VARIATIONS IN CHEMICAL COMPOSITION AND MOSQUITO REPELLENT EFFICACY OF ESSENTIAL OIL OF *CONYZA NEWII* FROM DIFFERENT REGIONS OF KENYA

BY

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TABLE OF CONTENTS

DECLARATION

This thesis is my original work and has never been presented for a degree in any other University.

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DEDICATION

To my father George, who always encouraged me to think big and pursue academic excellence. To my mother Susan, for having reminded me that education is the key. To my brothers and sisters, who were and are always true comrades. With love, gratitude and affection to Ruth Koech, who was always there for me, may God watch over her.

ACKNOWLEDGEMENT

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List of abbreviations

ANOVA – Analysis of Variance

BCED – Behavioral and Chemical Ecology Department

COSY – Correlation spectroscopy

DEET – *N, N* diethyl –*m*- toluamide

DEPT- Distortion enhanced by polarization transfer

DOPA- dihydroxylphenyl alanine

GC-MS – Gas chromatography mass spectrometry

GC-MS, CO – Gas chromatography mass spectrometry co-injection

HETCOR – Heterogenous correlation spectroscopy

ICIPE – International Center of Insect Physiology and Ecology

MIM – Multi-lateral Initiative on Malaria

MIV – Malaria Vaccine Initiative

NMR – Nuclear Magnetic Resonance

RBM – Roll Back Malaria

RD- Repellency Dose

SE – Standard Error

SNK – Student Newman's Kuels

TDR – Tropical Diseases Research

UN – United Nations

UNDP – United Nations Development Programme

UNICEF- United Nations Children Education Fund

WHO – World Health Organization

Abstract

In this research work, the chemical composition of essential oil of *Conyza newii* (Compositae) from seven regions within Kenya has been determined. The efficacy of three modes of application of the plant as a mosquito repellent is reported. The regions considered in the study are West Pokot, Kilome, Nyakach, Kericho, Naivasha, Nairobi and Webuye. The methods of application assessed are steam distilled oil, thermally expelled plant material and directly burned plant material. Vapors obtained from the plant by headspace trapping were also analyzed. Steam distillation was carried out using Dean and Stark apparatus while thermal expulsion and direct burning of plant material was undertaken, the vapors cooled and collected for bioassay. Headspace trapping was carried out using Porapak S adsorbent. Repellency bioassays were carried out according to established WHO protocols for field evaluation of insect repellents and pesticides. Identification of major compounds in the essential oil and smoke emanations from thermally expelled and directly burned plant material was carried by GC-MS and co-injection with authentic standards. ANOVA of protective efficacy with respect to geographical location of the plant and method of application of the products was carried out using the Student-Newman's Kuels test on SAS system for windows. Significant variation ($p = 0.001$) in the protective efficacy of essential oil of *Conyza newii* from different geographical locations was established. However, protective efficacy of smoke emanation of thermally expelled and directly burned plant material does not vary significantly with geographical location of the plant ($p = 0.001$ and 0.001, respectively). ANOVA revealed significant variations ($p = 0.001$) between the three modes of application, with steam-distilled oil showing the highest while direct burning had the lowest repellency. Major compounds identified in the essential oil were limonene, peril alcohol, peril aldehyde, geraniol, 1,8-cineole, *trans*-β-ocimene, α-caryophylene oxide, α-pinene and fenchyl alcohol. Most compounds in the essential oil were same as those identified in smoke emanations but in lower concentrations. Major compounds identified in the vapors from headspace trapping were *cis*-limonene oxide, *trans*-limonene oxide, *cis*-dihyrocarvone, 4-methoxyphenol and 3-methoxy-2-methylphenol. Repellency of individual compounds in the oil and their blends confirmed that the repellency of natural oil depends on the concentration of peril alcohol, geraniol, limonene and peril aldehyde.

CHAPTER 1
INTRODUCTION

1.1 The malaria burden

Malaria is the most important parasitic disease in the tropics killing 1-2 million people annually, with the majority being young children and pregnant mothers with low levels of immunity (WHO, 2000; 1996). Currently, malaria infection has become prevalent in areas where it had formerly been controlled with all age groups being vulnerable to the disease (WHO, 2000).

In all developing countries, 25–40% of all outpatient clinic visits are due to malaria in addition to 20-50% hospital admissions. With high fatality rates due to late diagnosis, inadequate management and lack of effective drugs, malaria is a major contributor to deaths among hospital inpatients (Mwageni, 2001). In Africa, malaria accounts for up to a third of all hospital admissions, and up to a quarter of all deaths of children under the age of five. Up to 800,000 infantile mortalities and a substantial number of miscarriages and very low birth weight babies per year due to the disease are reported (Woodruff *et al.*, 2002). Children who survive malaria may suffer long-term consequences of the infection. Reported episodes of fever and illness reduce appetite and restrict play, social interaction and educational opportunities thereby contributing to poor development. About 2% of children who recover from malaria infections suffer from learning impairments and disabilities due to brain damage including epilepsy and spasticity (WHO, 2002).

The poor are at the highest risk both of becoming infected with malaria and of becoming infected more frequently. Children mortality rates are known to be high in poor households and malaria is responsible for substantial proportion of these deaths (Mwageni, 2001). In a study carried out in the United Republic of Tanzania, under 5 mortality rates following acute fever was 39% higher in poor than in rich households (Mwageni, 2001). Poor families live in dwellings that offer little protection against mosquitoes and are less able to afford insecticide treated nets (Akazili, 2001). In Africa, a bout of malaria costs 10 working days (WHO, 2001). Malaria is responsible for a high

proportion of public health expenditure in most developing countries and substantial reductions in malaria incidence would free up available health resources, facilities and health worker's time to tackle other health problems (Akazili, 2001). In Dar-es-Salaam, Tanzania, estimates based on household interviews suggest that the 2.5 million residents spend as much as US$ 1 million per month on activities related to malaria control (Pates *et al.*, 2002). In many developing countries, malaria exerts a serious burden on lives, medical costs and labor days (WHO, 1996). Health experts estimate the cost of malaria management in Africa at US$ 3.6 billion annually. Statistics from 31 African countries during the period of 1980 to 1995 showed that the annual loss of economic growth due to malaria was as high as 1.3% per year (Brooke *et al.*, 2002).

1.1 Malaria parasite and life cycle

Human malaria is caused by four different parasite species: *Plasmodium vivax*, *P. falciparum*, *P. ovale* and *P. malariae* (Taubes, 2000). The parasite is transmitted by *Anopheles* mosquitoes and is found in Africa, New Guinea, Haiti, Asia, Oceania and South America (Taubes, 2000). *P. vivax* a less virulent strain is encountered in Indian sub-continent, Central America, Asia, Oceania and South America but has a low incidence in sub-Saharan Africa because of protective mutation (Duffy negativity) frequently found in the population (Taubes, 2000). *P. malariae* is the mildest form of malaria found in most regions where malaria is endemic (Taubes, 2000). Approximately 10-20% of the world's cases of *P. vivax* occur in Africa, south of the Sahara (Mendis *et al.*, 2001). In Eastern and Central Africa, *P. vivax* is responsible for 10% of malaria cases but less than 1% of cases in Western and Central Africa (Mendis *et al.*, 2001). Outside Africa, *P. vivax* accounts for >50% of all malaria cases with 80-90% cases occurring in the Middle East, Asia and Southern America (Mendis *et al.*, 2001). *P. ovale* is relatively uncommon in areas outside Africa while *P. falciparum* is the most prominent, frequent and virulent species occurring in sub-Sahara Africa. However, with relative ease and speed of travel and migration, cases of any kind of malaria can be imported into any region of the world (Taubes, 2000).

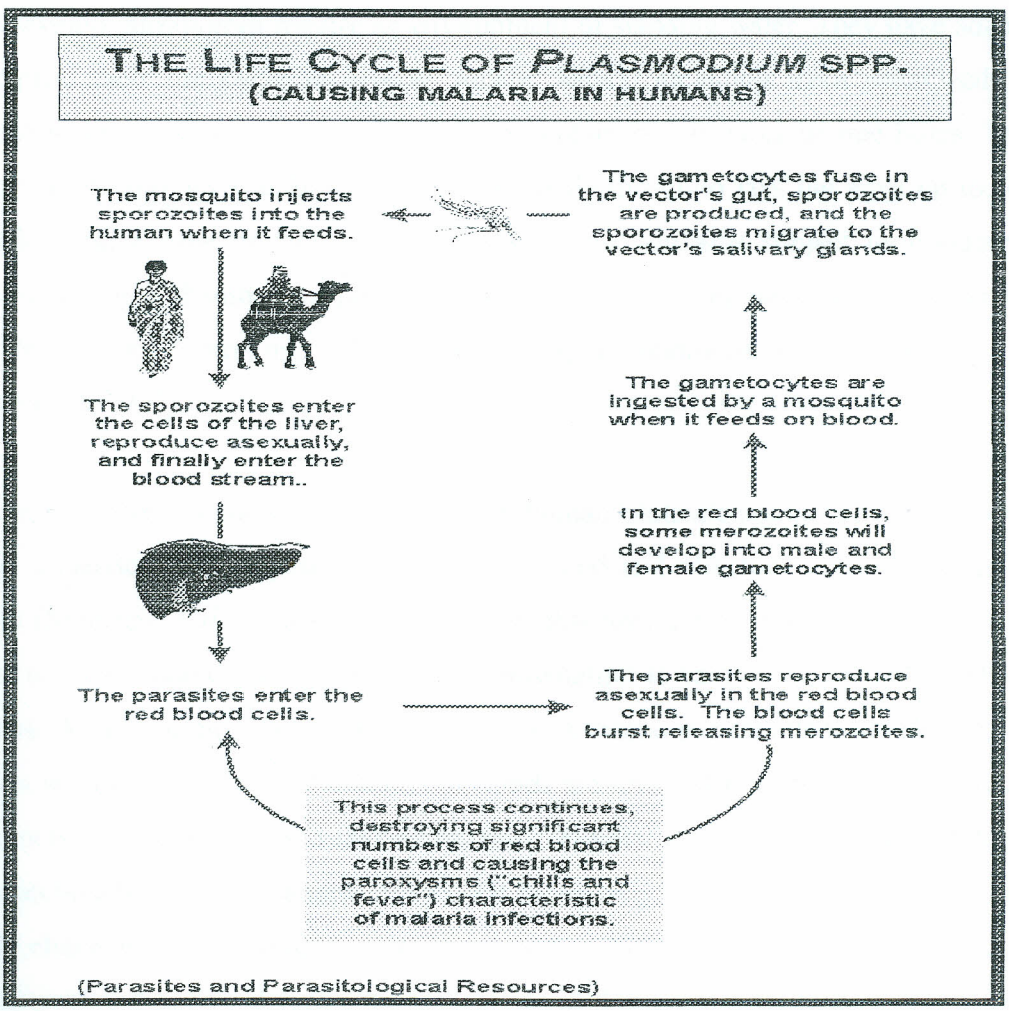
Following decoding the mapping DNA sequence of the parasite in 2002, it has been revealed that the parasite has about 5300 genes of which only 34% code for proteins with known functions (Gardener, 2003). More importantly, researchers have little idea which genes are switched on at what times. For example, *P. falciparum* goes through ten or more growth phases to survive inside the mosquito and in different parts of the human body (Garder, 2003). Genetic analysis has given clues to the function of about 1,000 new proteins. Particularly interesting is a sub-set of genes that seem to enable the parasite to cross from human bloodstream into the liver (Gardener, 2003).

The malaria parasite has a complex life cycle, which is split between a vertebrate host and the mosquito. Not all the *Plasmodium* species are parasites of man. However, the anopheline mosquito is the only known vector in man. Only 60 out of 380 species of mosquitoes can transmit malaria. The cycle begins when a mosquito bites an infected human host whose blood contains gametocytes. Within minutes of receiving the infected blood meal, the gametocytes are converted to male and female haploid gametes. The male fertilize the female gametes producing a diploid zygote that undergoes meiotic division to produce an ookinete, that migrates to the salivary glands of the mosquito. Three days after ingesting the blood meal, the ookinete starts to cross peritrophin, a semi-permeable membrane made up of chitin that forms around the blood meal. The parasite produces chitinase to help penetrate the membrane. Once the ookinete has crossed the peritrophic matrix, it undergoes another transformation and forms an oocyst in between outside the gut wall and the basal lamina. Depending on the species, the oocyst breaks and releases sporozoites into the hemocoele cavity within 24 hours of taking the blood meal. The sporozoites invade salivary glands of the mosquito.

