recorded under the same experimental conditions (Table 21).

Table 21: Transformed mean number* of mosquitoes responding to blend components

Blend†	Mean number responding ± SE
0	7.125 ± 0.666^{c}
1	1.625 ± 0.263^{f}
2	2.000 ± 0.423^{f}
10	1.625 ± 0.532^{f}
13	2.750 ± 0.313^{ef}
17	4.500 ± 0.654^{de}
34	3.875 ± 0.639^{def}
42	13.25 ± 1.359^{b}
43	4.750 ± 0.453^{de}
52	3.250 ± 0.453^{ef}
77	3.375 ± 0.461^{ef}
84	2.625 ± 0.324^{ef}
1,2	5.750 ± 1.065^{cd}
42,43	17.87 ± 1.540^{a}
42,43,52	16.87 ± 1.315^{a}

*Based on 8 replicates on number of mosquitoes responding to the tested blend. †Peak number of compound subtracted from the initial blend, A0 (contained all the 11 EAD active compounds). Means with the same letter are not significantly different (LSD test, $p<0.0001$).

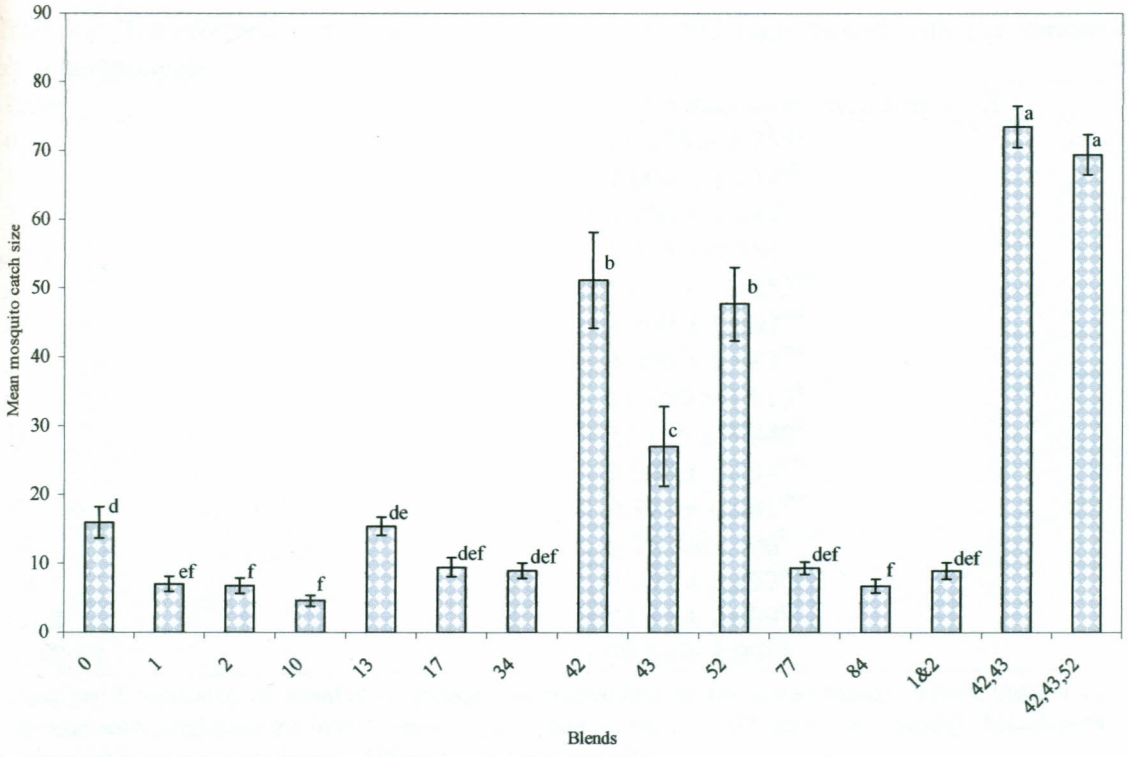
The blend 0, which constituted all the 11 EAD active components recorded higher number of mosquito landing or resting responses than any of the individual components (Table 20). Thus the 11 EAD active compounds when assayed as a blend, elicited much better behavioural response in the laboratory reared *An. gambiae* mosquitoes than when assayed singly. Blends 1, 2 and 10 resulted in reduced numbers of mosquitoes landing or resting near or on the test source compared to the blend 0. Blend 42 gave almost double the number of mosquitoes landing or resting near or on the test source as compared to the result obtained with the blend 0. Activity of blend 42 was significantly ($p=0.0001$) different from those of blends 42,43 and 42,43,52 (Table 21). The subtraction bioassay results indicated that compound 42 is not a kairomonal component of the human foot odour, while compounds 1, 2, 10, 13, 17, 34, 52, 77 and 84 appeared to be crucial attractive components of the human foot odour. The kairomonal and allomonal

behavioural activities of these compounds are being reported for the first time in this work.

5.3 Assay of behaviourally active components of the human foot odour under semi-field conditions

In the subtraction bioassays done under semi-field conditions, the largest drop in activity of the blends occurred in blend 10, which did not contain octanal (Fig. 9). The mean mosquito catch sizes for blends 1, 2, 10 and 84 were significantly lower ($p=0.0001$) than that for blend 0, which had all the 11 EAD active components. On the other hand, omission of 4-ethylacetophenone, 4-ethoxyacetophenone and undecanal in blends 42, 43 and 52 significantly increased the catch sizes of the blends compared to blend 0. There was no significant difference between the catch sizes due to blends 13 and 0. The results suggest that whereas isobutyric acid, isovaleric acid, octanal and tridecanal could be crucial kairomonal components of the human foot odour, 4-ethylacetophenone, 4-ethoxyacetophenone, undecanal and 2-methylcresol are not important components.

Figure 9: Transformed mean mosquito catches* in CFG traps baited with synthetic blends of EAD active foot odour components.



*Based on 8 replicates of mosquito catches per blend. Blends not sharing the same letters are significantly different (p = 0.0001).

The allomonal activity of 4-ethylacetophenone (42), 4-ethoxyacetophenone (43) and undecanal (52) was further confirmed in the bioassay of blends in which the two acetophenones (42,43) and both the acetophenones and undecanal (42,43,52) were omitted, respectively. The mean catches due to the two blends (42,43 and 42,43,52) were significantly higher (p=0.0001) than those obtained with the blends 42, 43, 52 and 0 (Table 22, Fig. 9).

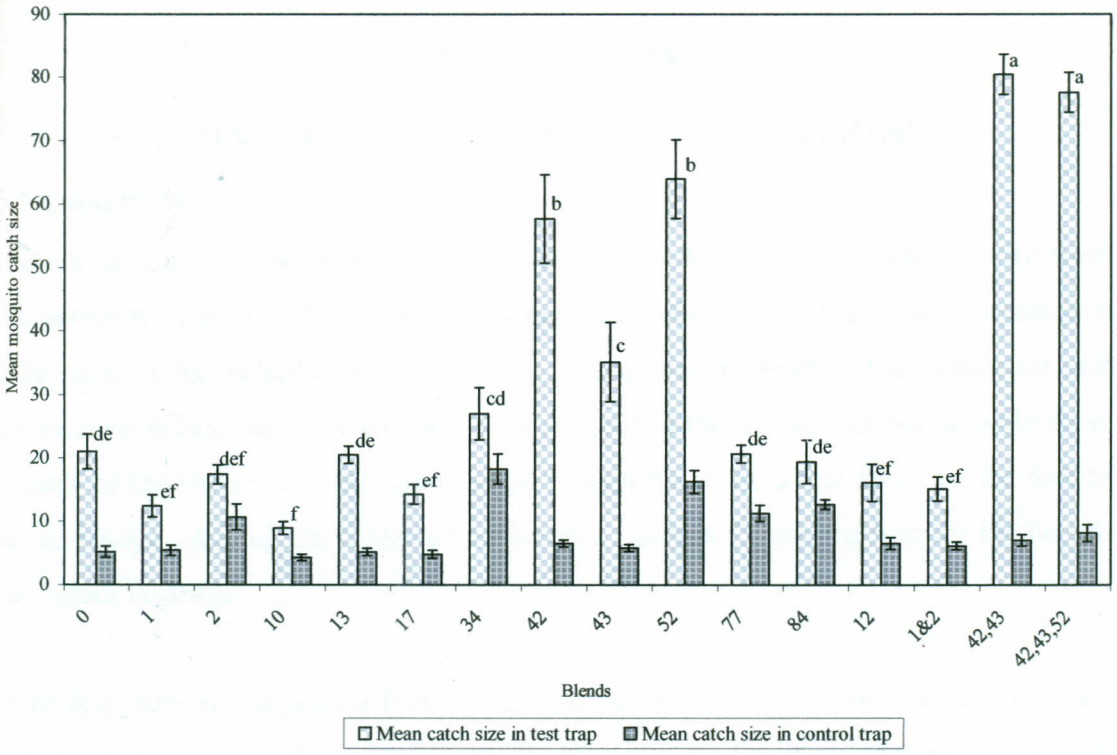
Table 22: Transformed mean mosquito catches* of CFG traps baited with the various blend components

Blend†	Mean mosquito catch size ± SE
0	15.875 ± 2.255^{d}
1	7.000 ± 1.134^{ef}
2	6.750 ± 1.146^{f}
10	4.625 ± 0.754^{f}
13	15.375 ± 1.252^{de}
17	9.500 ± 1.402^{def}
34	9.000 ± 1.982^{def}
42	51.250 ± 7.015^{b}
43	27.125 ± 5.848^{c}
52	9.500 ± 1.134^{def}
77	9.375 ± 0.981^{def}
84	6.750 ± 3.406^{f}
1,2	9.000 ± 2.252^{def}
42,43	73.50 ± 2.909^{a}
52,42,43	69.50 ± 2.994^{a}

*Based on 8 replicates on number of mosquitoes responding to the tested blend. †Peak number of compound subtracted from the initial blend, 0 (contained all the 11 EAD active compounds). Means with the same letter are not significantly different (LSD test, p=0.0001).

Comparison of the raw data of the mean mosquito catches in control and test traps are presented in figure 10. These results indicated that there was a significant difference (p=0.0001) in the number of mean mosquito catches obtained with the most attractive test blends compared to control. The use of kairomonal blend identified from the foot odour together with known mosquito repellents could reduce man-vector contact in a pull–push control strategy. Moreover, the attractive blends could be exploited to lure the vectors into a trap, where they can pick entomo-pathogens, die of hunger and/or desiccation. The results presented in this work underscore the relevance of subtraction assays in conferring activity to the components of the human foot odour blend.

Figure 10: Comparison of mean mosquito catches* in test and control CFG traps



*Based on 8 replicates of mosquito catches per blend. Blends not sharing the same letters are significantly different ($p = 0.0001$).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The attractiveness of *An. gambiae* s.s. mosquitoes to the human foot odour varied from one person to another. This variation is brought about by both qualitative and quantitative differences in the volatiles emanating from feet of human hosts. The qualitative and quantitative differences in the chemical compositions of the human foot odour might be as a result of the variation in the composition of skin micro flora and fauna on the feet of human beings. Among the evaluated adsorbents, porapak Q was the best for the human foot odour volatiles.

More than 60% of the human foot odour volatiles were carbonyl compounds. Ketones and esters formed the largest composition of the carbonyls from the most and least attractive feet, respectively. A combination of electrophysiological, behavioural and chemical studies led to the identification of key components of human foot odour responsible for mosquito attraction. It is noted that the use of GC-EAD reduced the behaviourally important compounds from about 300 to 11. There were 11 EAG active compounds in the volatiles of most and least attractive human feet. Of the 11 compounds, kairomonal components included C_4 and C_5 short chain carboxylic acids and C_8-C_{13} straight chain aldehydes excluding the C_{11} one. These compounds are not effective as attractants when presented to the mosquitoes individually, but become active as a blend in the approximate proportions in which they emanate from the human foot. Some mosquito allomones from the human foot odour included undecanal, 4-ethylacetophenone and 4-ethoxyacetophenone.

The identified attractive blends from human foot odour may be used to enhance the efficacy of entomo-pathogens, improve trap catches, monitor field mosquito populations or as decoys to reduce biting rates on humans. They may also replace human landing catches that are used in many scientific experiments. The identification of allomonal blends may be useful in keeping mosquitoes away from human hosts. A combination of

the kairomonal and allomonal blends may be useful in push-pull strategy in the control of malaria.

6.2 Recommendations

- More research should be carried out on the dose-response relationship of the attractive blend from the human foot odour so as to determine the minimum dose of the blend needed to obtain the maximum possible mosquito catch size in the semi-field conditions.
- There is need to evaluate the efficacy of the kairomonal blend from the human foot odour in the field on natural mosquito populations.
- The allomonal compounds from the foot odour should be exploited to control the malaria vectors along side the kairomones in a push–pull model, where the allomones are used to drive the insects away from man, as the kairomones lure them into a trap.
- Exploitation of the kairomonal compounds in mosquito surveillance and population dynamic studies should be tried.