During malaria transmission cycle, sporozoites from the mosquito salivary glands are injected into the human blood stream, during feeding, migrate to the liver and penetrate hepatocytes, where they remain for 9-16 days multiplying within cells. On release, they return to the blood stream and invade red blood cells and produce merozoites (micro-gametocytes) (Curtis, 1986). The merozoites released into the blood stream quickly invade other red blood cells while being protected from human immune system. Most of

the haploid merozoites enjoy a period of rapid mitotic amplification while feeding on hemoglobin in the red blood cells (Burdon & Kenneth, 1968). Each merozoite forms another schizont with 16-32 new merozoites inside. The schizont raptures after two days and the red blood cells burst releasing more merozoites, which quickly invade other red blood cells. The periodic fever and chills associated with malaria occur when red blood cells rapture and release the merozoites (Baker & Muller, 1992). A fraction of merozoites in the blood change to gametocytes, which are needed for the sexual stage of the parasite in the invertebrate stage of the parasite (Curtis, 1986). During the next blood meal, the cycle is repeated when a healthy mosquito feeds on an infected host. A diagrammatic representation of the life cycle is shown in fig. 1.

Figure. 1: Life cycle of *Plasmodium falciparum* (http//www.info.malaria.org)



1.3 The malaria vector

There are approximately 3200 species of mosquitoes in the family Culicidae. They transmit aboviruses responsible for dengue and hemorrhagic fever, polyathritis and several forms of encephalitis. The species responsible for human malaria transmission belong to the sub-family Anophelinae and the genus *Anopheles*. Of the known 250 species of anopheline mosquitoes only 60 species can transmit malaria parasites (Clements, 1992). Both sexes of anopheline mosquitoes feed on plant fluids and nectar. However, the females require a blood meal from a vertebrate host for production of a viable batch of eggs. In sub-Sahara Africa, the common vectors of malaria parasites are *An. gambiae*, *An. arabiensis* and *An. funestus* with *An. gambiae* being the most efficient species (Burgess, 1993).

To develop, mosquitoes require an environment of standing water. They have adapted to complete their life cycle in diverse aquatic habits, including fresh water, salt water marshes, brackish water or water found in containers, old tyres or tree holes. The life cycle of mosquitoes has four stages. The female mosquito lays her eggs up to several hundreds at a time on the surface of water or in an area subject to flooding. The unhatched eggs of some mosquito species can withstand long periods (weeks to months) of desiccation remaining viable until the right conditions before hatching occurs (Clements, 1992).

Factors involved in attracting mosquitoes to human hosts are complex and have not been fully understood. Mosquitoes use visual, thermal and olfactory stimuli to locate their hosts (Maibach *et al.*, 1966). For mosquitoes that feed during daytime, movement of the host in dark colored clothes may initiate orientation towards a person (Maibach *et al.*, 1966). Visual stimuli seem to be important as a mosquito nears its host (Gjullin, 1947). It has been estimated that 300-400 compounds are released from the human body as by-products of metabolism, and that more than 100 volatile compounds can be detected in human breath (Bock & Cardew, 1996). Of these, only a fraction have been isolated and fully characterized. Carbon dioxide and *L*-lactic acid are the two best-studied mosquito attractants and can be detected by mosquitoes at distances of up to 36 m from the host

(Gilles, 1980). The chemoreceptors on the antennae are stimulated by *L*-lactic acid and inhibited by *N, N*- diethyl-3-methylbenzamide based insect repellents (Davis & Sokolove, 1976).

1.4 Pathology and pathogenesis of malaria

The pathological changes in malaria are related to the development of the asexual parasites in the blood of the human host. In *P. falciparum* infections, the multi-faceted nature of the interaction between the erythrocyte, the host immune mechanisms and the parasite is central to the pathogenesis of malaria and results in mechanical and rheological changes to the infected erythrocyte. Parasitized erythrocytes bind to the host's molecules CD36, ICAM-1, thrombospondin, E-selectin VCAM-1 and chondochtrin sulphate (Hommel, 1993). The release of malaria antigens, pigments and toxins gives rise to a cascade of pathological events. Among these, the production of cytokines particularly tumor necrosis factor (TNFd) induced by the release of parasite products during schizont rapture appears to play a central role complemented by the effects of other circulating endogenous pyrogens such as interlukin 1 (IL-1) and IL-6. TNF or cachexin has been implicated as the cause of malaria fever. Although the nature of malaria toxins is still controversial, it is generally agreed that they are released at the time the schizonts rapture (Miller *et al.*, 1994). Death from *P. falciparum* in children living in areas of stable malaria is usually due to metabolic acidosis, cerebral malaria, malarial anaemia or a combination (Miller *et al.*, 1994). In non-immune persons, death is associated with acute renal insufficiency, cerebral malaria, pulmonary oedema and disseminated intravascular coagulation (Pasvol & Hogh, 1995).

1.5 Efforts to combat malaria

Four major strategies have been recommended by the WHO in effort to combat malaria: (i) early diagnosis and quick treatment of the disease; (ii) planning and implementation of selective and sustainable preventive measures including vector control and vaccine development; (iii) early detection for prevention and control of epidemics; and (iv) strengthening of research capacities to promote regular assessment of national malaria

situations such as ecological, social and economic determinants of the disease (WHO, 1998).

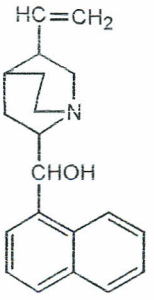
WHO realized that it requires the commitment of national health sector, affected communities, governmental organizations and the private sector to win the war against malaria (WHO, 1998). Consequently Roll Back Malaria, a global partnership seeking to halve malaria burden by the year 2010 was launched by WHO in conjunction with other UN agencies (UNDP, UNICEF and World Bank). Its major strategies have been to increase interventions to treat and prevent malaria in both stable and unstable situations (WHO, 2000). Other organizations from the public and private sector that have joined initiatives to control malaria include: Multilateral Initiative on Malaria (MIM) and Malaria Vaccine Initiative (MVI). WHO, UNDP/World Bank joined the task force to address the needs of malaria endemic regions and fund research activities on malaria control (WHO, 1998).

Several approaches including chemotherapy, vaccine development and vector control are available for management of malaria.

1.5.1 Chemotherapy

Chemotherapeutic approach in controlling malaria has been in existence for a long time. Quinoline containing anti-malarial drugs like quinine (**1**), chloroquine (**2**), amodiaquine (**3**) and mefloquine (**4**) have been used especially during the post-world war era (Baird *et al.*, 1995). Anti-foliates like chloroproguanil (**5**), pyrimethamine (**6**) and trimethoprim (**7**) have also been used. Other anti-malarial drugs include halofantrine (**8**), atovaquone (**9**) and artemisinin (**10**) and its derivatives (Baird *et al.*, 1995). Due to emergence of cross-resistance to new synthetic analogues eradication of malaria remains a challenge (WHO, 2000). *In vivo* resistance has been reported against all anti-malarial drugs except artemisinin and its derivatives (Zucker & Campbell, 1992). The problem of drug resistance may be attributed to increased selection pressures on *P. falciparum* from indiscriminate use and incomplete doses for self treatment (Zucker & Campbell, 1992). Resistance to common anti-malarial drugs such as chloroquine and decreasing efficacy of

quinine has been reported in all strains of malaria (Wernsdofer, 1979). Many factors and mechanisms account for changes in drug sensitivity of malaria parasites (Zucker & Campbell, 1992). Pressure on previously existing drug resistant parasite population under spontaneous mutation and existence of plasmid like factors have been implicated in resistance development (WHO, 1998). Many factors and mechanisms account for changes in drug sensitivity of malaria parasites (WHO, 1998). Due to this chronic resistance to drugs like proguanil (**11**), sulphodoxine (**12**) and mefloquine (**4**) have been used in the treatment of malaria (Fredena *et al.*, 1997). Since resistance has been developed against all available drugs and their existing combinations, alternatives to chemotherapy have been investigated.



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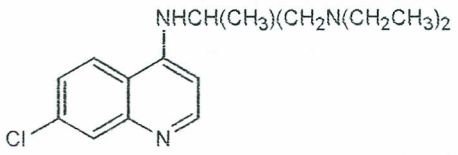

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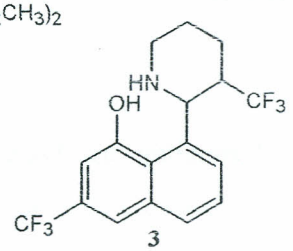

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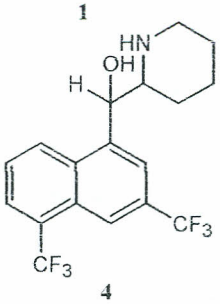

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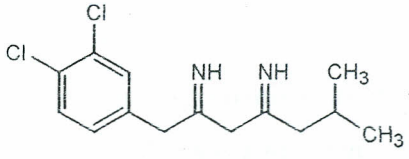

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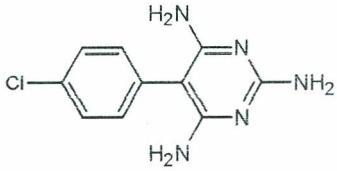

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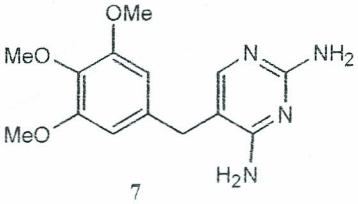

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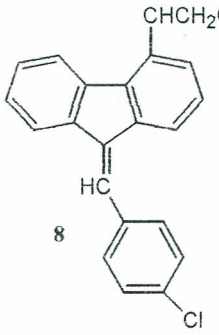

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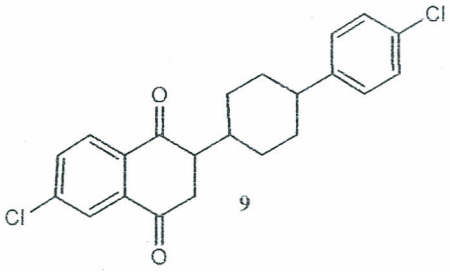

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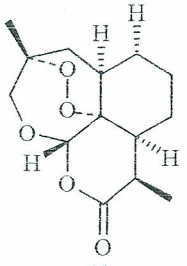

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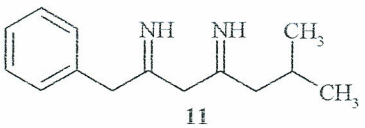

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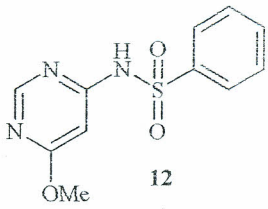

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1.5.2 Vaccine development

In the past decade, there has been progress in efforts to develop vaccines for malaria with focus on three types of vaccine (Franke *et al.*, 1999). The development of such vaccines has been made difficult by the ability of the parasite to change its immunological identity thus evading host immune mechanisms (Curtis & Lines, 1985).

Sporozoite antigens have been considered as one of the major candidate antigens for malaria vaccines (Wakelin, 1996). Such vaccines include anti-sporozoite vaccine; anti-asexual blood stage vaccine; and transmission blocking vaccines designed to prevent infection; severe manifestation of the disease and development of the parasite by blocking transformation of gametocytes into sporozoites, respectively (WHO, 1998). The simplest vaccine constitutes inactivated sporozoite that once injected into host protects them from subsequent infections by the parasite (Hoffman & Doolan, 2000). Test animals immunized with Liver Stage Antigen-3 (LSA-3) produce anti-bodies, which can activate the system to eliminate the parasite (Hoffman & Doolan, 2000). The advantage of LSA-3 is that the protein shape does not vary much from one parasite strain to another as in other sporozoite proteins. Molecular configuration being crucial in protein anti-body interaction, the vaccine is effective to all strains of malaria parasite (Hoffman & Doolan, 2000).

Circumsporozoite surface protein (CSP) and the second sporozoite surface protein (SSP-2) as well as cytotoxic T-cells have been demonstrated to stimulate immunity against pre-erythrocytic stages of malaria *in-vitro* (Wakelin, 1996). Cytotoxic mechanisms of the

two vaccines have been shown to target parasite antigens expressed on the surface of infected hepatocytes (Wakelin, 1996). SSP66, a sporozoites surface protein-based vaccine has been evaluated as a vaccine for *P. falciparum* malaria, with protective efficacy of 1.6-48.44% (Margarita *et al.*, 1998). The malaria vaccine candidate RTS, formulated with an oil-in-water emulsion plus immuno-stimulants, monophosphoryl A and a saponin derivative QS21, has showed superior efficacy over all other existing vaccines under trial (Staute *et al.*, 1998). It targets sporozoite development. Recent studies reveal that RTS, S/AS02A, an RTS derived vaccine, is effective in all age groups including children aged under 24 months (Alonso, 2004). At six months after administration of the vaccine, *P. falciparum* infection was 87% lower in RTS, S/AS02A recipients than in controls. It appears safe and immunogenic in young children who are at the highest risk of malaria infection. The vaccine has also shown constant efficacy against all strains of malaria (Alonso, 2004).

Despite several decades of research, no functional and fully protective vaccine against malaria has been found thus increasing the need for alternative control strategies.

1.5.3 Vector control

The main goal of vector control is the reduction of malaria morbidity and mortality by lowering the level of transmission via manipulation of anopheline mosquito population. Consequently, vector control strategies aim at reducing population as well as human vector contacts. Major vector control strategies include environmental management and the use of biological control agents, larvicides, insecticides and repellents.

1.5.3.1 Environmental management

The WHO defines environmental management for vector control as the planning, organization, carrying out and monitoring of activities for modification and manipulation of environmental factors or their interaction with man with a view of preventing or minimizing vector multiplication and reducing man-vector contact (WHO, 2004). The vectors of malaria are linked to aquatic ecosystems like, natural water bodies, man-made water bodies and water bodies in human settlements and house hold environment (WHO,

2004). The creation of man-made water bodies often result in hydrological changes that favor intensified vector breeding. Appropriate management of such ecosystems will go along way in controlling diseases transmitted by such vectors. Examples of environmental management for malaria vector control include activities like building dams at higher altitudes or away from human settlements, draining water in permanent (swamps) and temporary breeding habitats (tyres, tins, water storage tanks), intermittent irrigation, desiccation by planting trees and improved housing (Ghebreyesus *et al.*, 1999).

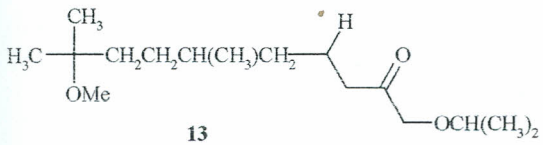
1.5.3.2 Biological control agents

Biological control agents involve the use of predators, parasites and pathogens to control disease vectors. Several factors are important for an organism to be considered as a suitable biological control agent. The organism must have high virulence to target species but harmless to non-target organism. It must be easily produced and stored for long periods of time without loss of virulence. The best-known vertebrate biological control agent for mosquitoes is the mosquito fish, *Gambussia affinis*, a native of USA, which has been introduced in many tropical and sub-Saharan regions to control mosquito larvae (Edoh *et al.*, 1997). Inveterbrate agents include entomo-parasitic nematodes such as *Romenomermis culicivorax* have been used to control mosquito larvae (Rawlings, 1998). Fungal mosquito bio-control agents like *Entomophaga maimaga*, *Beauveria bassiana*, *Mediga sariva* and *Cordyceps militaris* have also been developed to control mosquito larvae (Brown & Knodson, 1994). Bacterial larvicides have also been identified and developed in the last 10 years. *Bacillus thurengiensis var israelensis* (sero-type H14) has been tested as mosquito larvaicides and demonstrated to be effective under field and laboratory conditions. Toxins produced by the bacteria show high virulence and selective activity against larval instars (Berry *et al.*, 1987). *B. sphaericus*, a commercially produced biological control agent for mosquitoes has been shown to persist in polluted habitats and has a very narrow host range. Viral control agents like bacoloviruses have been developed in the past decade to control mosquito larvae (Brown & Knodson, 1994). Biological control of malaria vector has several limitations. A major draw back of *B. thurengiensis* has been lack of residual activity and persistence for months in larval habitats and cadaveurs (Berry *et al.*, 1987). Bacterial agents have slow

mode of action compared to chemical insecticides and are sensitive to ultra-violet light. The requirement of a living system for production has made viral mosquito control agents against expensive. Although inoculation of insect populations with entomophagic fungi has provided a classical biological control of mosquitoes, most species of the fungi are difficult to produce and their primary conidia are short lived making timing of inoculative application ineffective (Weiss *et al.*, 1994). Besides, infecting mosquitoes with fungi in the field may present a serious difficulty requiring incorporation of odour/food baits. Use of nematodes to control mosquitoes has not been effective especially in developing countries where the technology involved cannot be attained (Rawlings, 1998).

1.5.3.3 Insect growth regulators

Insect growth inhibitors (IGI) or regulators (IGRs) are materials that interrupt or inhibit the life cycle of an insect. Several IGRs have been employed in control of mosquitoes. Methoprene (**13**), an active ingredient in precor™, precor 2000™ and Extinguish Fire Ant Bait™ (EFAB), prevents the egg and larval stages of mosquitoes from developing (Weiss *et al.*, 1994). It has 3-7 month residual and minimal environmental hazards. Methoprene granules have become an important tool in reducing mosquitoes in areas where there is standing water or other permanent mosquito breeding habitats (Weiss *et al.*, 1994). Hydroprene (**14**) is used as the active ingredient in Gentrol™, Gentrol Point Source™ and Gentrol™ aerosol. It is not photostable and therefore recommended for indoor use only (Weiss *et al.*, 1994).



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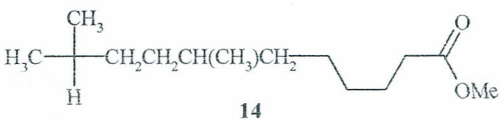
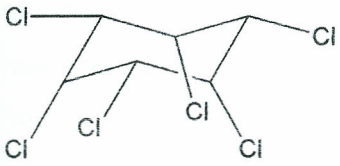


14

1.5.3.4 Chemical larvicides

The control of mosquito larvae using chemicals dates back to 1379 AD when mosquito larvae were killed by applying oil on stagnant water surfaces (Wiggleworth, 1976). It was demonstrated that the hydrocarbons killed the larvae by interfering with the normal physiology of the tracheal system of the mosquito larvae (Murry, 1939). Synthetic inorganic compounds such as Paris green [$Cu(H_2O)_2.3Cu(AsO_2)_2$] and copper

metarsenite, $[Cu(AsO_2)_2]$ have also been used as larvicides (Metcalf & Flint, 1962). Chlorinated synthetic organic compounds like lindane (**15**), dialdrin (**16**), chlorodane (**17**) and DDT (**18**) have also been used as larvicides. However, they are not biodegradable and have caused serious environmental pollution (Metcalf & Flint, 1962).



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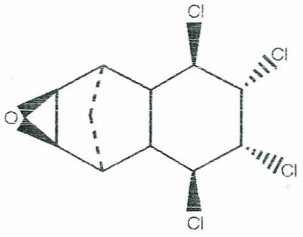

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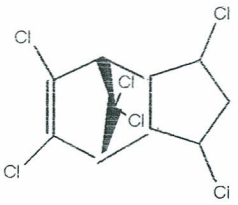

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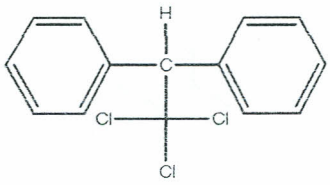

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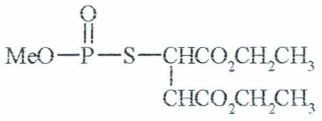
Organophosphates such as methyl parathion (**19**) have also been used as larvicides. Although bio-degradable, they are not selective in their mode of action (Metcalf & Flint, 1962). Resistance to organophoshates has been reported in certain species of mosquitoes (Matsumara & Brown, 1961).



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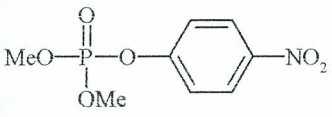
1.5.3.5 Adulticides

Insecticides currently in use include chlorinated hydrocarbons, organophosphates, organocarbamates, synthetic pyrethroids and organic insecticides of plant origin (Matsumara & Brown, 1961). Chlorinated hydrocarbons are probably the most widely used class of insecticides (Matsumara & Brown, 1961). Organophosphates, malathion

(**20**) and diazinon (**21**), are highly toxic to mammals including man and other non-target species but are much less stable than organochlorines (Motoyama *et al.*, 1997). They have also been used as adulticides in control of mosquitoes. Other organophosphates that have been used to control mosquitoes include phosphorothioic acid (**22**) and methoxyphosphorothioic acid (**23**).

Carbamates were originally extracted from the calabar bean, which grows in West Africa. The extracts of this bean contain physostigimed, a methylcarbamate ester. Carbamates are derivatives of carbimic acid (Matsumara & Brown, 1961). Carbamates that have been used in mosquito control include: 2, 2-dimethyl-1, 3-benzodioxol carbamate (**24**), and methyl carbamate (**25**).



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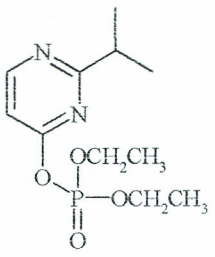

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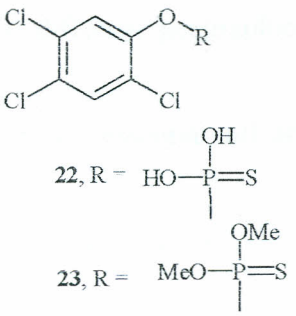
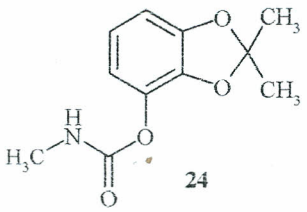


22, R =

23, R =



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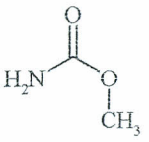

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Statement of the problem

The variation of mosquito repellent activity and chemical composition of essential oil of *C. newii* with geographical location and extraction method need to be investigated before it is recommended as a mosquito repellent for malaria control.

Hypothesis

The chemical composition and mosquito repellency of essential oil of *C. newii* varies with geographical location of the plant and method of extraction, respectively.

Objectives

(i) To compare the chemical profiles of *C. newii* essential oil obtained by various extraction methods.

(ii) To compare the repellency of *C. newii* essential oil from different geographical regions within Kenya.

(iii) To establish the differences in chemical profiles of *C. newii* essential oil and correlate this to repellent activity.

(iv) To determine the best mode of deployment of *C. newii* as a mosquito repellent.

CHAPTER 2

LITERATURE REVIEW

2.1 Repellents

The first recorded use of insect repellents dates back to 1379 AD (Kreier, 1983). Most plants contain metabolites, which are toxins to other organisms (Palsson, 1999). Traditionally, rural communities have used plants in many parts of the world to control medical vectors and agricultural pests (Merhil *et al.*, 1976). Such plants and their products have been used widely to keep away potential disease vectors from humans and livestock (Seyoum *et al.*, 2002). In Jaypore and Madras regions of India, *Curcoma longa lar* mixed with vegetable oil has been used for protection against mosquitoes and other blood-sucking insects (Gupta & Luois, 1994). To date women in some parts of Mexico apply *Bixa orelana* in vegetable or animal oil on their men to protect them from mosquito bites when they go hunting (Gupta & Louis, 1994).

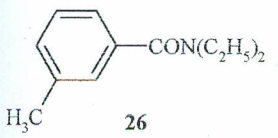
Repellents are chemical substances that protect animals, plants, or materials such as fabrics, grains and timber from insect attack by rendering them unattractive, unpalatable or offensive (Metcalf & Flint 1962). To be effective a repellent substance must be capable of stimulating some sensory system other than that which has influence on locomotion or feeding since response of an organism depends on the sensory system that has been placed in operation (Dethier, 1956). The properties of a good repellent against blood-sucking insects include: effective protection of the treated area for several hours; complete freedom from toxicity and irritation; freedom from unpleasant smell; low cost; and availability (Shambaugh & Brown, 1958).