CHAPTER SEVEN

EXPERIMENTAL

7.1 General procedure

All the glassware used was soaked in chromic acid, cleaned thoroughly with water, rinsed with distilled water, acetone and dried in the oven at 110 °C. Solvents and other liquids reagents were obtained from Sigma-Aldrich Chemical Company (Milwaukee, WI USA) and purified by distillation where necessary. Solvents and reagents for dry reactions were all kept in bottles with molecular sieves immediately after distillation. All the glass syringes and stainless steel needles used in dry reaction were well washed and rinsed with distilled water before leaving them in the oven at 110 °C where they were only removed when needed and cooled under inert atmosphere of argon. Argon and dry ice were obtained locally from BOC and Carbacid, respectively. Polygram precoated silica gel G_{60} F_{254} plates (5 x 10 cm x 0.20 mm thickness and 20 x 20 cm x 0.25 mm thickness) with fluorescent indicator were used for analytical and preparative TLC, respectively. Silica gel G60 was used for column chromatography. NMR and MS spectra were obtained on Varian Gemini 200 at the University of Nairobi and Fission Platform 11 Mass Spectrometer (ICIPE), respectively.

7.2 Insects

An. gambiae s.s. mosquitoes that were used in the semi-field experiments were from a colony reared at ambient temperature (26-28 °C) and humidity (70-80% R.H.) at the International Centre for Insect Physiology and Ecology (ICIPE), Thomas Odhiambo Campus, on the southern shores of the Winam Gulf of Lake Victoria in Nyanza Province, Kenya (00° 25' S, 34° 13' E). The colony had been established from specimens collected in 1998 from Njage Village, 70 km from Ifakara, south-east Tanzania. Adult female mosquitoes were routinely offered human arm to feed upon. The larvae were fed on TetraMin® fish food (Tetra GmbH, Germany) three times per day. They were reared in plastic basins (50 x 16 cm diameter and height) filled to a depth of 3 cm with fresh water from Lake Victoria. The basins contained 200-250 larvae. Pupae were collected daily

and kept in mesh-covered cages (30 x 30 x 30 cm) containing 6% glucose solution in filter paper wicks. Adult females were used for the experiments when 5-8 days old and with no prior access to a blood meal.

The mosquitoes used in the laboratory assays were laboratory reared female *An. gambiae s.s.* (cultured in 1998 from specimens originally obtained from Njage, 70 km from Ifakara, south-east Tanzania). The insects were reared according to the WHO (1996) protocol at ICIPE, Nairobi, Kenya. The larvae were reared at 32-36 °C and fed on TetraMin® (Tetra GmbH, Germany). The adults were maintained on 6% glucose solution and the females fed on human blood thrice a week. Rearing temperatures and relative humidity in the adult insectaria were 26-28 °C and 70-80%, respectively.

7.3 Preparation of adsorbents

Porapak Q (80-100 mesh), activated charcoal (80-100 mesh), reverse phase octadecyl (C_{18}) bonded silica and reverse phase octyl (C_8) bonded silica both of 25-40 µm particle sizes were used as the adsorbents for the feet odour.

Filter paper (Whatman No. 1) was used to make the pockets for packing the adsorbents. The papers were first cut into small strips (10 x 3.0 cm), folded into two equal halves (5 x 3.0 cm) and then along the sides to form pockets (5 x 2 cm). One short side and the two long ones were sewn using white cotton thread on a sewing machine to form the adsorbent pockets/containers. Similar pockets were made using cream wire mesh sheets (Estal Mono, 120T mesh; Swiss Silk Company, Switzerland), but with the three folded edges stapled. The cream wire mesh pockets were used for activated charcoal, while the filter paper pockets were used for porapak Q, reverse phase C_{18} and C_8 silica adsorbents.

The paper pockets were packed with 500 mg of adsorbent (Porapak Q, C_{18} or C_8 reverse-phase silica), the open end folded twice and stapled. The filled paper pockets were enclosed in the cream mesh sheets to protect them from getting torn. In the case of the charcoal, same amount of the powder was packed in the pockets made from the cream

wire mesh sheets. The pockets containing adsorbents were cleaned in a soxhlet extractor using dichloromethane for 72 h, removed using a pair of forceps and dried in an oven for 2 days at 40 °C. Porapak Q, C_8 and C_{18} reverse-phase silica were activated under an inert atmosphere of N_2 in a GC oven at 60 °C for 5 h, while charcoal was activated at 250 °C for the same period of time.

Cotton and polyester socks were used for bulk adsorption of the feet odours. The socks were bought from the same batch, washed well with bar soap, rinsed and air dried before usage.

7.4 Foot odour collection

Odour collection was done at night when the volunteers were sleeping. This was carried out in two huts (3.2 x 2.7 x 2.4 m) within a screen house (11.5 x 7.1 x 3 m) at ICIPE (Mbita Point Field Station). Soxhlet-cleaned (dichloromethane) cotton thread was used to tie the adsorbent sachets around the phalanges of the feet of human subjects at 8.30 pm (local time), (Plate 1). The subjects were then provided with new soxhlet-cleaned (dichloromethane) white cotton and polyester socks to put on for 10-12 h while they slept. The sachets were removed at 7.00 am in the morning (local time) of the following day, packed in glass bottles (100 ml) with quick fit stoppers and stored in a cool box from where they were transferred into a freezer (-20 °C) until the desorption of the volatiles was carried out.

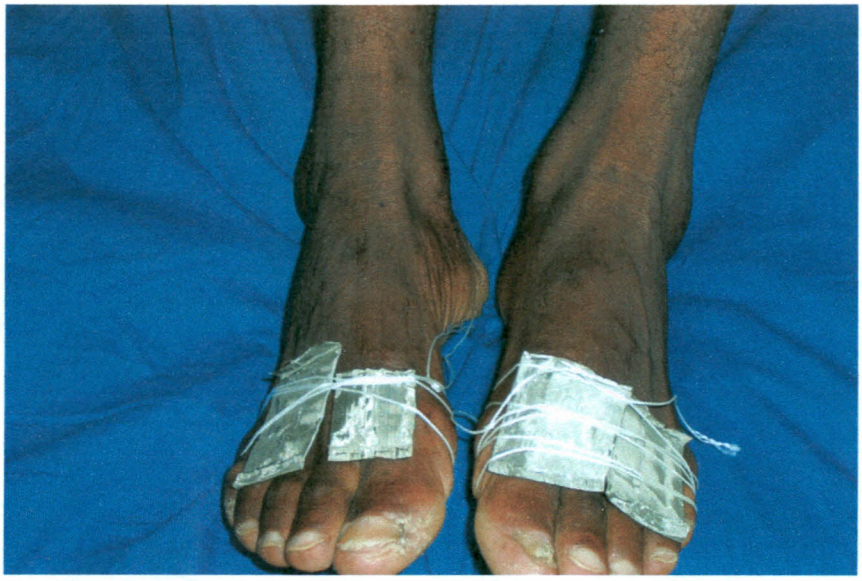


Plate 1: Adsorbent sachets on the feet of the human subject

7.5 Extraction of volatiles from the adsorbents

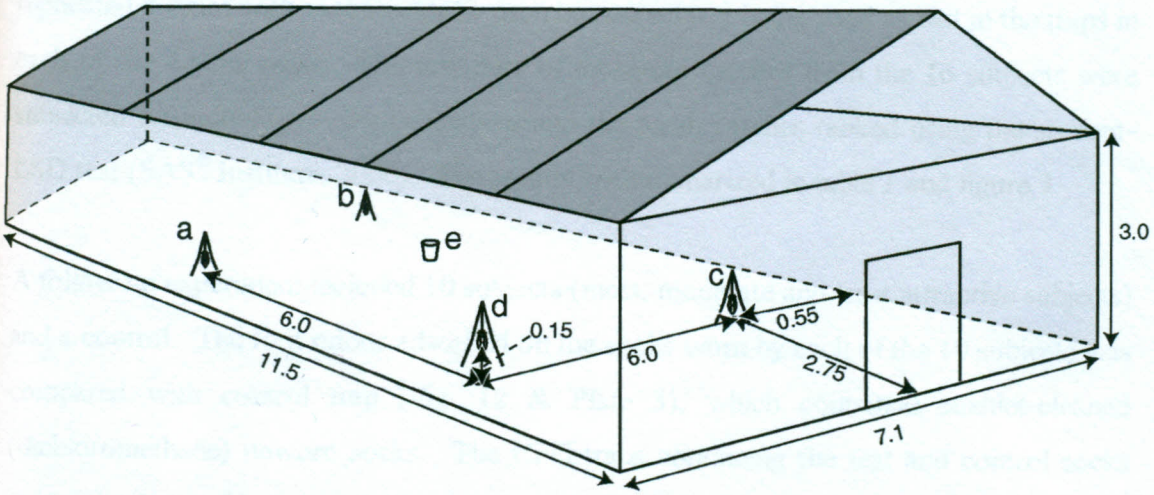
The adsorbent materials in the pockets were carefully transferred into Pasteur pipettes, eluted with 2 ml dichloromethane (99.9% HPLC grade, Aldrich Chem. Co., UK) and the eluent collected in clean (4 ml) vials under ice salt mixture (-4° C). The dichloromethane extracts were concentrated under ice salt mixture (-4 °C) using a gentle stream of white spot nitrogen (99%) (Wanzala *et al.*, 2004; Gikonyo *et al.*, 2002) and stored in the freezer until when required for bioassays and chromatographic analyses.

7.6 Ranking volunteers for attractiveness of their feet odours to *An. gambiae*

Sixteen (16) male subjects were randomly put into 4 groups and odour collections done on socks and adsorbents in the screen house described earlier. Four subjects slept in the two huts (2 per hut), while putting on cotton and polyester socks for 8 consecutive nights. A new set of socks was used in each replicate for every night. The socks worn in the night were kept in glass bottles during the day and used the following night as baits in CFG traps to catch mosquitoes in another screen house of the same dimensions as the one described earlier. The pair of socks from each subject were placed in each of the four CFG traps and used every night to trap 200 previously starved (6 h) 5 day old female *An.*

gambiae s.s, released from a cup placed at the center of the screen house and equidistant from each trap (Fig. 11 and Plate 2).

Figure 11: Sketch of the arrangement of CFG traps in the screen house



a, b, c, and d represent the 4 CFG traps at the 4 different locations with the cup (e) at equidistant point from each of the traps in the screen house. All the dimensions are in meters.

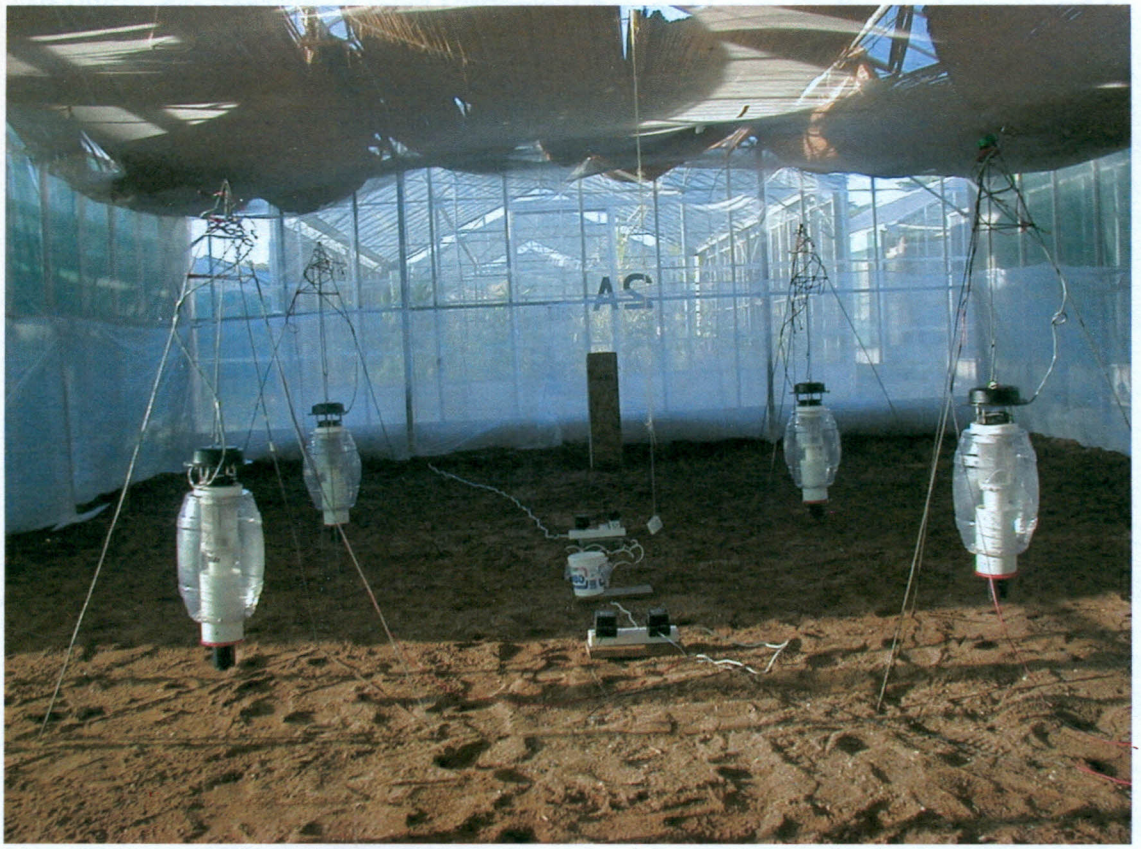
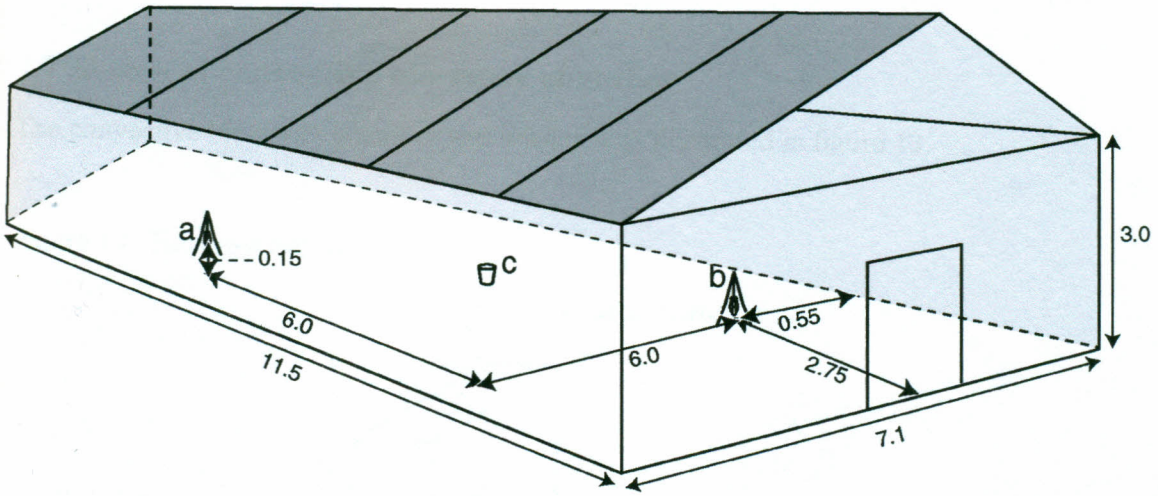


Plate 2: Photograph of the experimental layout of the CFG traps in the screen house

The CFG traps were removed from the screen house the following morning and put in the freezer for 10 min to immobilize and count the trapped mosquitoes. The experiment was replicated 8 times with socks worn by each human subject being used as bait in the traps in each of the 4 sites twice. The raw data of mosquito catches from the 16 subjects were subjected to multivariate cluster analysis and the mean catches ranked using the student-LSD test (SAS® Institute, 2000). The results are summarized in table 1 and figure 3.

A follow up experiment included 10 subjects (most, moderate and least attractive subjects) and a control. The foot odour adsorbed on the socks worn by each of the 10 subjects was compared with control trap (Fig. 12 & Plate 3), which contained soxhlet-cleaned (dichloromethane) unworn socks. The CFG traps containing the test and control socks were interchanged between the two sites A and B every two successive nights such that, 4 replicates were done at each of the sites A and B for both the test and control socks. The experiment was replicated 8 times (between 9 p.m. and 6 a.m. for 8 nights) for each of the 10 male human subjects. The number of mosquito caught by the foot odour bait from each of the 10 subjects was computed using the equation: $N = T - C$; where by N = number of catches due to foot odour, T = number of mosquitoes in test trap and C = number of mosquitoes in the control trap (Mehr *et al.*, 1985). The resulting transformed data for the mosquito caught was subjected to statistical analysis of the mean using the student-LSD test (SAS® Institute, 2000). Table 2 summarises the results.

Figure 12: Sketch of diagonal arrangement of test and control CFG traps in screen house



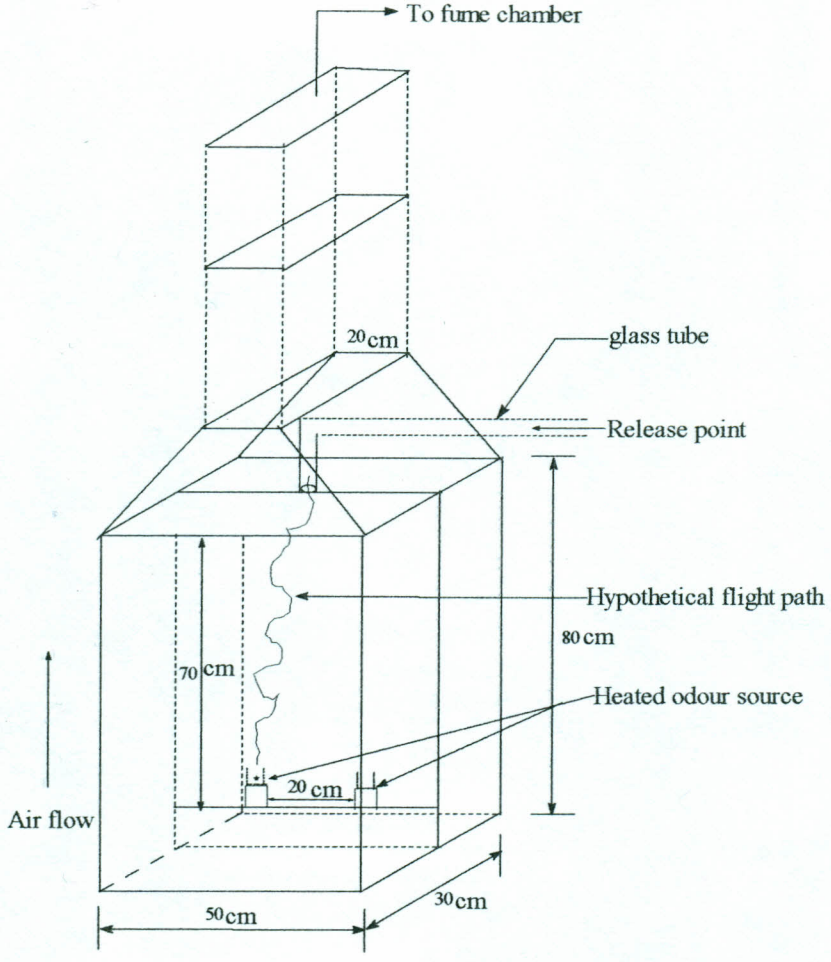
a and b represent the test and control CFG traps at the 2 different locations with the cup (c) at equidistant point from each of the traps in the screen house. All the dimensions are in meters.

Plate 3: A photograph of the diagonal arrangement of test and control CFG traps in the screen house

7.7 Sketch of convective bio-assay chamber

The convective bio-assay chamber was designed as illustrated in figure 13.

Figure 13: The bio-assay cage



The bottom and the pyramidal frustum shaped top portions were made of aluminium. The front face was made of transparent glass window (Pyrex), while the remaining sides were made of wire gauze with very small openings that could not allow the insects to escape from the chamber (Plate 4). The bio-assay cage was then connected to the fume hood. Two small metal block heaters (6 cm diameter) on the floor of the cage regulated the temperature at 38-39 °C (slightly above normal human body temperature). The heaters allowed a flow of steady upward convection current from the odour source. The whole

chamber was enclosed in an air tight jacket whose front face was made of Pyrex. Plates 4 and 5 show the bio-assay cage and the heaters, respectively.

Plate 4: Photograph of convective bio-assay chamber

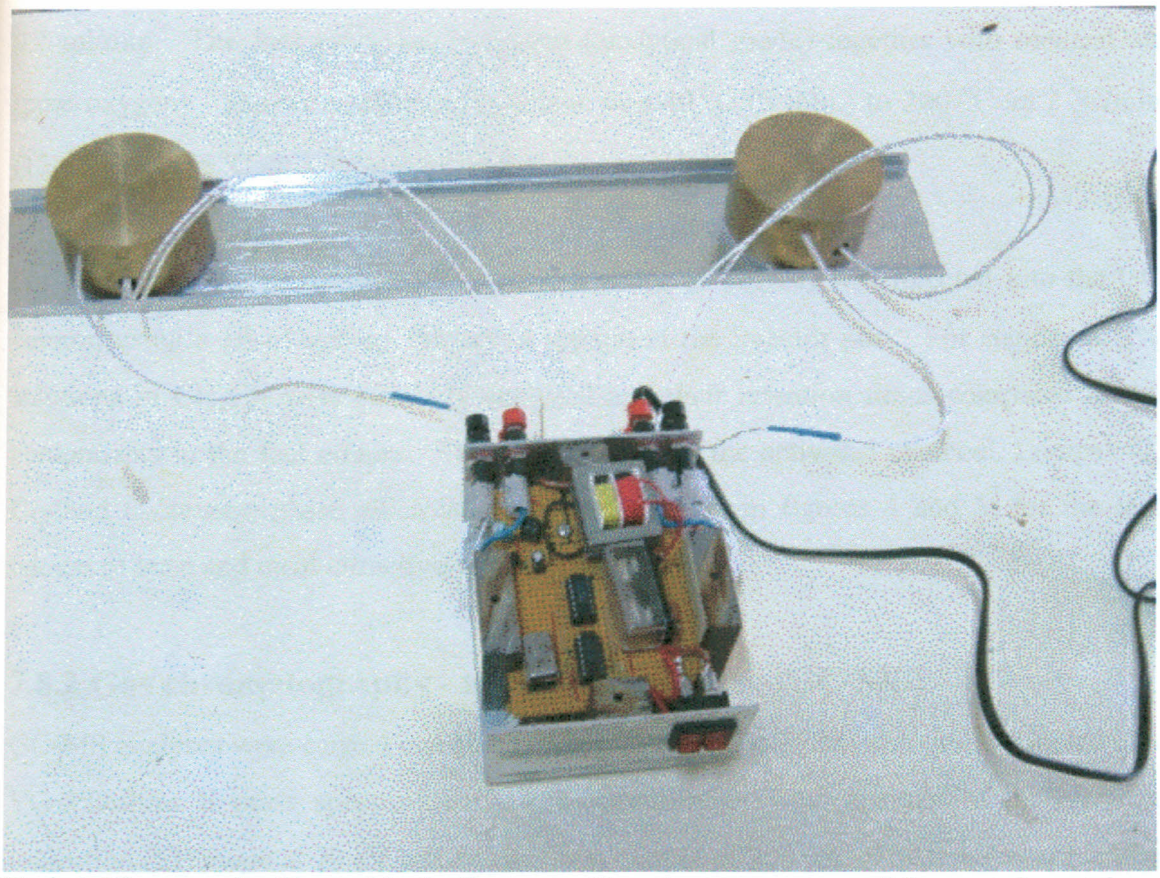


Plate 5: Photograph of brass blocks for controlling the temperature of the experimental samples

7.8 Chemical analysis and identification of the volatile components

Characterization, identification and determination of the volatile components of the feet odours was done by gas chromatograph (GC), gas chromatograph linked to mass spectrometer (GC-MS), and GC co-injection with authentic standards (Gikonyo *et al.*, 2002).

7.8.1 Gas chromatography (GC)

Gas chromatographic separation was performed on a Hewlett Packard (HP) model 5890 Series II capillary gas chromatograph equipped with a splitless capillary injector system, a flame ionization detector (FID) coupled to an integrator (HP 3393A Series II). The resolution was done on a HP cross-linked methylsilicone capillary column, 50 m x 0.2 mm (i.d) x 0.33 μm (film thickness). Carrier gas was white spot nitrogen at a flow rate of

0.7 ml/min. The fuel used was hydrogen (analytical grade) together with medical air (pure oxygen). The temperature programme was 40 °C (5 min.) to 200 °C @ 2 °C/min (15 min.) to 285 °C @ 3 °C/min (75 min).

For GC analysis 2 µl solution of the eluted odour in CH_2Cl_2 was injected into the GC column using a 10 µl syringe through a septum at the injector port. For standards, 2% solutions in CH_2Cl_2 were similarly analysed and their retention times compared to the components in the feet odours. The GC profiles for the activated charcoal, Porapak Q, C_{18} and C_8 reverse-phase silica adsorbents are shown in figures 4 and 5, for the feet odours of least and most attractive subjects, respectively.

7.8.2 Gas chromatography - mass spectrometry (GC-MS)

GC-MS analyses were carried out on a HP 8060 Series II gas chromatograph coupled to a VG Platform II mass spectrometer. The spectrometer was operated in the electron ionization (EI) mode at 70 eV and an emission current of 200 µA. The temperature of the source was held at 180 °C and the multiplier voltage was 300 V. The pressure of the ion source was held at 9.4×10^{-6} mBar, while that of the analyser (MS detector) was 1.4×10^{-5} mBar. The spectrometer had a scan cycle of 1.5 sec. (scan duration of 1 sec. and interscan delay of 0.5 sec). The mass range was set at *m/z* 1-1400. The scan range for the samples was however from *m/z* 38-650. The instrument was calibrated using heptacosafluorotributylamine $[CF_3(CF_2)_3]_3N$ (Apollo Scientific Ltd. UK). The GC column used was the same as the one described for the GC analysis except for the film thickness of 0.5 µm. The temperature programme was the same as that for the GC analysis.

The GC-MS was linked to a computer with MS data library (NIST and WILEY). The compounds were identified by comparing their MS with those of authentic samples or with MS library data. This was further confirmed by GC co-injection.

7.8.3 GC co-injection

Identity of the components of the feet odours was confirmed by peak enhancement upon GC co-injection of the feet odours with authentic standards.

7.9 Synthesis

7.9.1 2-Ethylacetophenone (85)

This compound was synthesized from *o*-ethylaniline via ethylbenzene diazonium chloride salt, which was converted to 1-bromo-2-ethylbenzene, lithiated and finally acetylated into the compound of interest.