Both synthetic and naturally occurring repellents have been used to control mosquitoes.

2.1.1 Synthetic repellents

N, *N*-Diethyl-*m*-toluamide (DEET) **(26)** remains the standard for commercial insect repellents. It is a broad spectrum repellent with activity against mosquitoes, biting flies, jiggers, fleas and ticks. To date, more than 20,000 other compounds have been tested and there is no better repellent than DEET. DEET is available in 5-10% concentration in multiple formulations including solutions, lotions, creams, gels, aeresol, pump sprays and

impregnated towelettes (Shreck, 1995). DEET has had a remarkable safety profile during more than 40 years of its use as an insect repellent world wide (Shreck, 1995). However, it can damage plastics, synthetic fibers, leather, painted surfaces and nylon (Herms & James, 1961). Radioautographic studies have revealed that DEET is absorbed promptly from the skin and distributed to all organs including the brain and the feotus (Gryboski *et al.*, 1994). High concentrations of DEET in vital organs has been implicated to cause cancer and accelerate tumor development (Gryboski *et al.*, 1994). High levels of DEET are found in liver, kidney, lacrimal glands and nasal mucosa 4 hours after injection. Many DEET analogues have been developed to control mosquitoes. Synthetic repellents closely related to DEET include, *N*-methyl succinimide (**27**), *N*-ethly succinimide (**28**), *N*-butylsuccinimide (**29**) and *N*-ethyl-*N*-phenyl acetamide (**30**) (Gryboski *et al.*, 1994). The mosquito repellent activity of diethyl acetamide (DEA) (**31**) has been reported (Kumar *et al.*, 2002). In a comparative study carried out, DEET exhibited 93 and 38% repellency after 2 and 8 hours, respectively, while DEA at the same concentration exhibited 90 and 71% after similar periods (Kumar *et al.*, 2002). Another synthetic repellent closely related to DEET is IR3535 (**32**) (Fradin & Day, 2002).



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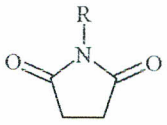

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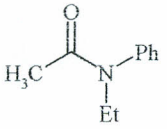
28, R = CH_2CH_3

29, R = $CH_2CH_2CH_2CH_3$



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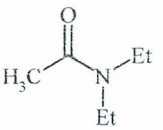

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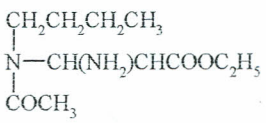

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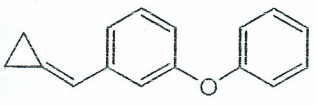
Permethrin (**33**) is a synthetic repellent pyrethroid that causes toxicity to the central nervous system leading to death or knockdown of insects. It has low toxicity in mammals, is poorly absorbed by the skin and rapidly inactivated by ether hydrolysis (Lindsay *et al.*, 1991). It maintains its potency for at least two weeks. A combination of

permethrin-treated clothing and skin application of a DEET based repellent creates a formidable barrier against mosquito bites (Kline & Shreck, 1989). In a field trial conducted in Alaska, persons wearing permethrin-treated uniforms and polymer based 35% DEET product had more than 99.9% protection for over 8 hours (Kline & Shreck, 1989). Other synthetic pyrethroids that have been used to control mosquitoes include allethrin (**34**), bioallethrin (**35**), cymethrin (**36**) and tetramethrin (**37**) (Knox *et al.*, 2003). On the shelf, pyrethroids are usually available as dusts or liquids combined with hydrocarbon bases (Knox *et al.*, 2003).



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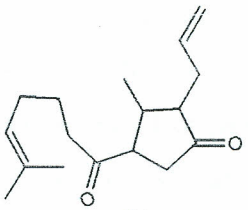

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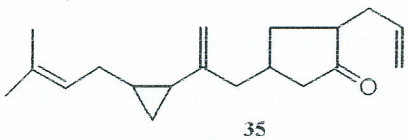

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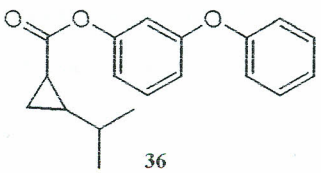

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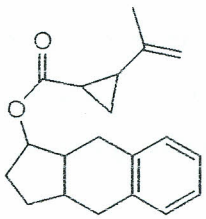

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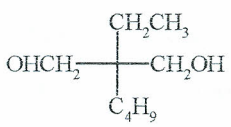
Other synthetic repellents that have been used to control mosquito bite include, 2-butyl-2-ethyl-1, 3-propanediol (**38**), 2-phenylcyclohexanol (**39**) and dimethyl phthalate (**40**) (Knox *et al.*, 2003).



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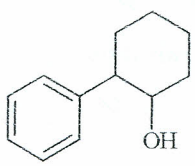

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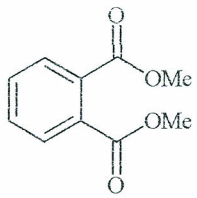

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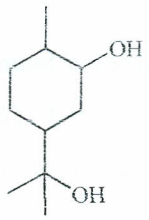
Due to adverse effects of synthetic repellents, the search for natural products with the same properties continues.

2.1.2 Repellent plants

Plants such as *Ocimum spp* (White, 1973), *Azadiracta indica* (Shell, 1997), *Ajuga remota* (Curtis, 1986), *Lippia javanica* (Shell, 1997), *Lantana camara* (Taubes, 2000), *Cymbogon nardus* (Shell, 1997) and *Lipia ukambensis* (Taubes, 1997) among others are used to repel mosquitoes. These plants are usually smouldered to produce chemical compounds that repel mosquitoes (White, 1973). In China, burning of herbs like *Artemisia* and *Calamus* by the rural population helps to keep away mosquitoes and other blood-sucking insects (Marbiah *et al.*, 1988). In East Africa, *Ocimum basillicum* (Labiatae) is traditionally used by the Luo community to drive away mosquitoes and other blood-sucking insects by laying branches in their houses (Seyoum *et al.*, 2002). In some villages in Tanzania, women use herbs like *Ocimum* and *Hyptis* species as natural repellents (Edoh *et al.*, 1997). Perfumed resins and wood kernels from the tree *Danieli oliveri* and *Hyptis suaveolens* kept indoors at night significantly repelled mosquitoes (Sukumar *et al.*, 1991). Plants whose essential oils have been reported to have repellent activity include citronella, cedar, verbena, geranium, lavender, pine, cajeput, cinnamon and peppermint (Sukumar *et al.*, 1991). Repellent plants have contributed significantly in pest and vector control with some like pyrethrum being commercialized (Edoh *et al.*, 1997).

The oil of eucalyptus plant *Eucalyptus citriodora* has repellent activity against mosquitoes (Zhuang *et al.*, 1974). Its main ingredients are citronellal, citronellol, geraniol, isopulegol, δ-pinene and sesquiterpenes (Zhuang *et al.*, 1974). The activity of the plant as a mosquito repellent is not attributed to the oil but to a waxy material containing high concentration of *p*-menthane-3, 8-diol (**41**). It is sold in 30-40% alcoholic solutions. Mosquito nets treated with the wax containing *p*-menthane-3, 8-diol are effective for 8 days on average (Trigg, 1996).



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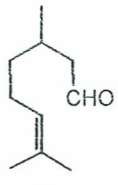
2.1.3 Plant derived repellents

Thousands of plants have been tested as sources of insect repellents (Wright, 1975). However, none of the plant-derived repellents tested to date demonstrate spectrum effectiveness and duration of DEET. The most common plant derived repellents include citronellal (**42**), geraniol (**43**), pyrethrins (**44a-f**) (Wright, 1975) and *p*-menthane-1, 8-diol (**45**). Other repellents derived from plants include: α- pinene (**46**), geraniol (**43**), linalool (**47**), cineol (**48**), *p*-menthane-1, 3- diol (**49**) (Barasa *et al.*, 2002) and camphor (**50**).



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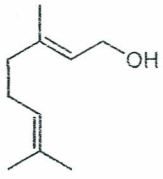

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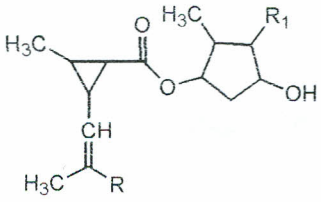

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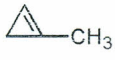
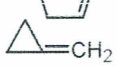
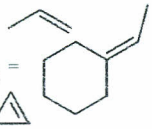


44b. R = $COOCH_3$, R_1 =

44c. R = CH_3, R_1 =

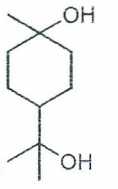


44d. R = CH_3, R_1 =



44e. R = $COOCH_3$, R_1 =

44f. R = CH_3, R_1 =



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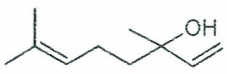

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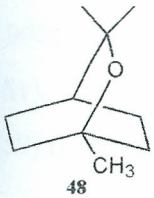

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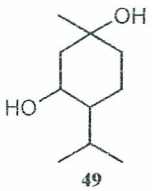

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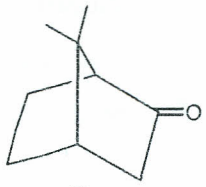

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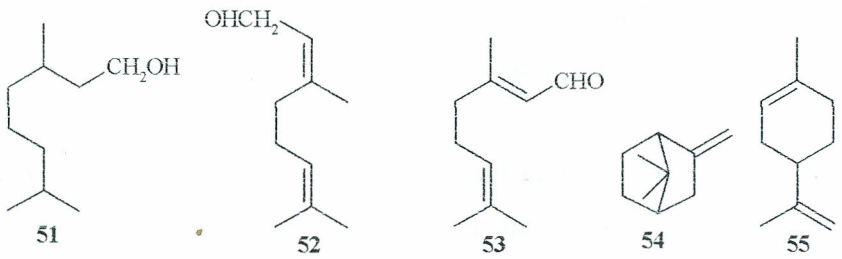
2.1.3.1 Citronella

Citronellal (**42**) is an active ingredient most commonly found in herbal insect repellents in most parts of the world (Wright, 1975). Citronella oil has a lemon scent and was first extracted from the lemon grass *Cymbogon nardus* (Wright, 1975). Studies reveal that citronella oil is an effective repellent of a wide range of blood-sucking insects (Wright, 1975). The oil provides short complete protection time as compared to other known DEET-based repellents (Wright, 1975). Natrapel® a commercial repellent with 10% of citronella oil reduced mosquito bites by 84% during a 4-minute test (Wright, 1975). Buzz Away®, formulation with 5% citronella oil, provided an average protection time of 1.9 hours against *Aedes aegypti* under field conditions (Wright, 1975). In another field experiment Buzz Away® containing 90% citronella oil, provided an average protection of 88% repellency during a 2-hour exposure. Citronella candles have been promoted as an effective way to repel mosquitoes in the backyard (Wright, 1975).

The main ingredients of citronella oil have been identified as citronellol (**51**), citronellal (**42**), geraniol (**43**), nerol (**52**), citral (**53**), camphene (**54**) and limonene (**55**) (Lindsay *et al.*, 1990).



51 52 53 54 55

2.1.3.2 Bite blocker

Geranium oil has been shown to posses mosquito repellent activity (Lindsay *et al.*, 1990). The major compounds identified in geranium oil include geraniol (**43**), citronellol (**51**), citronellyl formate (**56**), linalool (**47**), eugenol (**57**), myrtenol (**58**) and terpinoel (**59**) (Lindsay *et al.*, 1990).