7.9.1.1 1-Bromo-2-ethylbenzene (84)

o-Ethylaniline (0.5 ml, ~ 4 mmol) was put in a 100 ml flask to which a mixture of 10 ml of concentrated HCl (37% W/W in water) and 10 ml water was added and swirled gently to dissolve the aniline. The mixture was cooled in ice-salt for 20 min and a solution of 0.34 g $NaNO_2$ in 4 ml of water added slowly to the flask contents, while keeping the temperature below 10 °C. Lastly, a solution of 0.6 g KBr in 10 ml water was added to the ethylbenzene diazonium chloride salt in the flask, allowing the temperature of the mixture to rise above 10 °C. The flask containing the reaction mixture was transferred into a cold water bath and then refluxed for 30 min. After steam distillation, the distillate obtained was extracted with 3 x 5 ml portions of diethyl ether in a separatory funnel. The combined organic extracts were washed with 5 ml of 10% NaOH solution, followed by 5 ml water. The organic layer was dried with anhydrous sodium sulphate, filtered and concentrated. Column chromatography using silica gel and dichloromethane as eluent gave 0.452 g (58%) as a colourless liquid (R.I. 1.5475). Observed ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.27 (t, 3H), 2.80 (q, 2H), 7.45 – 7.5 (m, 4H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 14.2 (d, $-CH_3$), 29.4 (s, $-CH_2-$), 124.3 (s, C-1), 127.3 (d, C-4), 127.5 (d, C-5), 129.5 (d, C-3), 132.7 (d, C-6), 143.2 (s, C-2); The EIMS *m/z* 77 (50%), 90 (30%), 105 (100%) [M-Br], 170 (60%), 183 (38%) [M^+], 185 (38%) [M^+].

7.9.1.2 2-Ethylacetophenone (85)

To a solution of 1-bromo-2-ethylbenzene (**84**) (0.7 ml, 5.67 mmol) in 50 ml diethyl ether at -78 °C under argon, *n*-BuLi (4.5 ml, 11.4 mmol) was added. The reaction mixture was stirred at -78 °C for 30 min and then at room temperature for a further 30 min. The mixture was then cooled to -78 °C and acetyl chloride (1.6 ml, 22.7mmol) added drop-wise for 30 min while stirring. The reaction mixture was allowed to warm to room temperature, and quenched with saturated aqueous NH_4Cl (2 x 100 ml). The organic layer was washed with water (2 x 100 ml), dried with anhydrous $NaSO_4$, filtered and evaporated under reduced pressure. The crude extract was purified by preparative TLC on silica gel using DCM-hexane (8: 2) and analysed by GC. However, the final product (P3) still had contamination of other reaction products (Fig. 7). The experimental procedure was repeated but with the inclusion of dry molecular sieves in the reaction flask before the introduction of the solvent and reagents. The inclusion of molecular sieves improved the yield of 2-ethylacetophenone (**85**) (R_t 26.10 min) (Fig. 7). Comparison of R_t of 2-ethylacetophenone (**85**) with the EAG-active peak revealed that it was not the active compound and hence no further purification of the mixture was done.

7.10 Bioassays

7.10.1 GC-EAD analysis of human foot volatiles

Antennae of 3-4 days-old female *An. gambiae s.s.* mosquitoes were used for the coupled gas chromatography-electroantennographic detector (GC-EAD) analysis. A glass micro-pipette containing Beadle-Ephrussi saline (Ephrussi & Beadle, 1936) was inserted through the inter-segmental membrane between the abdomen and the thorax of the insect. The fine tip of the micro-pipette was pushed through the thorax, then the neck and into the head. The other end of the micropipette was sheathed over a silver wire, the recording electrode, which was connected to the input of a universal AC/DC UN-05 amplifier (Syntech, The Netherlands). To complete the circuit, the distal end of the antenna was nipped off with a scalpel and the open end inserted into a similar glass micropipette containing the saline and was also sheathed over a silver wire electrode that was grounded. GC-EAD tests were performed on HP 5890 Series II gas chromatograph equipped with a flame ionisation

detector (FID) and HP Ultra 1 (cross-linked methyl silicone gum) capillary column (50 m × 0.2 mm × 0.33 μm) using nitrogen at a flow rate of 0.80 ml/min as the carrier gas. The oven temperature was maintained at 60 °C for 5 min after injection of sample and then programmed at 5 °C/min to 280 °C, where it was held for 15 min. The effluent from the capillary column was split in ratio of 1:1 into two 50 cm long deactivated silica columns, one connected to the FID and the other connected to a stainless steel tube (5 mm i.d.) that was focused onto the antennal preparation. A make-up gas (40 ml/min) was added just before the split point to accelerate the effluent through the deactivated columns. The deactivated transfer line carrying the effluent over to the antennal preparation was maintained at 150 °C by a THC-3 temperature control unit (Syntech, The Netherlands). Aliquots (6-8 μl) of the human foot volatile extracts were analysed by the GC. EAD and GC signals were monitored synchronously using a programme on a GC/EAD interface card (Syntech, The Netherlands) installed in a PC (Harvard Professional Computer, American Megatrends Inc.).

7.10.2 Laboratory assay of the EAD active components of the foot odour

The 11 EAD active foot odour components were assayed both individually and as blends in the convective bioassay chamber (Plate 4). In order to determine the amount of each of the EAD active components to be used in both the individual component and blends assays, 0.2 μl of *p*-cymene (99% GC grade Aldrich Chem. Co., UK) was added to 2 ml (2000 μl) of the foot odour extract in dichloromethane (99.9% HPLC grade, Aldrich Chem. Co., UK). This implied that 1 μl of the mixture had 0.0001 μl of the internal standard (*p*-cymene). The mixture (2 μl) was injected into the GC column using a 10 μl syringe through the septum at the injector port. The GC machine used, the column and conditions of analysis were the same as previously described. The relative peak area (0.678) of the internal standard was correlated to the amount of the compound (0.0002 μl) in the 2 μl mixture injected into the GC column. The result obtained was then used to calculate the amount of each of the EAD active compounds present in the foot odour extract by multiplying relative area of each of the EAD-active components by a factor 0.000295 (0.0002 μl /0.678). The resulting quantities of each of the EAD active

components of the foot odour were then multiplied by a factor of 25 to obtain the measurable amounts of each of the components and blends required to prepare 50 ml of the test solution in acetone (99.9% HPLC grade, Aldrich Chem. Co., UK).

In the individual component assays, 5 µl of the prepared solution was evenly applied on a 10 mg piece of DCM cleaned cotton wool placed in an aluminium dish (5 cm diameter). In another aluminium dish containing same mass of DCM cleaned cotton wool, 5 µl acetone was dispensed evenly to serve as control. Both aluminium dishes containing test and control samples were left in the open air for 10 min, to allow excess acetone to evaporate, before being placed on the two metal block heaters and the temperature control circuit put on. The fume hood was also switched on to allow vertical movement of air current. Starved female *An. gambiae* mosquitoes (50) were then carefully introduced into the top part of the chamber through a glass tube (Fig. 11). Their resting/landing pattern/preference was monitored and recorded after 10 min from the time of the introduction of mosquitoes into the chamber. The experiment was replicated 8 times for each component, interchanging the test and control dishes between the two metal block heaters. The number of mosquitoes preferring to rest/land on/near the test and control dishes on the metal bocks was recorded and the control data used to transform the data as follows: N = T - C where by N = number of mosquitoes responding to the test chemical, T = number of mosquitoes preferring to rest/land on/near the test source and C = number of mosquitoes preferring to rest/land on/near the control source (Mehr *et al.*, 1985). The resulting transformed data for the number of mosquitoes responding to the test chemical was subjected to statistical analysis of the means using the student-LSD test (SAS® Institute, 2000). The results are summarised in table 20.

The 11 EAD active compounds were also assayed in blends through subtraction bioassays. The first blend constituted a mixture of all the 11 EAD active compounds in the proportion in which they occurred in the most attractive human foot odour. The next 11 blends had one specific component omitted in each one of them. The rest of the blends had either two or three components omitted from the initial mixture of the 11 EAD active

compounds. The preparation of the blend solutions was done by dissolving the different amounts of EAD active compounds calculated in 50 ml acetone (99.9% HPLC grade, Aldrich Chem. Co., UK) as earlier illustrated. For each blend, 5 µl of the solution was assayed against *An. gambiae s.s.* mosquitoes as described earlier. The number of mosquitoes preferring to rest/land on/near the test and control dishes on the metal bocks was recorded and the control data used to transform the data as described earlier. The means of the transformed data were determined through statistical analysis as previously described. The results are summarized in table 21.

7.10.3 Semi field assay of the EAD active components of the foot odour

All the EAD active components were again subjected to subtraction bioassays as blends under semi field conditions. The first blend had all the 11 EAD active components, while the rest of the blends had a total of 10 components, each blend missing one particular component that would be present in the rest. Initially, a total of 12 blends were prepared and assayed in 8 replicates each (between 8 p.m. and 6 a.m. for 8 nights). Control experiments contained 99.9% HPLC grade acetone only. Three additional blends were similarly prepared by omitting more than one component from the rest. The amount of each of the EAD active components incorporated in the blends was calculated from their relative peak areas in the most attractive foot odour as described earlier. The resulting amounts of the EAD active components of the foot odour were then multiplied by a factor of 25 to obtain the amounts required to prepare 50 ml of each of the blends in acetone.

A 5 ml syringe was used to apply 3 ml of a blend solution evenly onto cleaned cotton socks, which was then used as the bait in a CFG trap after being left in the open for 30 min to allow acetone (solvent) to evaporate. The same amount of acetone was dispensed on another set of cleaned socks also given 30 min to evaporate before being put in another CFG trap to act as the control. The test and control CFG traps were arranged diagonally in the screen house and 200 starved female *An. gambiae* mosquitoes released from a cup placed at the central point as previously illustrated (Fig. 10). The test and control CFG traps were interchanged between the two sites A and B every two successive nights such

that, 4 replicates were done at each of the sites A and B for both the test and control CFG traps. The experiment was replicated 8 times for each of the EAD active components. The number of mosquitoes caught by each of the 12 blends was computed using the equation: N = T - C where by N = mosquito catch size due to a blend, T = number of mosquitoes in test trap and C = number of mosquitoes in control trap (Mehr *et al.*, 1985). The resulting transformed data for the mosquito catch sizes was subjected to statistical analysis of the means as previously described. The results are summarised in table 22.

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APPENDICES

Figure 14: Mass spectrum of Isobutyric acid

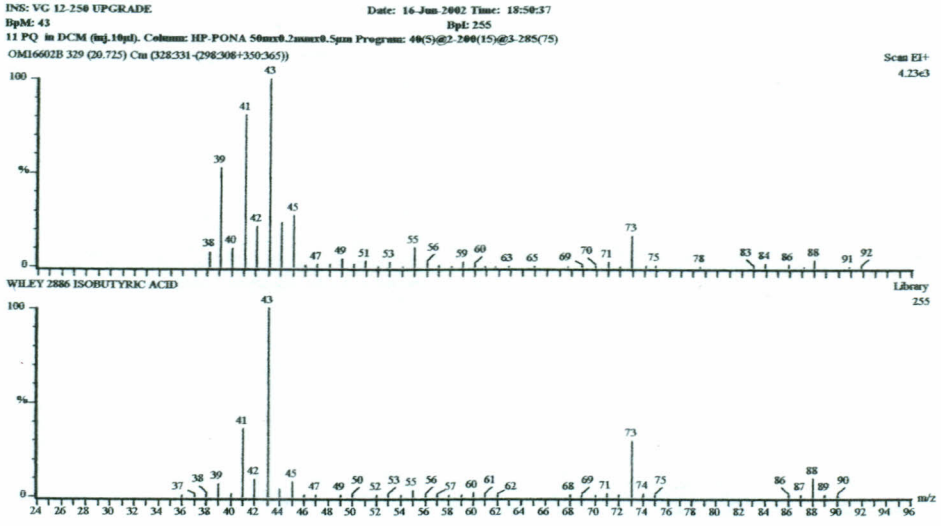


Figure 15: Mass spectrum of 3-methylbutyric acid

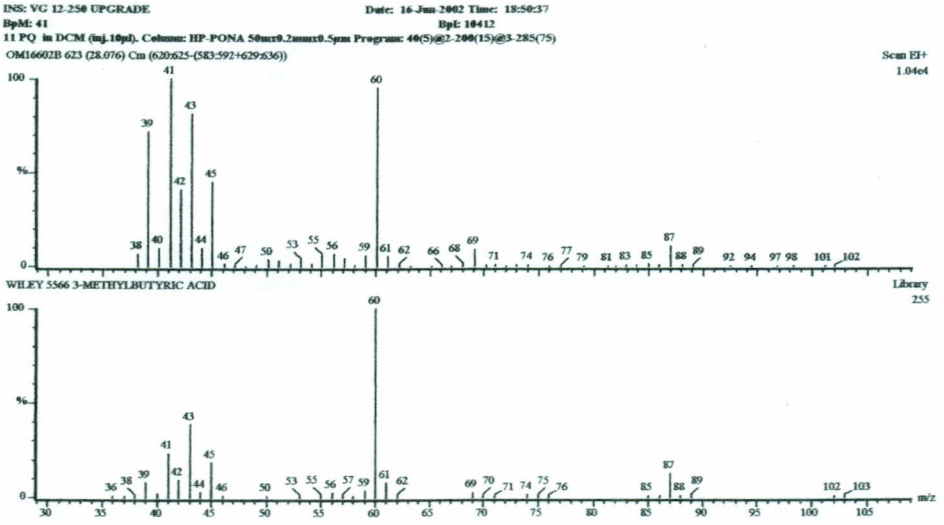


Figure 16: Mass spectrum of *cis*-3-hexenyl phenylacetate

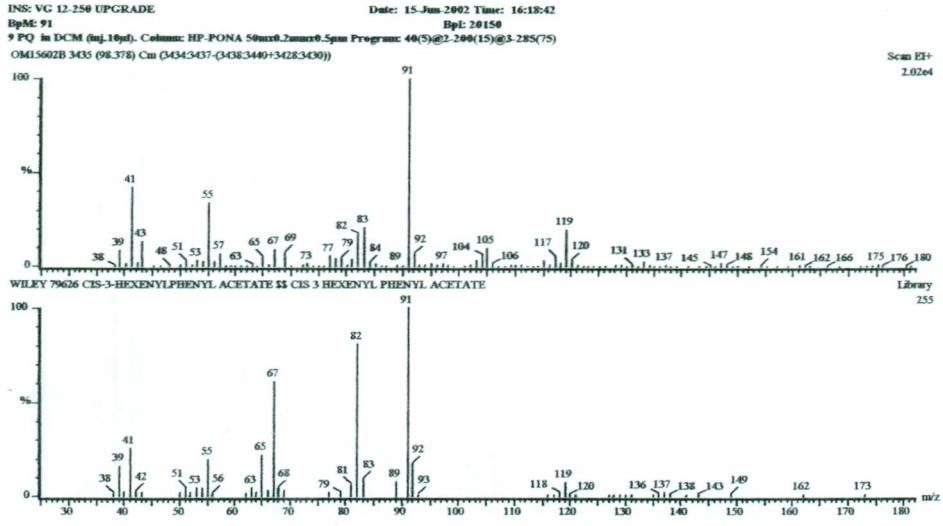


Figure 17: Mass spectrum of diisobutyl phthalate

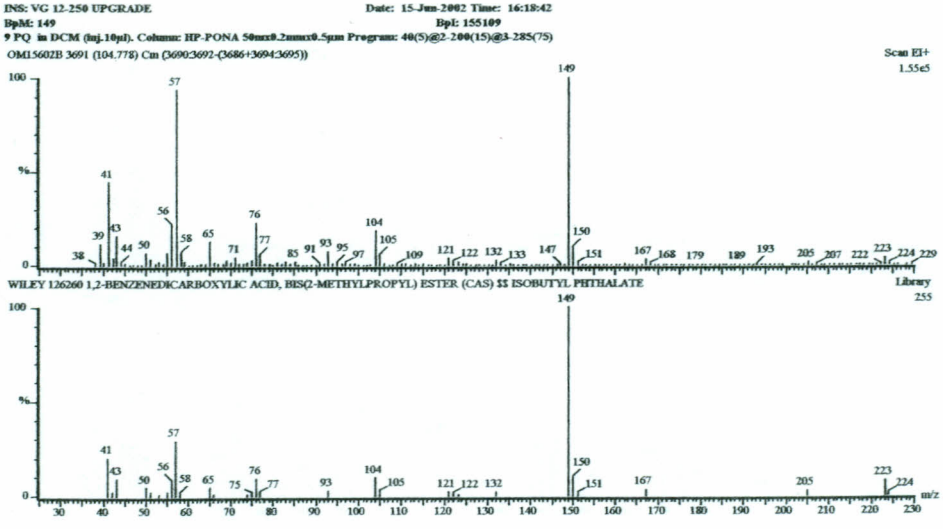


Figure 18: Mass spectrum of *n*-octanal

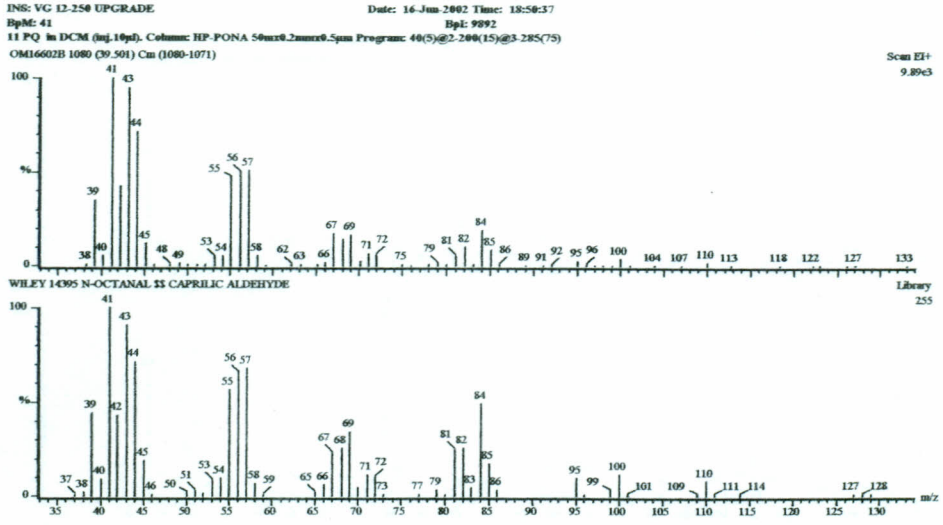


Figure 19: Mass spectrum of *n*-decanal

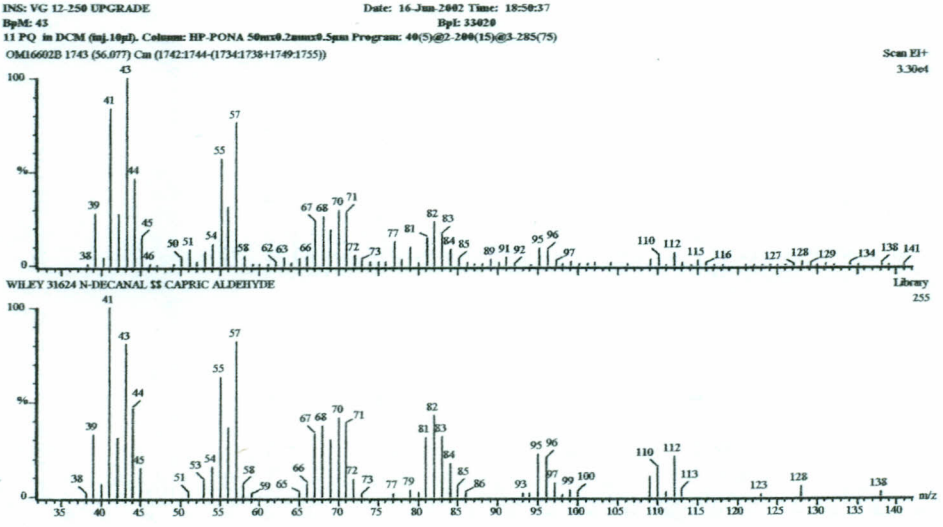


Figure 20: Mass spectrum of 4-(1-hydroxyethyl)benzaldehyde

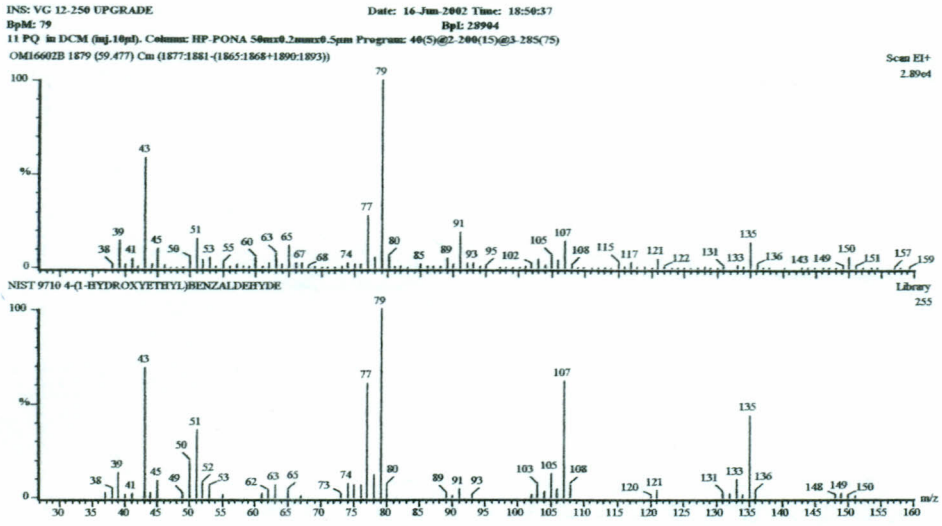


Figure 21: Mass spectrum of 6-methyl-5-hepten-2-one