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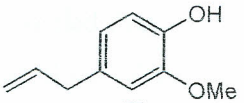

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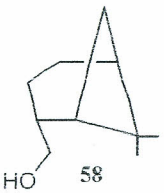

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Geraniol (**43**) has been used in production of commercial mosquito repellents like Bite Blocker® (Fradin & Day, 2002), that was released in the United States in 1997 (Lindsay *et al.*, 1990). Bite blocker, a commercial repellent containing 90% geraniol has demonstrated protective efficacy of up to 93% for 3 hours against *An. gambiae* mosquitoes in field experiments (Wright, 1975). It gives 97% protection against *Ae.* mosquitoes under field conditions even 3.5 hours after application (Lindsay *et al.*, 1990). The product combines soybean oil, geranium oil, and coconut oil in formulation that has been available in Europe for several years (Lindsay *et al.*, 1990).

2.1.3.3 Pyrethrins

Pyrethrins are natural repellents produced by certain species of *Chrysanthemum* plant. The flowers are harvested shortly after blooming, dried and powdered or the essential oil extracted with organic solvents. The pyrethrin containing dusts and extracts usually have active ingredient content of about 30 and 65%, respectively. The insecticidal components are collectively known as pyrethrins (Sukumar *et al.*, 1991). Natural pyrethrins are contact poisons, which quickly penetrate the insect nervous system. Examples of natural pyrethrins include pyrethrin I (**44a**), pyrethrin II (**44b**), cinerin I (**44c**), cinerin II (**44d**), jasmmolin I (**44e**), and jasmolin II (**44f**).

Pyrethrin and pyrethroid insecticides are generally safe to non-target organisms in the environment and degrade quickly. They have low toxicity to mammals, are non-toxic to birds but have slight toxicity to young children and bees. They break down in the environment at high temperatures and accelerated by sunlight (Song & Narahash, 1995).

2.1.3.4 *p*-menthane-3, 8- diols

p-Menthane-3,8-diol has 4 stereoisomers extracted from *E. citriodora* (Barasa *et al.*, 2002). Repellency assays revealed that all the four compounds are equally active against *An. gambiae s.s.* The natural blend of the stereoisomers is repellent against *An. gambiae* (Barasa *et al.*, 2002). Commercial mosquito repellent products that incorporate these isomers include Mosiguard® and Mozigone® .

2.2 Application of repellents

Different modes have been employed to deploy repellents as a strategy to control malaria. Such modes include: smoking of houses at night, use of lotions or creams containing repellent material, hanging branches of repellent plants in the houses, burning repellent plant material, use of mosquito coils, candles and vaporizing mats.

The practice of fumigating houses to repel mosquitoes is wide spread among many rural communities in the tropics (Bockarie *et al.*, 1994). In Sierra Leone, some houses where wood smoke was used during the night consistently had higher number of mosquitoes in window exit traps than houses that did not contain smoke at night (Bockarie *et al.*, 1994). Separate studies have revealed that during wet season, when mosquito numbers are highest, there is abundance of resins and wood for sale in the local markets in Gambia (Bockarie *et al.*, 1994). Smoke produced by Churai, which is composed of several resins and wood mainly from the tree *D. oliveri,* effectively reduced the number of mosquitoes coming to bite (Bockrie *et al.*, 1994).

Methods like use of lotions containing repellent substances are now being used due to environmental hazards posed by smoke emanations during burning of traditional lamps (Pates *et al.*, 2002).

2.2.1 Smoking of repellent fuels

This tactic involves the use of traditional lamps to burn kerosene oil mixed with repellent volatiles producing smoke that repels the mosquitoes (Pates *et al.*, 2002). It is a simple low cost method of vaporizing repellents for use as spatial repellents against mosquitoes.

As a source of light, traditional lamps are cheaper and much common in rural areas. A traditional lamp is made locally to hold 100-200 ml of kerosene, with a metal chimney and a cotton rag or string wick. A repellent material is mixed with kerosene and dispersed as it burns. Protective efficacy of 0.1-99.5% has been achieved using this method as a mode of applying repellents against mosquitoes (Pates *et al.*, 2002).

Incense and Joss sticks have been used since the time of ancient Egyptians and Assyrians to control malaria vectors. Pyrethrum flower mosquito stick and Agarbaltis (incense sticks) are made with natural pyrethrum to repel mosquitoes and kill midges. The stick burns for over an hour giving off sweet fragrant smell and at the same time deploying the repellent. The stick contain no synthetic toxicants and hence ideal for use in the home or outdoors (Paru *et al.*, 1995).

Burning of coils has also been used to control malaria vectors. Mosquito coils consist of an insecticide/repellent, organic filler capable of burning with smoldering, binder and additives such as synergists, dyes and fungicide (Robert *et al.*, 2003). DEET, geranium oil, citronella oil and geraniol have been used in such coils to control mosquitoes. In Indonesia, estimated seven billion coils are purchased annually for mosquito control activities (Robert *et al.*, 2003). In China, coils containing octachlorodipropyl ether as a synergist or active ingredient have been used to repel mosquitoes (Robert *et al.*, 2003).

Citronella candles have been promoted as an effective way to repel mosquitoes. The ability of commercially available 3% citronella candles, 5% citronella incense and plain candles to prevent bites by *Aedes* mosquitoes under field conditions were compared. Persons using the citronella candles had 42% fewer bites than controls (Wright, 1975). However, burning ordinary candles reduced the number of bites by 23% (Wright, 1975).

2.2.2 Use of repellent lotions

The use of lotions containing repellent material to deter mosquitoes from landing on human hosts has been a common practice in most parts of the world as a malaria control strategy (Gupta & Louis, 1994). The use of tumeric by girls in India was believed to

explain the low rates of spleenomegaly in girls as compared to boys who did not use tumeric (Gupta & Louis, 1994). The use of sulfur and molasses in lotions as mosquito repellents in Transvaal and Natal provinces of South Africa has been documented (Lindsay & Gibson, 1988). In the United States, DEET is available in 5-95% formulations including solutions, lotions, creams, gels, aerosol and pump sprays and impregnated towellets (Curtis & Lines, 1985). Standard mosquito repellents containing up to 75% DEET in an alcohol base have been used to achieve 100% protective efficacy against mosquitoes. A polymer based lotion containing 35% DEET has also been developed to control mosquitoes (Curtis, 1986). At the ICIPE, Mozigone® lotion has been developed from *p*-menthane-3, 8- diol.

2.2.3 Vaporizers

Vaporizers have been used to dispense repellent containing substances to achieve protection against mosquitoes. It is the most effective way to keep a room free of mosquitoes and other small pests. Application of heat releases smokeless natural pyrethrin into the air, killing mosquitoes in the room and repelling those outside (Mark & Fradin, 2003). Moss Chips® is a typical example of a vaporizer containing refined natural pyrethrum extract. It is available in a range of fragrances (Regular, Sandal, Floral and Lavender). Each chip contains 60 mg of pyrethrum for maximum efficiency. The pyrethrins are vaporized by a 2 pin cordless plastic type vaporizer with pilot lights 220-240 V (Mark & Fradin, 2003).

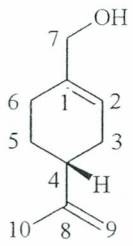
2.2.4 Thermal expulsion

Thermal expulsion is another method of deploying insect repellents. The effectiveness of thermal expulsion of *Ocimum americanum* as a mosquito repellent has been established (Seyoum *et al.*, 2002). Studies have revealed that all plant species tested show significant repellency against *An. gambiae* deployment by thermal expulsion (Seyoum *et al.*, 2002). Since early studies in the research group show that *C. newii* has mosquito repellent activity, it is possible that this activity can be enhanced by thermal expulsion of the essential oil.

2.3 *Conyza newii*

The plant *Conyza newii* is also called *Kisegeyo* by the Shamba (Kokwaro, 1993). The plant is a herb with thin leaves, dented on their margins and pointed apex. The leaf veins are branched and the flowers are yellow. Boiled root decotion is used in the treatment of stomach pains (Kokwaro, 1993). The leaves produce a pungent smell and are chewed for chest troubles (Kokwaro, 1993). The plant is abundantly distributed in Kenya.

The major compounds identified in the essential oil include peril aldehyde (**60**), limonene (**55**), cineole (**48**), peril alcohol (**61**), and geraniol (**43**) (Omolo, 2001). Variations in chemical composition of the oil with respect to geographical distribution, mode of application and mosquito repellent activity has not been investigated.



60, R = CHO

61, R = CH_2OH

CHAPTER 3
MATERIALS AND METHODS

3.1 General procedures

3.1.1 Solvent purification

All the solvents (acetone, dichloromethane, ethylacetate and *n*-hexane) were GC grade analytical reagents (Aldrich Chemical Co. Inc).

3.1.2 Glassware preparation

All glassware used in the study was soaked in concentrated nitric acid for 24 h, washed with warm soapy water, rinsed with distilled water and dried in an oven at 50 °C for 12 hrs.

3.1.3 Mass spectrometry analysis

Mass spectrometry analysis of all compounds was carried out on a HP 8060 series MS machine on VG plat form.

3.1.4 NMR analysis

^{1}H, ^{13}C, DEPT, COSY and HETCOR analyses of the isolated compound was carried out on a Varian Gemini 200 MHz NMR machine in deutriated chloroform.

3.2 Detailed procedures

3.2.1 Collection and cultivation of *Conyza newii*

Plants were collected from five regions (West Pokot, Kilome, Nyakach, Naivasha and Kericho). Plant seedlings were also collected from West Pokot and cultivated in Webuye and Nairobi. Propagated plants were harvested after 10 weeks for extraction of repellents materials.

3.2.2 Extraction

Extraction of essential oils from plant samples was done by steam distillation, direct burning, thermal expulsion, headspace trapping and solvent extraction. Traditional stoves

locally known as *Jikos* were modified and used for direct burning and thermal expulsion experiments (Seyoum *et al.*, 2002). Charcoal (250 g) was used to light the stove to burn or thermally expel the plant material (Seyoum *et al.*, 2002).

3.2.3 Direct burning

Direct burning was done by placing the material (500 g) directly on the burning charcoal in a stove. Fresh material (50 g) was added after every 5 minutes. Smoke emanations given off was directed into a funnel hanging above by a stream of white spot nitrogen and collected in 10 ml of DCM (Seyoum *et al.*, 2002). The solution was dried with sodium sulfate, the yield recorded and sample stored at -4 °C for GC analysis and bioassay. Direct burning was carried out using modified traditional stoves (Fig. 2).

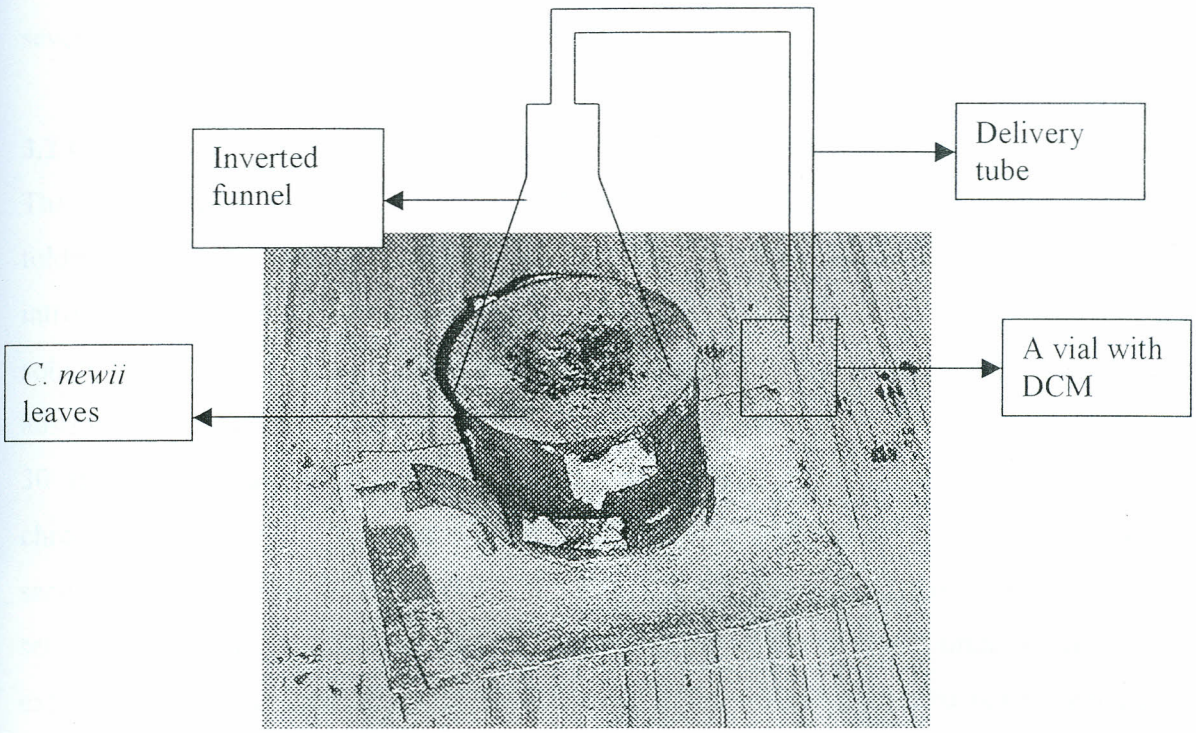


Figure 2: A modified traditional stove used to carry out direct burning experiments

3.2.4 Thermal expulsion

Thermal expulsion was done by placing the material (500 g) on a hot plate above burning charcoal (Fig. 2), but not in direct contact with the burning charcoal. Fresh material (50 g) was added after every 5 minutes. Smoke emanation was directed into a funnel hanging above by a stream of white spot nitrogen cooled using a condenser and collected in 10 ml of DCM. The solution was dried with sodium sulfate, the yield recorded and the sample stored at -4 °C for GC analysis and bioassay.