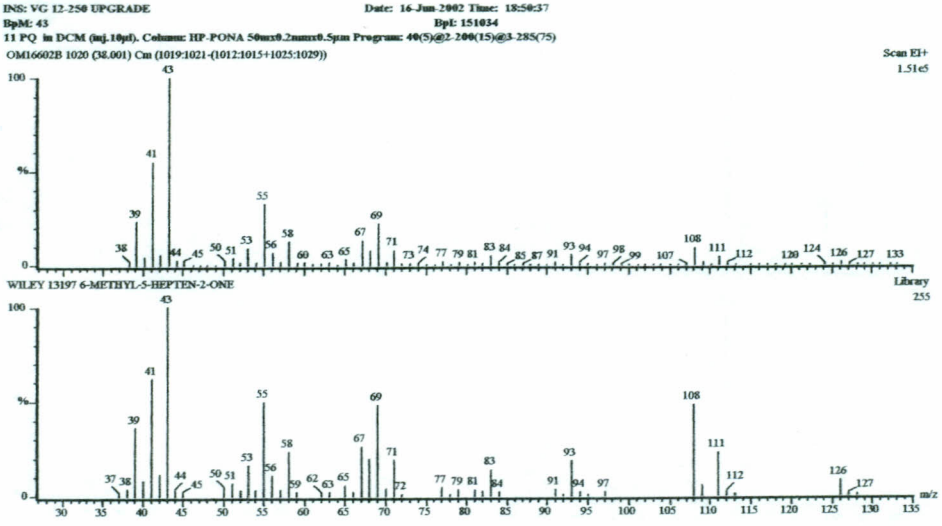


Figure 22: Mass spectrum of 3,5,5-trimethylcyclohex-2-ene-1-one

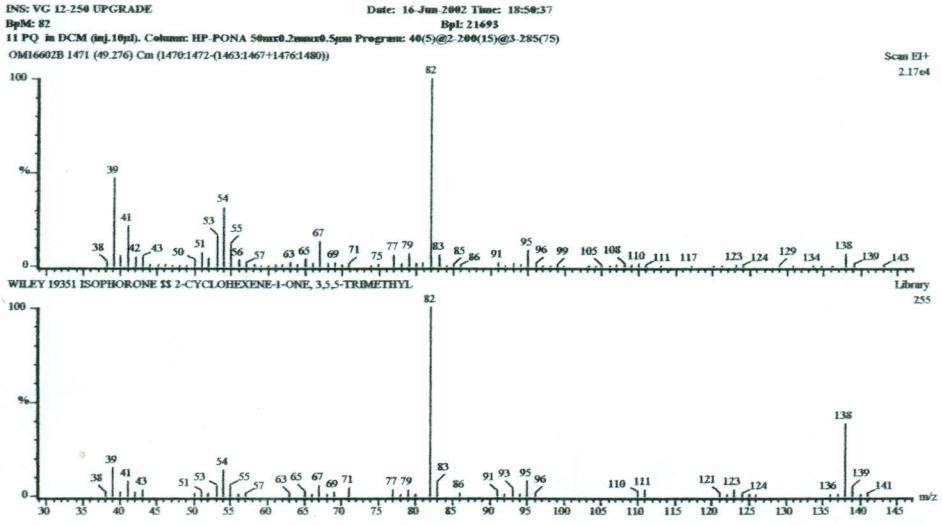


Figure 23: Mass spectrum of 4-ethylacetophenone

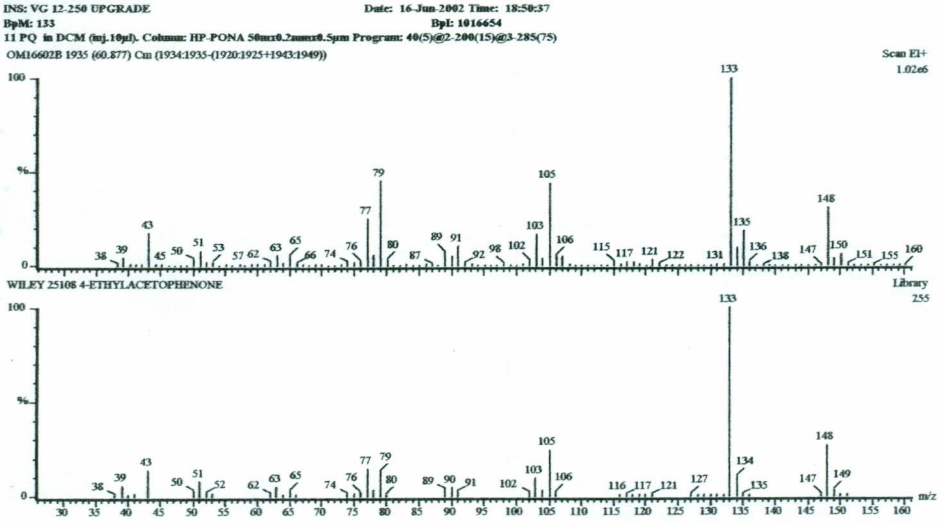


Figure 24: Mass spectrum of 2-methylphenol

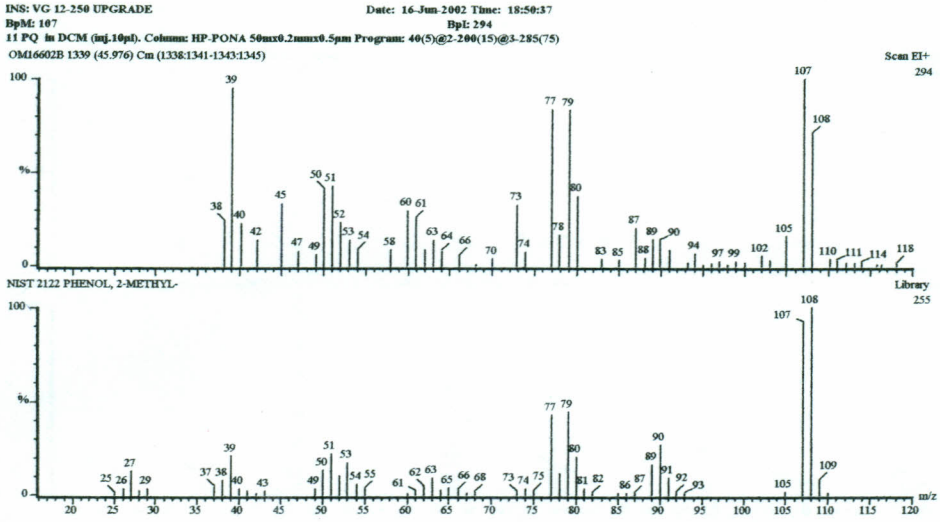


Figure 25: Mass spectrum of 2-phenoxyethanol

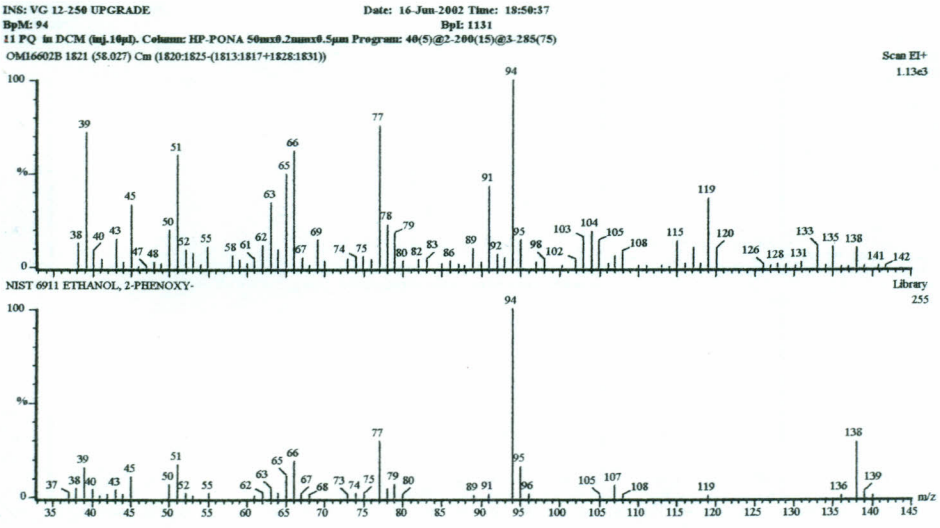


Figure 26: Mass spectrum of4-*tert*-butylcyclohexanol

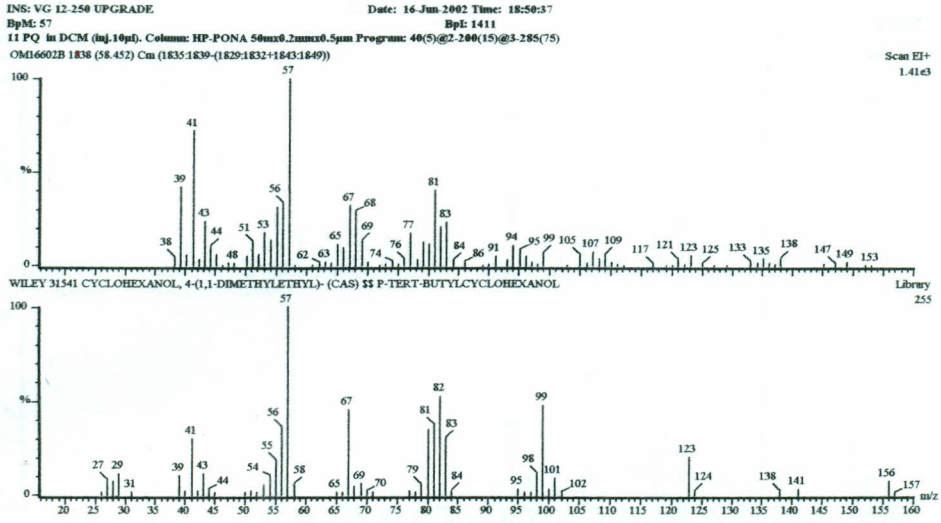


Figure 27: Mass spectrum of *O-tert*-butylphenol

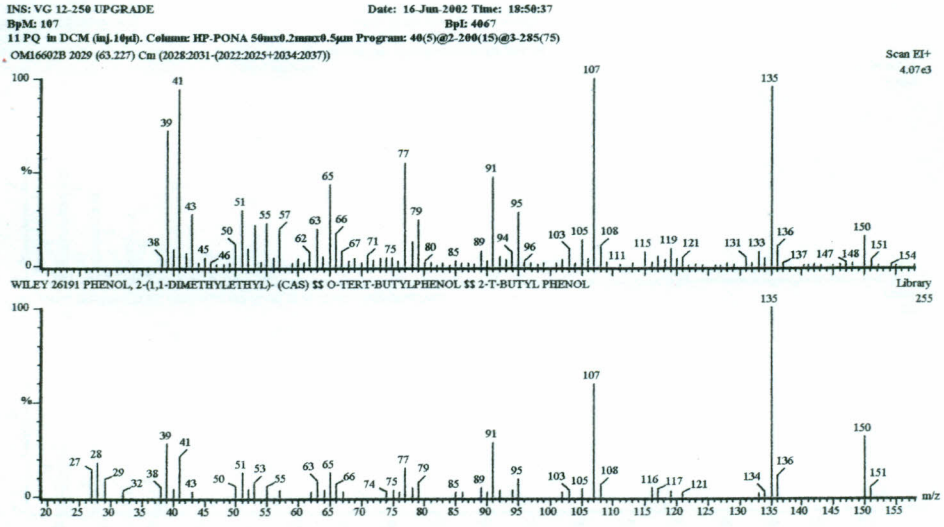


Figure 28: Mass spectrum of 1-ethyl-4-isopropylbenzene

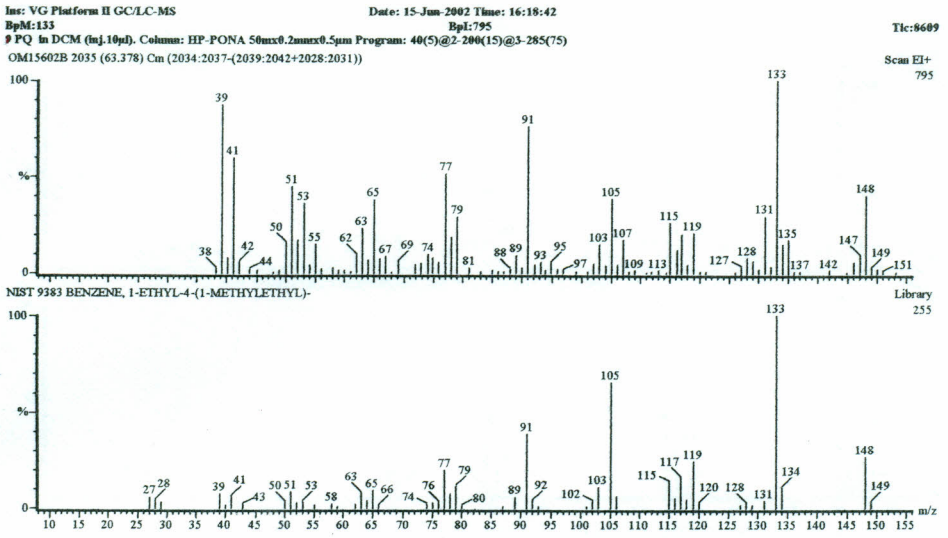


Figure 29: Mass spectrum of 2-decyldodecylbenzene

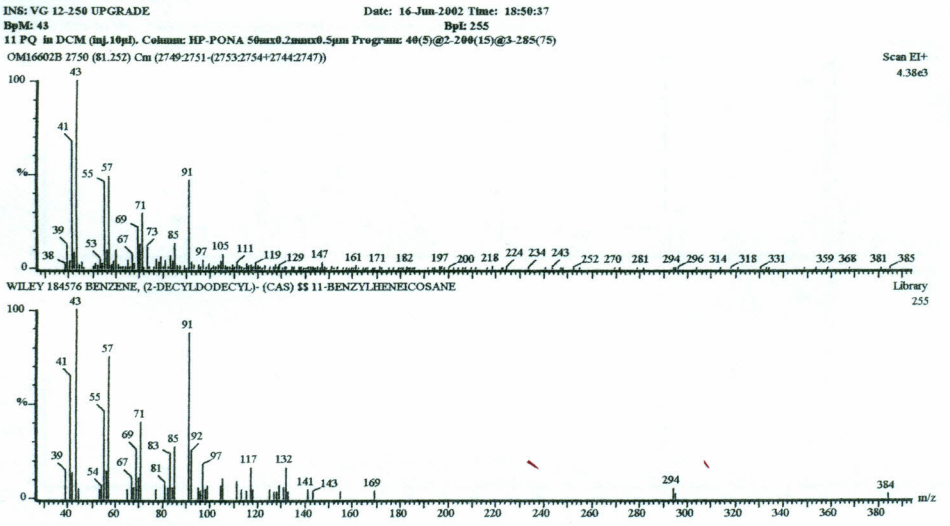