3.2.5 Steam distillation

Steam distillation of plant material was done using Dean-Stark apparatus. Plant material was put into a 5 litre round-bottom flask and 150 ml of tap water added. The flask was fitted with Dean-Stark apparatus and a double pocket condenser. The plant material was distilled for 8 hours until no more oil was being collected. The essential oil was collected

on water, separated and dried over Na_2SO_4. The yield of the oil was recorded and the oil stored in a dark colored vial at -4 °C until when required for use. Plant material from the seven different regions was distilled separately.

3.2.6 Headspace trapping

The volatiles from *C. newii* were obtained through headspace trapping. Wire mesh was folded to make adsorbent pockets measuring 2.5 × 4.0 cm. Porapak S (200 mg) was introduced into the sachets and sealed with staples. The adsorbent was cleaned in a sohxlet using HPLC grade dichloromethane for 4 days. The cleaned sachets containing adsorbent material were handled with a pair of forceps and dried in the oven at 30 °C for 30 min. The adsorbents were activated under nitrogen in a purpose-adapted gas chromatograph oven. A tripod stand was used to hold an inverted funnel and a suspended sachet of adsorbent material just above the leaves of the plant. A control experiment was set up in the same way at the same site but with no *C. newii* plants underneath. The experiment was stopped after 12 h and repeated for several plants to collect enough material for analysis. The test and control sachets were eluted separately with 2 ml of HPLC grade dichloromethane and the eluate collected. Both eluants were kept at -4 °C until required for GC analysis. Apparatus for headspace trapping of volatiles were assembled as shown below (Fig. 4).

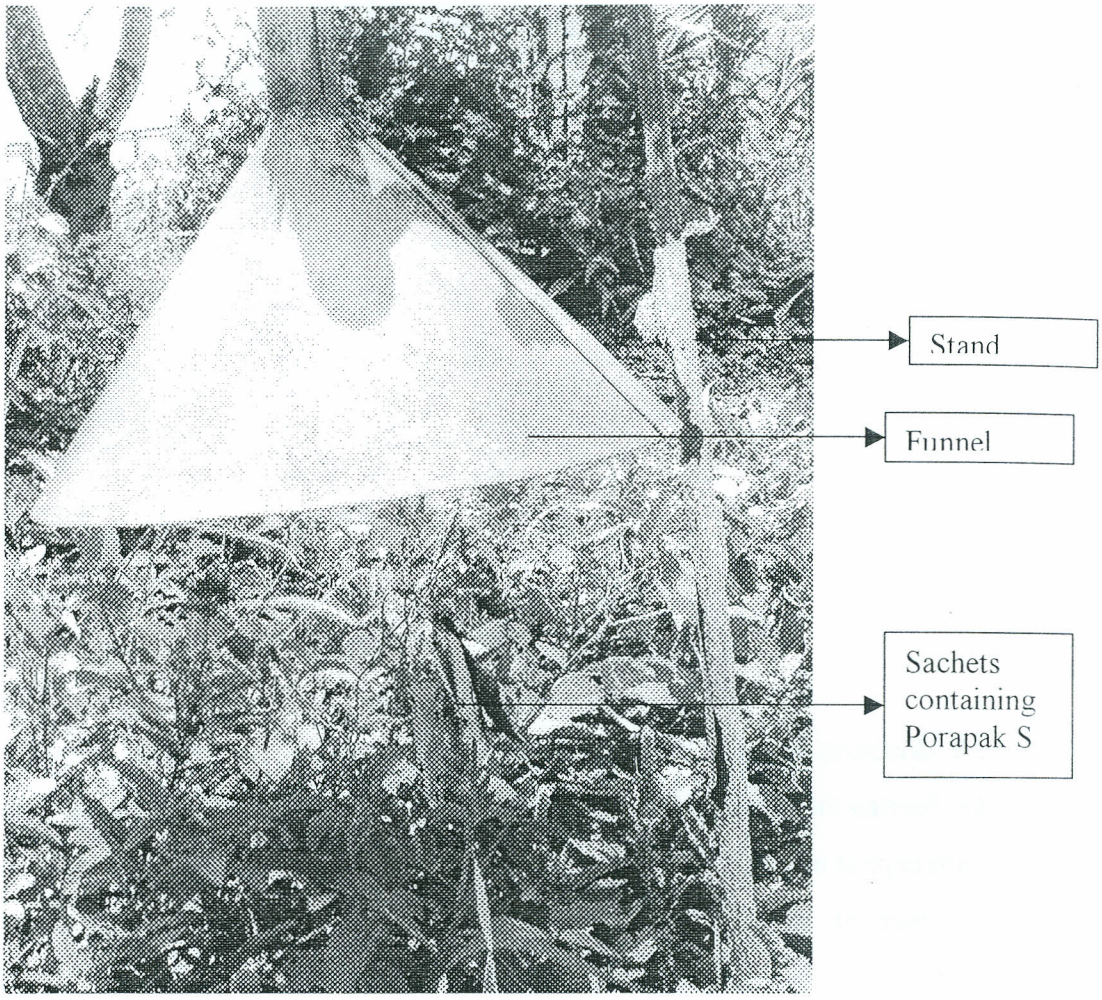


Fig 4: Apparatus for headspace trapping

3.3 Solvent extraction

Leaves of *C. newii* from seven regions were dried under shade for 7 days. Leaves (500 g) from each region were ground separately and sequentially extracted with hexane. The mixture was filtered and excess solvent removed by rotatory evaporation. Thin layer chromatography of crude extracts from the seven regions was carried out on silica gel plates (5 × 10 cm, AlumGRAM Sil G/UV_{254} Sigma Aldrich) using hexane-ethyl acetate (8:2) in a closed chamber and visualized under UV at λ_{max} 254 nm. Fractionation of the crude extract was carried out by Kieselgel 60 (230 – 400 mesh) packed to make 2 × 40 cm length of stationary phase in a glass column. Crude extract (15 g) was introduced into the column and eluted with hexane–ethyl acetate mixture in increasing polarity. Pure fractions obtained through column chromatography were stored at -4 °C for analysis.

3.4 Chemical composition of *Conyza newii* essential oil

Characterization and determination of compounds in essential oils were done by column chromatography, mass spectroscopy, GC-MS and GC-co-injection with authentic standards. Separation was done on a Hewlett Packard (HP) 5890 series II capillary GC, equipped with split-less capillary injection system and a flame ionization detector (FID) coupled with an integrator (HP 3393 A series II). Resolution was done on 50 m × 2 mm (i.d) × 0.33 µm (film thickness) cross–linked methylsilicone capillary column. White spot nitrogen was used as a carrier gas at a flow rate of 0.7 ml/min. The temperature program was 50 (5 min) - 280 (10 min) at 5 °C/min. GC-MS analysis was carried out on a HP 8060 series II GC linked to VG Platform MS. The temperature of the source was set at 180 °C and the multiplier voltage 300 V.

3.5 Bioassays

Mosquito repellency assays were carried out according to WHO (1996) protocols for laboratory and field evaluation of insecticides and repellents. Bioassays were carried out in a dark room with red light as the only source of illumination. The room temperature and humidity was artificially set to mimic the feeding conditions of female *An. gambiae s.s* (27-35 °C, RH≥ 65%). All repellency tests were carried out with 5-7 day old female *An. gambiae s.s* mosquitoes. Six human volunteers were used in repellency bioassays using 0.01, 0.1, 1.0 and 10% solutions of essential oils in acetone. A total of 18 cages each measuring 50 × 50 cm were used with each containing 25 starved female *An. gambiae s.s* mosquitoes. Test solution (10 ml) was dispensed on one forearm of a volunteer from the elbow to the wrist. The rest of the arm was covered with a glove to make it unattractive to the mosquitoes. Acetone (10 ml) was dispensed on the other arm in the same way to act as a control. The control arm was placed into the cage immediately after releasing 25 insects and kept there for 3 min. The number of mosquitoes landing on both the treated and control arm were recorded separately. The screening was done sequentially beginning with the lowest dose (control) and ending with the highest dose (10%). Each concentration was screened with a fresh batch of mosquitoes. After bioassay of each concentration, the arms were washed with bar soap,

rinsed well with tap water and dried using a clean towel for 15-20 min. before application of next dose of sample. The % protective efficacy (PE) was calculated as follows:

$PE = (PCM - PTM)/ PCM \times 100.$

Where PCM and PTM is the % control and treated means, respectively (WHO, 1996).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction

4.1.1 Yields of *Conyza newii* essential oils

The yields of steam-distilled essential oil were the highest compared to thermal expulsion and direct burning (Table 1).

Table 1: The yield in of volatiles from *Conyza newii* from different parts of Kenya

Region	Yield (g)		
	Steam distilate	Thermal expulsion	Direct burning
West Pokot	17.1[a] (14.25%)	0.41[f] (0.34%)	0.94[k] (0.78%)
Webuye	15.09[b] (12.58%)	0.28[g] (0.28%)	0.74[l] (0.62%)
Nairobi	14.38[c] (11.98%)	0.56[h] (0.47%)	0.84[l] (0.7%)
Nyakach	13.45[c] (11.21%)	0.74[i] (0.82%)	0.82[l] (0.68%)
Naivasha	12.94[d] (10.78%)	0.29[g] (0.24%)	0.71[l] (0.59%)
Kilome	12.2[d] (10.17)	0.29[g] (0.24%)	1.24[m] (1.03)
Kericho	11.92[e] (9.93%)	0.24[j] (0.18%)	0.76[l] (0.63%)

Means with the same letter are not significantly different

Conyza newii collected from West Pokot had the highest yield followed by Webuye and Nairobi with yields of 17.1g (14.25%) 15.09 g (12.58%) and 14.38 g (11.98%), respectively. This trend in yield is closely related to geographical location of the plant because the *C. newii* grown in Webuye and Nairobi were from seedlings collected from West Pokot. This again correlates positively with close geographical location of West Pokot and Webuye. Direct burning gave significantly higher yields than thermal expulsion in all the 7 regions but much less than steam distillation. Due to the small amount of the volatiles obtained from headspace, they were analyzed directly without quantification or bioassay.

4.2 Bioassays

4.2.1 Bioassay of steam distilled essential oil from *Conyza newii*

There was considerable variation in protective efficacy of *C. newii* oil from the seven regions of Kenya (Table 2).