Figure 30: Mass spectrum of 1,3,5-trichlorobenzene

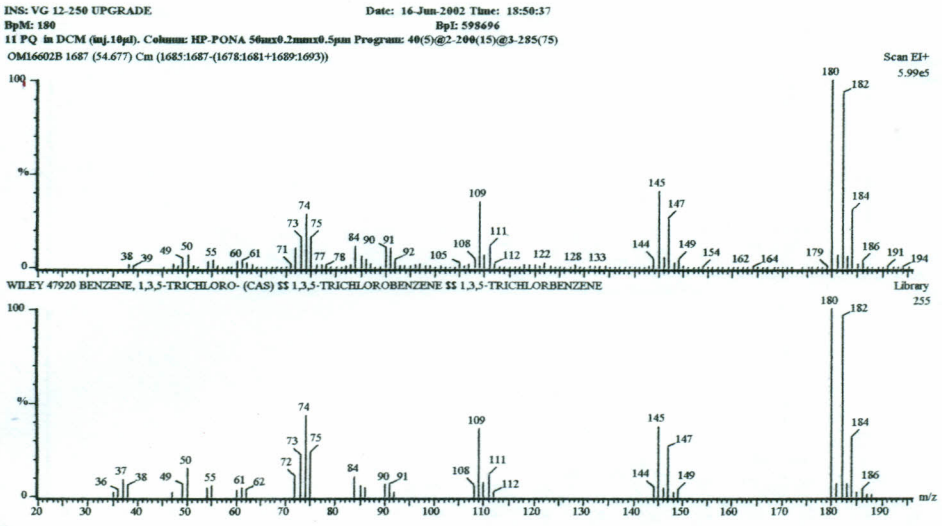


Figure 31: Mass spectrum of 2-methylnaphthalene

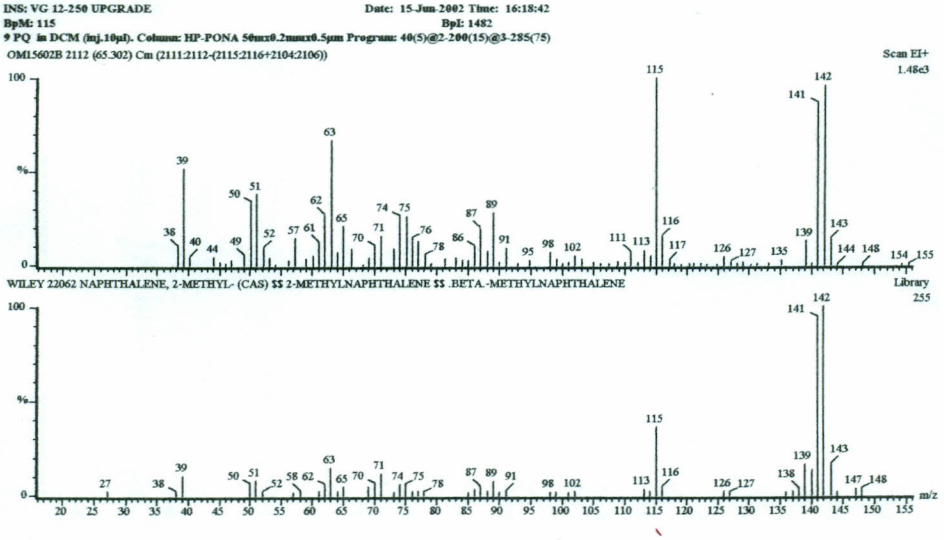


Figure 32: Mass spectrum of 1-isopropylnaphthalene

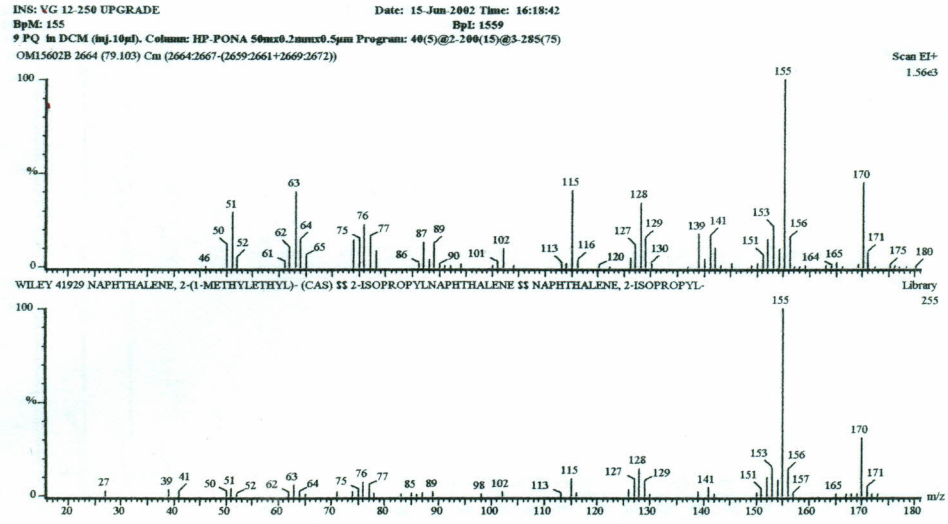


Figure 33: Mass spectrum of *n*-tetradecane

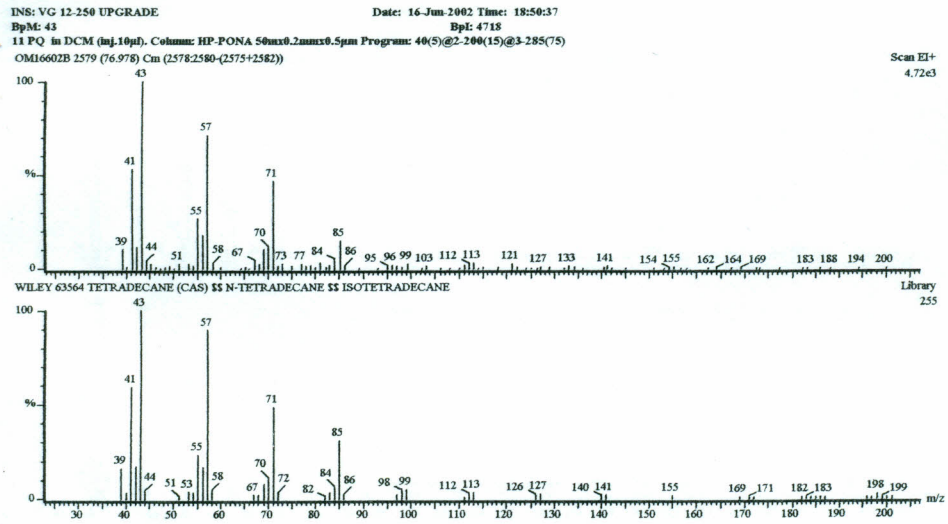


Figure 34: Mass spectrum of *n*-octacosane

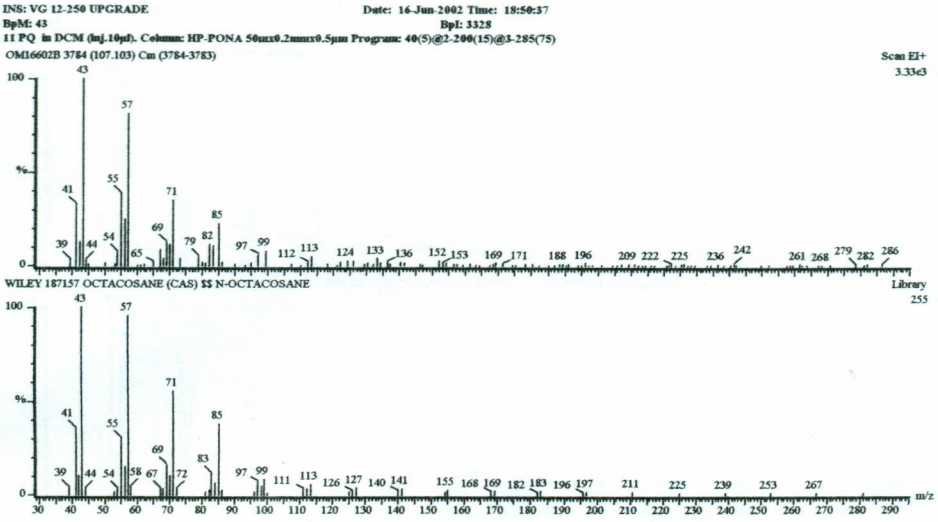


Figure 35: Mass spectrum of *n*-eicosane

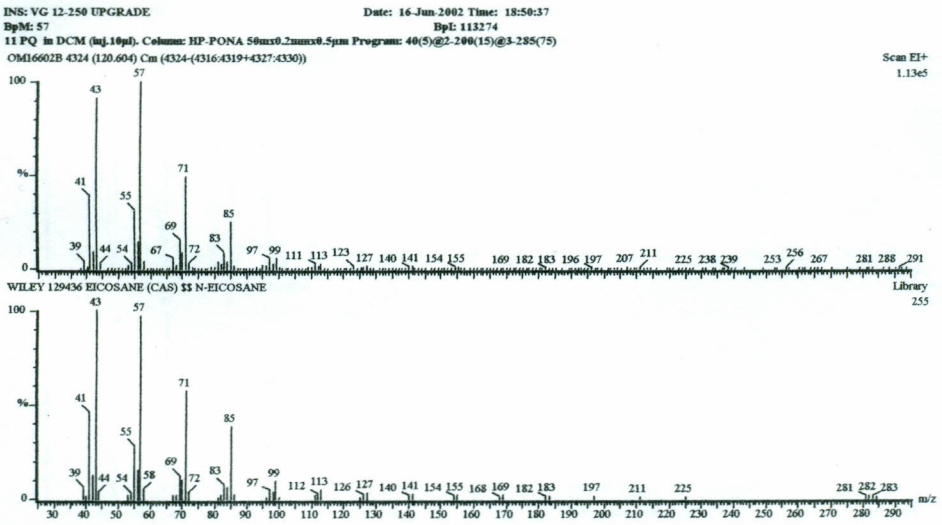


Figure 36: Mass spectrum of 1-tetradecene

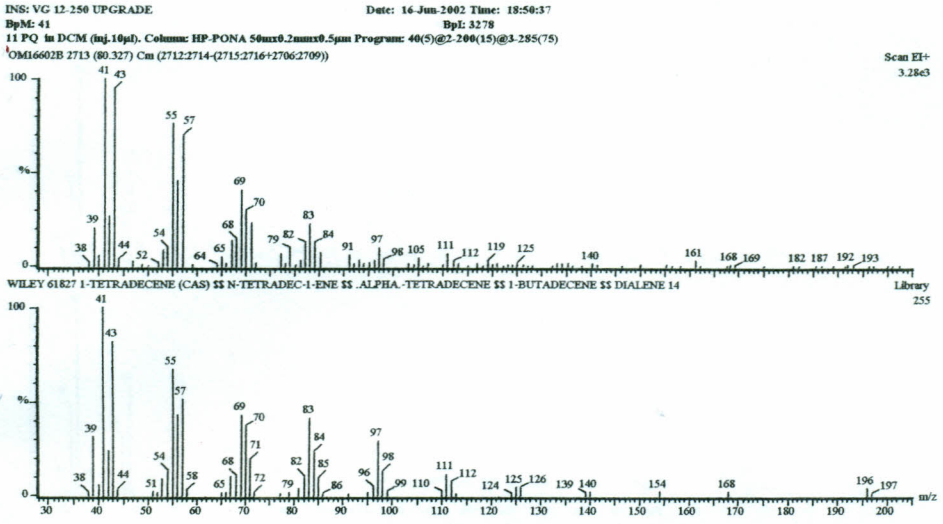


Figure 37: Mass spectrum of 2,6,10,14-tetramethylheptadecane

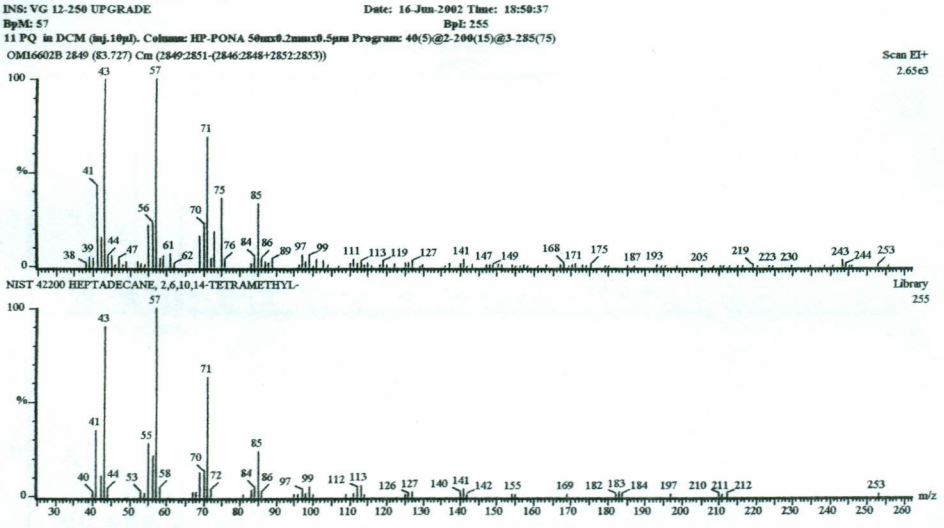


Figure 38: Mass spectrum of 4-methyloctadecane