Table 2: ANOVA and SNK grouping of mean % protective efficacy of steam-distilled essential oil of *Conyza newii* from different parts of Kenya

	Mean % protective efficacy ± SE			
Region/Conc	0.01	0.1	1	10
West Pokot	$23.99^{a} \pm 0.42$	$67.59^{f} \pm 0.21$	$95.67^{i} \pm 0.30$	100^{m}
Kilome	$10.33^{b} \pm 0.37$	$17.37^{c} \pm 0.31$	$79.53^{j} \pm 0.78$	100^{m}
Naivasha	$16.63^{c} \pm 0.46$	$54.7^{g} \pm 0.56$	$92^{k} \pm 0.53$	100^{m}
Webuye	$10.58^{b} \pm 0.16$	$41.42^{e} \pm 0.64$	$84^{l} \pm 0.74$	100^{m}
Nyakach	$19.25^{d} \pm 0.45$	$56.45^{g} \pm 0.1$	$94.38^{i} \pm 0.48$	100^{m}
Kericho	$38.73^{e} \pm 0.46$	$68.13^{f} \pm 0.47$	100^{m}	100^{m}
Nairobi	$17.22^{c} \pm 0.22$	$44.30^{h} \pm 0.14$	$91.25^{k} \pm 0.33$	100^{m}

Means with the same letter are not significantly different

At 0.01% concentration, essential oil from Kericho shows the highest protective efficacy of 38.73 ± 0:46%, a trend that is repeated at all the tested concentrations. The essential oil from Kericho achieved 100% protective efficacy at 1% concentration unlike all the regions. The lowest protective efficacy at 0.01% was found in steam distilled oil of *C. newii* from Kilome and Webuye and was repeated at all other concentrations tested. West Pokot, Nyakach, Nairobi and Naivasha types gave moderate protective efficacies. At 10% concentration, all the oils from the seven regions achieved 100% protective efficacy.

Analysis of variance in mean protective efficacy of *C. newii* essential oil from different ecological zones revealed that variations are more pronounced at low concentrations (Table 2). At 0.01% concentration for example, there is appreciable variation with a total of five SNK group of means (a, b, c, d, e). At 0.1% concentration, there is also variation in protective efficacy with respect to ecological zones with a total of five SNK group of means (f, c, g, e, h). The same trend in variation is also observed at 1.0% concentration of the oil with a total of five SNK group of means, showing significant variation (i, j, k, l, m). There was no significant variation in protective efficacy with respect to ecological zones at 10% concentration of the oil ($p = 0.001$) with only one SNK group of means (m). Generally, there is significant variation ($p = 0.001$) between ecological zone and protective efficacy. The general trend was: Kericho > West Pokot > Nyakach > Nairobi > Naivasha > Webuye > Kilome.

4.2.2 Bioassay of thermally expelled essential oil from *Conyza newii*

Although variation in protective efficacy of essential oil obtained by thermal expulsion was observed with regions, it is not as pronounced as for steam-distilled oil (Table 3).

Table 3: ANOVA and SNK grouping of protective efficacy of essential oil from thermal expulsion.

	Mean % protective efficacy ± SE			
Region/Conc	0.01	0.1	1	10
Kericho	$21.46^{a} \pm 0.54$	$31.86^{e} \pm 0.18$	$60.69^{h} \pm 0.46$	$93.28^{m} \pm 0.36$
Naivasha	$16.91^{b} \pm 0.45$	$30.46^{e} \pm 0.47$	$45.06^{i} \pm 0.65$	$61.92^{h} \pm 6.13$
Nairobi	$14.97^{c} \pm 0.22$	$21.75^{a} \pm 0.48$	$44.16^{i} \pm 0.83$	$59.41^{h} \pm 6.09$
West Pokot	$14.05^{c} \pm 0.30$	$24.28^{f} \pm 0.47$	$51.48^{j} \pm 0.29$	$72.47^{n} \pm 5.99$
Webuye	$14.03^{c} \pm 0.85$	$20.19^{a} \pm 0.17$	$28.99^{k} \pm 0.28$	$57.10^{o} \pm 6.39$
Nyakach	$13.51^{d} \pm 0.23$	$31.80^{e} \pm 0.28$	$39.51^{l} \pm 0.42$	$63.65^{p} \pm 4.11$
Kilome	$16.60^{b} \pm 0.12$	$18.93^{g} \pm 0.28$	$39.51^{l} \pm 0.42$	$68.48^{q} \pm 4.78$

Means with the same letter are not significantly different

Thermally expelled oil of *C. newii* from Kericho still had the highest mean protective efficacy (93.28 ± 0.36% at 10% concentration and 21.46 ± 5.44% at 0.01%). West Pokot oil was the next best at 72.47 ± 5.99% at 10% concentration followed by Kilome, Nyakach and Nairobi essential oils. The materials from Naivasha and Webuye had the lowest performance

Analysis of variance in mean protective efficacy of thermally expelled *C. newii* reveals lack of significant variation ($p = 0.001$) with ecological zones at the same concentration (Table 3). At 0.01 and 0.1% concentration of thermally expelled essential oil there are only 4 SNK group of means (a, b, c, d and a, e, f, g), respectively. Note that in the case of the steam-distilled essential oil, the variation in protective efficacy with respect to ecological zones was more pronounced at low concentrations. At 1 and 10%, there is very little variation still. Note that 100% efficacy is not achieved even at 10% concentrations.

4.2.3 Bioassay of essential oil from directly burned *Conyza newii*

The protective efficacy of directly burned *C. newii* from seven regions show a slightly different trend from that obtained by steam distillation and thermal expulsion (Table 4).

Table 4: ANOVA and SNK grouping of protective efficacy of essential oils from direct burning

	Mean % protective efficacy			
Region/Conc	0.01	0.1	1	10
Kericho	$9.87^{a} \pm 0.97$	$15.51^{e} \pm 0.18$	$25.71^{g} \pm 0.32$	$61.91^{l} \pm 0.44$
Naivasha	$11.49^{b} \pm 0.28$	$20.69^{f} \pm 0.19$	$30.98^{i} \pm 0.28$	$57.68^{m} \pm 0.43$
Nairobi	$9.24^{a} \pm 0.12$	$25.80^{g} \pm 0.38$	$30.94^{i} \pm 0.33$	$47.86^{n} \pm 0.17$
West Pokot	$6.28^{c} \pm 0.32$	$13.17^{d} \pm 0.34$	$33.91^{j} \pm 0.38$	$61.81^{l} \pm 0.84$
Webuye	$5.64^{c} \pm 0.61$	$17.91^{h} \pm 0.31$	$32.13^{j} \pm 0.39$	$52.48^{o} \pm 0.31$
Nyakach	$9.67^{a} \pm 0.18$	$15.08^{e} \pm 0.91$	$21.27^{f} \pm 0.13$	$54.55^{p} \pm 0.66$
Kilome	$13.52^{d} \pm 0.19$	$26.69^{g} \pm 0.34$	$37.91^{k} \pm 0.35$	$67.22^{q} \pm 0.17$

Means with the same letter are not significantly different

Conyza newii material collected from Kilome shows the highest protective efficacy of 13.52 ± 0.19 at 0.01%. This trend is repeated for all concentrations tested. However, the performance of essential oil from West Pokot and Kilome are closely related at 10%. This observation is interesting because in the case of steam distilled essential oil, *C. newii* from Kilome showed the lowest protective efficacy at all the four concentrations. On the other hand, essential oil from Kericho exhibited the highest protective efficacy, which is not the case in direct burning. The plant material from the rest of the regions exhibited similar efficacy levels.

4.2.4 Bioassay of the crude hexane extract

The crude hexane extract exhibited a higher activity compared to steam distilled oil, thermal expulsion and direct burning (Table 5).

Table 5: The % mean protective efficacy of crude hexane extract

Concentration	Mean % protective efficacy ± SE
0.01	51.6 ± 0.21
0.1	100
1	100
10	100

The steam distilled essential oil from West Pokot the protective efficacy is 23.99 ± 0.42% compared to 51.6 ± 0.21% for the solvent extract. This trend is the same for all regions at all the four concentrations. The crude hexane extract achieved 100% protective efficacy at 0.1% compared to the essential oil from steam distillation at 10%. Thermal expulsion and direct burning did not achieve 100% PE even at 10% concentration.

4.2.5 Bioassay of the isolated pure compound

The compound isolated from the crude hexane extract was assayed against female *An. gambiae* mosquitoes. The compound exhibited a high protective activity, slightly higher than the crude extract (Table 6).

Table 6: The % mean protective efficacy of the isolated pure compound

Concentration	Mean protective efficacy ± SE
0.01	76.5 ± 0.11
0.1	100
1	100
10	100

At 0.01%, the isolated compound exhibited a protective efficacy of 76.5 ± 0.11 compared to 51.6 ± 0.21 in the case of crude extract at the same concentration. Like the crude extract, it achieved 100% protective efficacy at 0.1% concentration.

Due to very small amount of volatiles obtained by headspace trapping, they were not enough for bioassay.

4.2.6 Repellent activity (RD_{50}) of extract and pure compound

From probit analysis of the repellency data and the derived regression equation, the RD_{50} values from the different methods of extraction of *C. newii* are as summarized in Table 7.

Table 7: Repellent efficacy (RD_{50}) from different extraction methods of *Conyza newii* from different parts of Kenya

	RD_{50} (mg/cm^2)				
	Essential oil	Thermal expulsion	Direct burning	Hexane extract	Pure compound
West Pokot	2.86×10^{-3}	5.9×10^{-3}	5.8×10^{-3}	2.14×10^{-3}	2.11×10^{-3}
Kilome	3.08×10^{-3}	6.0×10^{-3}	5.4×10^{-3}	-	-
Naivasha	2.88×10^{-3}	5.8×10^{-3}	5.7×10^{-3}	-	-
Webuye	3.06×10^{-3}	6.2×10^{-3}	6.2×10^{-3}	-	-
Nyakach	2.70×10^{-3}	5.4×10^{-3}	7.2×10^{-3}	-	-
Kericho	2.85×10^{-3}	5.91×10^{-3}	7.9×10^{-3}	-	-
Nairobi	2.68×10^{-3}	5.6×10^{-3}	5.8×10^{-3}	-	-

Key: (-) - not assayed

The different RD_{50} values for the different extraction methods and ecological zones may be explained by qualitative variation of the repellent components of the essential oils. From the RD_{50} values, no significant difference was observed between thermal expulsion and direct burning of *C. newii* from the same ecological zone. It was noted that steam distillates were twice as active as thermally expelled or directly burned essential oils. Hexane extract was slightly better than the steam-distilled essential oil and as good as the pure isolated compound. This can be explained by the relatively high concentration of repellent compounds in steam distilates and the hexane extract.

4.3 Chemical composition of *Conyza newii*

4.3.1 Steam distilled *Conyza newii* oil

In all the seven regions, the major compounds identified from steam distilled oil were limonene (**55**), peril alcohol (**61**), peril aldehyde (**60**), geraniol (**43**), and 1, 8-cineol (**48**) (Table 8).