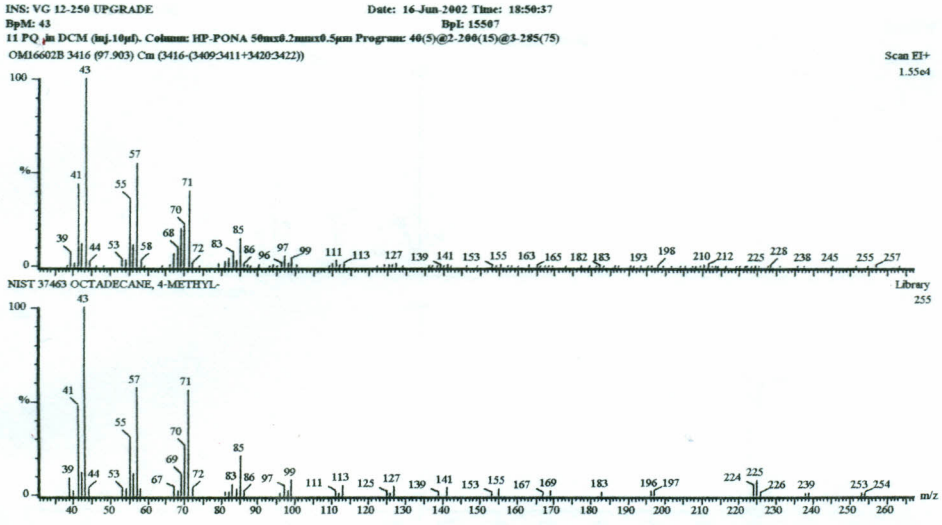


Figure 39: Mass spectrum of 7-hexyleicosane

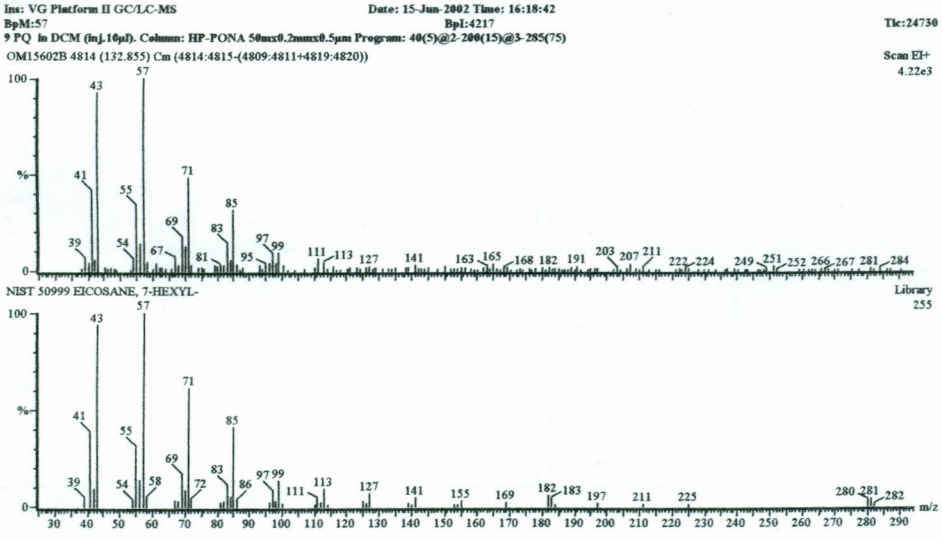


Figure 40: Mass spectrum of 1-bromo-2-ethylbenzene

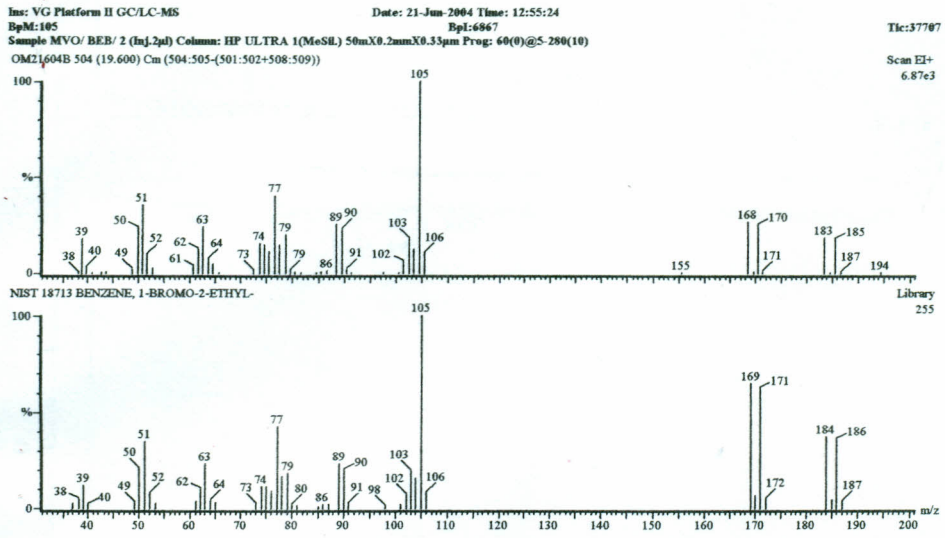


Figure 41: Mass spectrum of 2-ethylacetophenone

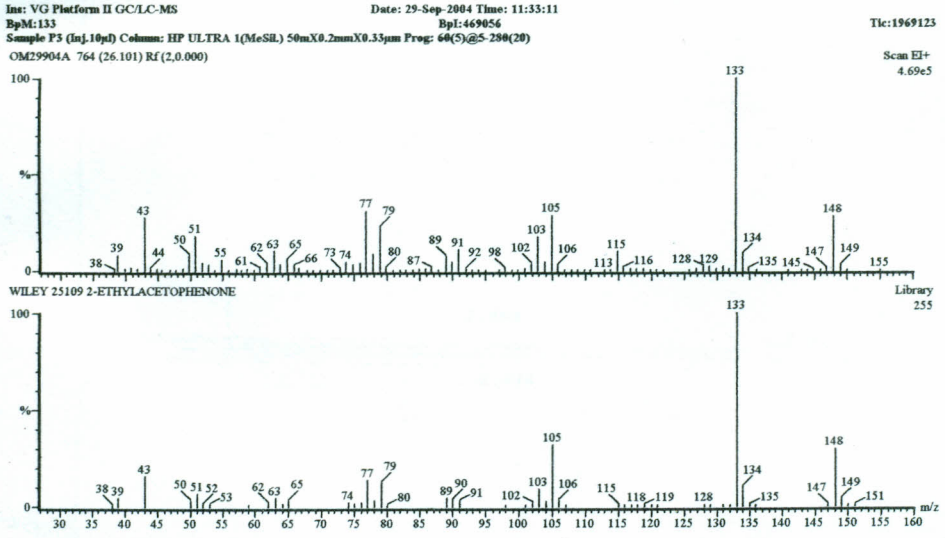
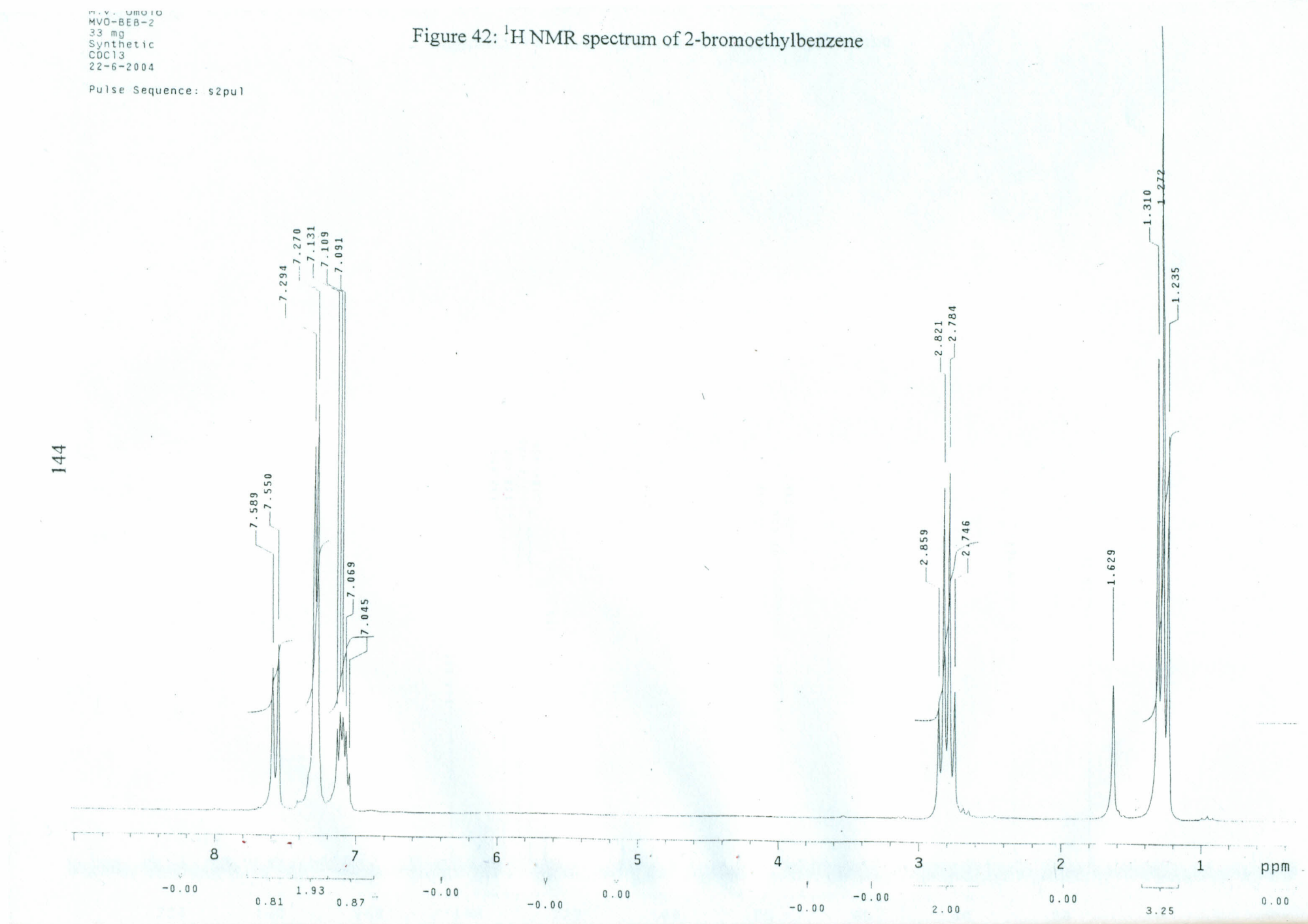


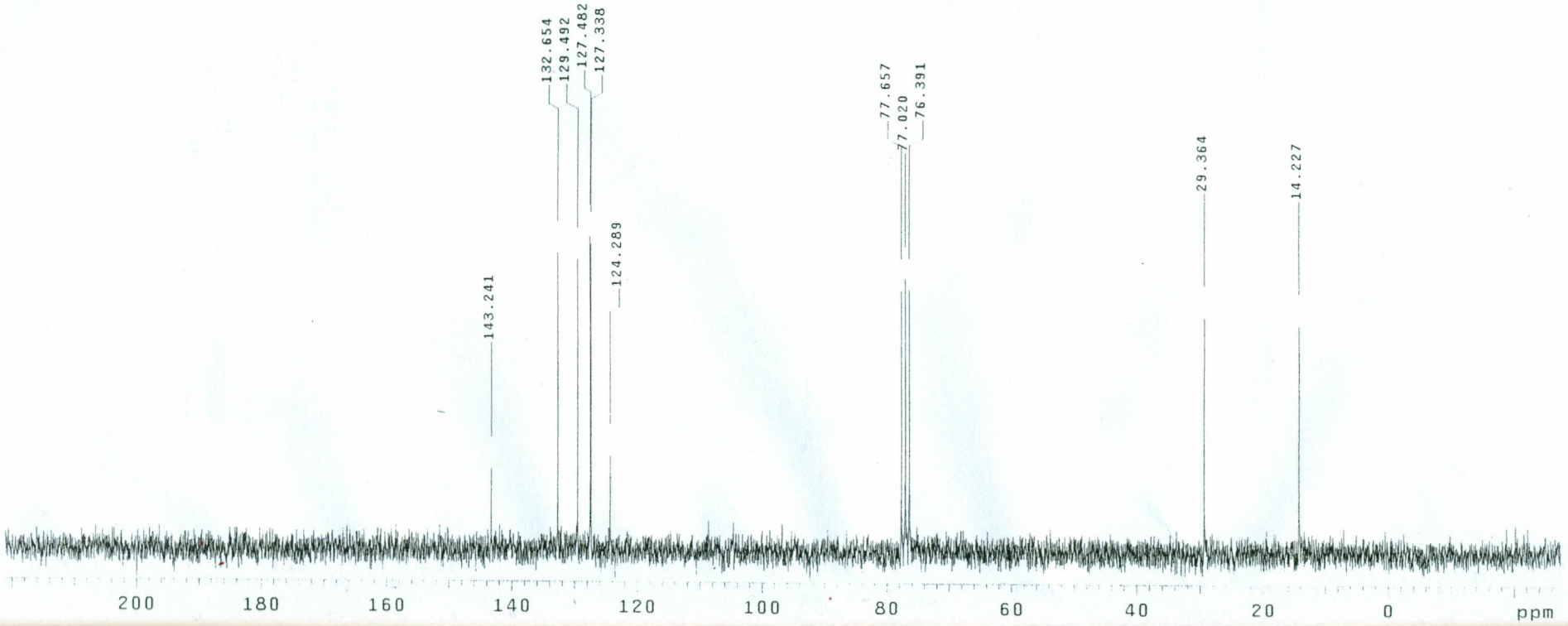
Figure 42: ^{1}H NMR spectrum of 2-bromoethylbenzene



M.V. Umolo
MVO-BEB-2
33 mg
Synthetic
CDCl3
22-6-2004

Pulse Sequence: s2pul

Figure 43: ^{13}C NMR spectrum of 2-bromoethylbenzene



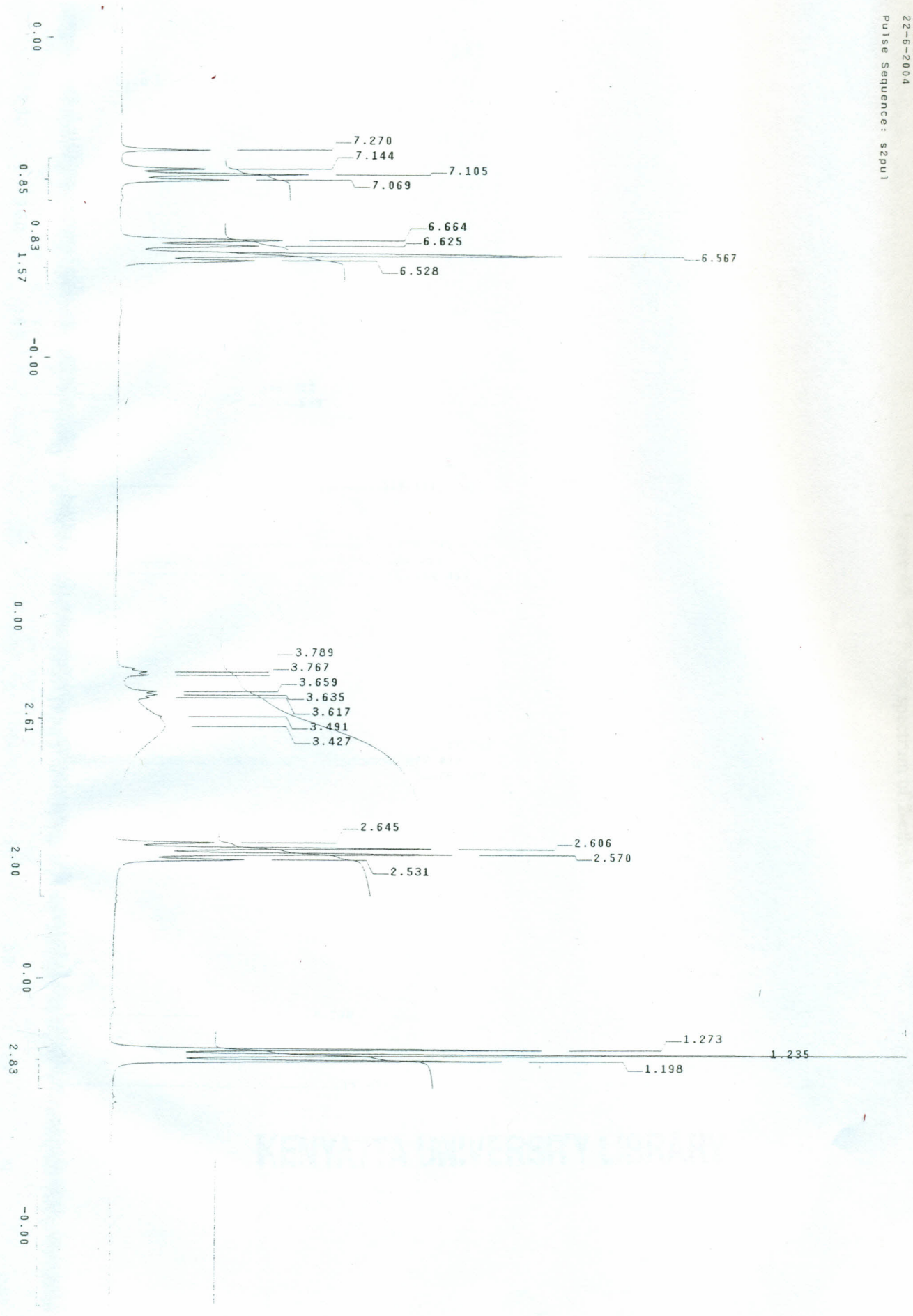


Figure 44: ^{1}H NMR spectrum of 2-ethylaniline

29 mg
Ethyl aniline
CDCl3
22-6-2004

Pulse Sequence: s2pul

Figure 45: ^{13}C NMR spectrum of 2-ethylaniline