Table 8: Compounds in essential oils of steam distilled *Conyza newii*

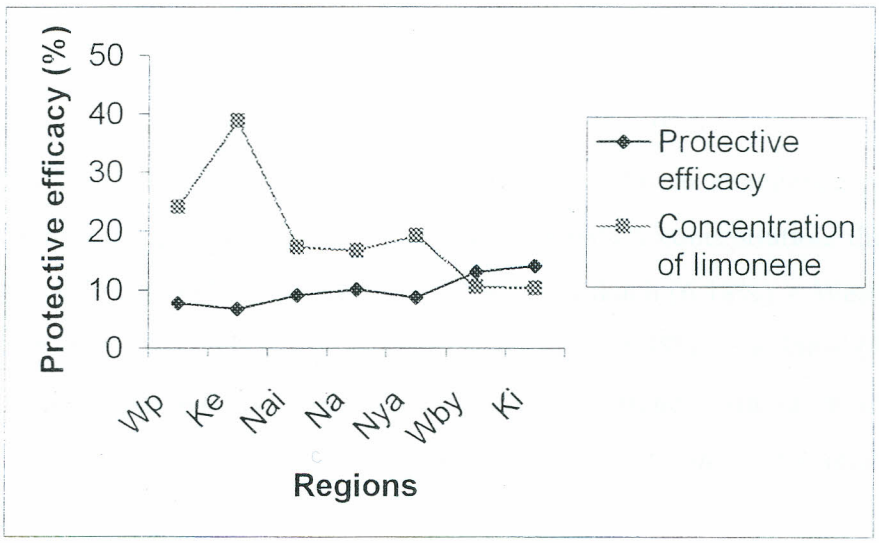
		Relative abundance (%)						
Peak	Compound	West Pokot	Webuye	Kilome	Nairobi	Naivasha	Kericho	Nyakach
1	δ-4-carene	t	t	t	t	t	t	t
2	α-pinene	0.43	0.39	0.41	0.44	0.35	0.36	0.31
3	limonene	7.81	13.1	14.08	9.1	10.09	6.78	8.82
4	α-caryophylene	0.46	0.41	0.41	t	t	0.38	t
5	*trans*-β-ocimene	2.22	2.21	2.19	2.24	2.21	2.23	2.2
6	geraniol	28.44	18.14	16.56	23.48	20.19	30.42	25.21
7	geranyl acetate	t	t	t	t	t	t	t
8	β-myrcene	t	t	t	0.41	t	0.13	t
9	peril aldehyde	28.53	30.42	31.06	30.15	29.42	31.88	30.42
10	carvone	t	t	t	t	t	t	t
11	germacrene B	0.19	0.15	0.13	0.19	t	t	t
12	α-amorphene	0.91	1.28	1.22	1.19	1.14	1.11	1.29
13	1, 8 cineole	4.48	5.08	4.42	5.87	5.04	4.49	5.17
14	α-terpeneol	t	t	t	t	t	t	t
15	fenchyl alcohol	0.88	0.84	0.87	0.87	0.89	0.86	0.88
16	peril alcohol	5.21	4.68	3.11	5.12	4.68	7.12	6.18
17	germacrene D	t	t	t	t	t	0.48	t
18	camphor		t	t	t	t	t	t
19	isocaryophylene	t	t	t	t	t	t	t

t = trace

The relative abundance of peril aldehyde (**60**) was high in all the seven regions but highest in the essential oil from Kericho (31.88%) and was lowest in the essential oil of *C. newii* from West Pokot (28.53%). Geraniol was the second most abundant compound in the essential oil. Its concentration was also highest in Kericho oil (30.42%) followed by West Pokot, Nyakach, Nairobi and Naivasha and lowest in Webuye and Kilome oil. Limonene (**55**) was the third most abundant compound in the essential oil. Its relative abundance was highest in *C. newii* oil from Kilome. From this observation, it is noted that although limonene is one of major compounds in the oil, there is no direct correlation of protective efficacy of the oil with the relative abundance of limonene. This was observed in the essential oil of *C. newii* from Kilome, which exhibited the lowest protective efficacy yet it had the highest limonene content. The other compounds present in reasonable amounts were peril alcohol (**61**) and 1, 8-cineole (**48**). For peril alcohol, the highest amount was found in Kericho oil followed by Nyakach, West Pokot, Nairobi, Naivasha and Webuye in that order. The lowest amount was found in Kilome oil. There was minimal variation in 1, 8-cinoele concentration in oils from all the regions.

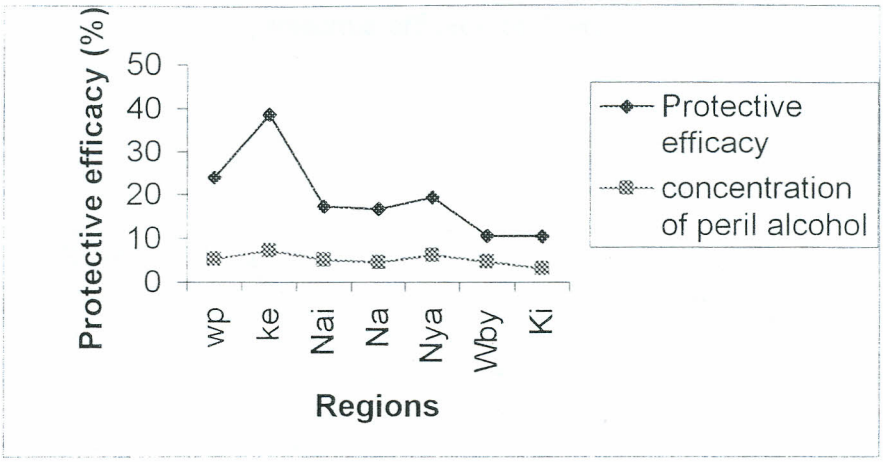
From previous studies (Omollo *et al.*, 2001), it has been shown that out of all the compounds identified, peril aldehyde, peril alcohol and geraniol exhibit 100% protective efficacy at 0.01 g/ml concentration. Previous studies have also suggested that limonene lowers the activity of peril alcohol, peril aldehyde and geraniol (Purcell & Bulcinus, 1994). In this study, a relationship between protective efficacy and some of the major components of the essential oil was investigated. The compounds chosen were limonene, peril alcohol, peril aldehyde and geraniol. The results are summarized in figures 5-8.

Figure 5: Comparison of protective efficacy of *Conyza newii* oil and concentration of limonene



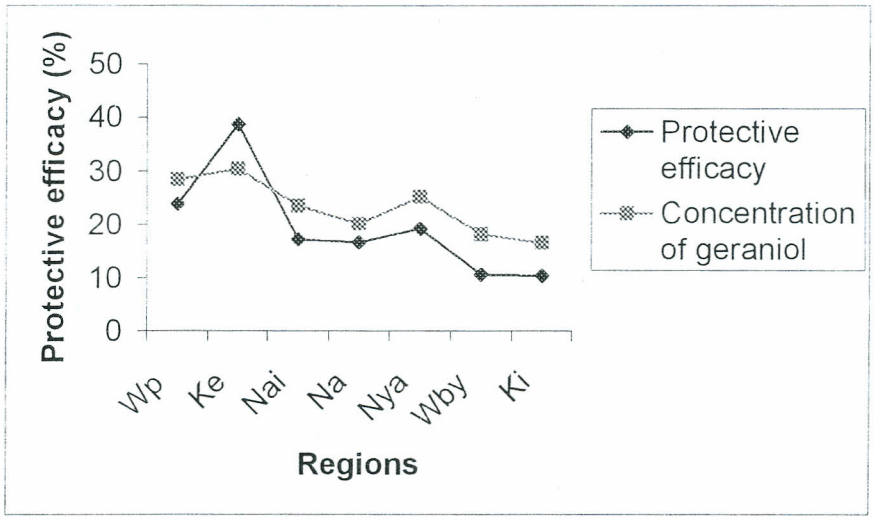
There was an inverse relationship between the relative abundance of limonene and protective efficacy of essential oil of *C. newii*. For example, the trend in protective efficacy at 0.01% concentration is Kericho (6.78%) > West Pokot (7.81%) > Nyakach (8.82%) > Nairobi (9.1%) > Naivasha (10.09%) > Webuye (13.10%) > Kilome (14.08%), with the relative abundance of limonene in parentheses. From this, it is noted that regions with *C. newii* essential oil with high relative abundance of limonene have low protective efficacy.

Figure 6: Comparison of protective efficacy of *Conyza newii* oil and peril alcohol concentration.



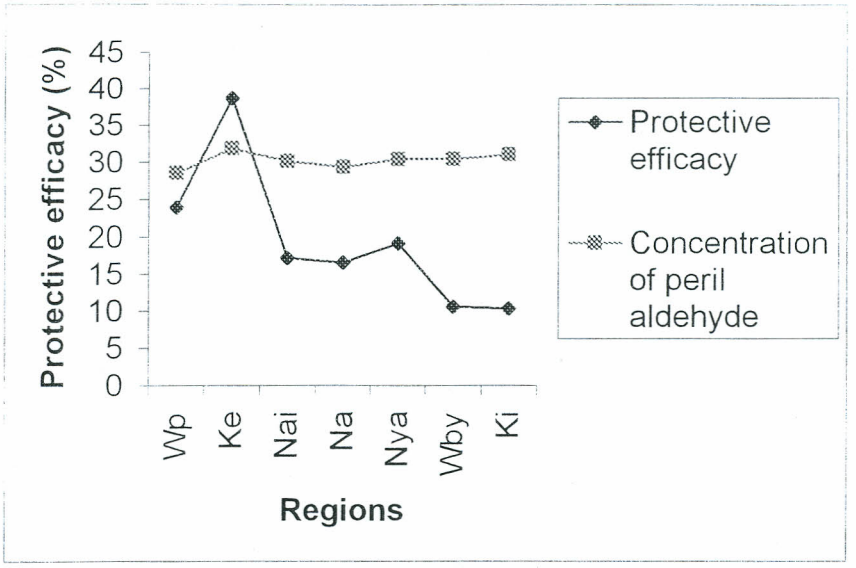
For peril alcohol, there is a positive correlation between protective efficacy and its relative abundance. Regions with *C. newii* having high relative abundance of peril alcohol exhibit high protective efficacy. For example, at 0.01% concentration, the trend in protective efficacy is as follows: Kericho (7.12%) > Nyakach (6.18%) > West pokot (5.21%) > Nairobi (5.12%) > Naivasha (4.68%) > Webuye (4.48%) > Kilome (3.11%) with relative abundance of peril alcohol in parentheses. This trend is similar at all other concentrations tested. This suggests that repellent activity of *C. newii* essential oil is closely dependent on the level of peril alcohol in the oil.

Figure 7: Comparison of protective efficacy of *Conyza newii* oil and concentration of geraniol



The positive correlation between protective efficacy and relative abundance of individual compounds was observed in the case of geraniol. *C. newii* essential oil having high relative abundance of geraniol exhibited high protective efficacy. At 0.01% concentration of the oil, the trend in protective efficacy was similar to that of peril alcohol and was as follows: Kericho (30.41%) > Nyakach (28.44%) > West Pokot (25.21%) > Nairobi (23.48%) > Naivasha (20.19%) > Webuye (18.14%) > Kilome (16.56%) with relative abundance of geraniol in parentheses.

Figure. 8: Comparison of protective efficacy of *Conyza newii* oil and concentration of peril aldehyde



There was no consistent relationship between the relative abundance of peril aldehyde and the protective efficacy of the oil. Its concentration is high in all the seven regions but highest in the essential oil from Nyakach (30.42%). The concentration of peril aldehyde is lowest in the essential oil of *C. newii* from West Pokot (28.53%).

4.4 Bioassay of blends

Ten blends were prepared and bioassayed to confirm the natural trend observed in protective efficacy of *C. newii* oil against *An. gambiae* (Table 9). The first blend (blend 1) contained peril aldehyde, peril alcohol, geraniol and limonene (5:1:5:1). The second blend (blend 2) contained peril aldehyde, peril alcohol and geraniol (5:1:5). The third blend (blend 3) contained limonene, peril alcohol and geraniol (1:1:5), while the fourth blend (blend 4) contained peril alcohol and geraniol (1:5). The fifth blend (blend 5), contained peril aldehyde and geraniol (1:1) the sixth blend (blend 6) contained peril aldehyde and peril alcohol (5:1) the seventh blend (blend 7) contained peril aldehyde and limonene (5:1) the eighth blend (blend 8), contained geraniol and limonene (5:1), blend 9

contained peril alcohol and limonene (1:1) and blend 10 contained peril aldehyde, peril alcohol and limonene (5: 1: 1)

The trend in protective efficacy of blends is similar to that observed in the case of the plant oil (Table 9).

Table 9: Bioassay of blends of major components of *Conyza newii* oil

	Mean % protective efficacy				
Concentration (%)	0.01	0.1	1	10	RD_{50} (mg/cm^2)
Plant oil	$38.73^{a} \pm 4.62$	$68.13^{c} \pm 4.69$	100^{k}	100^{k}	2.68×10^{-3}
Blend 1	$50.51^{b} \pm 2.38$	$91.5^{i} \pm 2.1$	100^{k}	100^{k}	1.72×10^{-3}
Blend 2	$69.4^{c} \pm 3.11$	$90.2^{i} \pm 3.2$	100^{k}	100^{k}	1.70×10^{-3}
Blend 3	$44.9^{d} \pm 3.08$	$62.9^{h} \pm 1.8$	$85.8^{l} \pm 1.12$	100^{k}	3.31×10^{-3}
Blend 4	$67^{e} \pm 2.14$	$91.8^{i} \pm 2.41$	100^{k}	100^{k}	1.72×10^{-3}
Blend 5	$72.31^{f} \pm 2.08$	$98.22^{j} \pm 1.34$	100^{k}	100^{k}	1.09×10^{-3}
Blend 6	$70.68^{g} \pm 1.14$	$94.99^{j} \pm 1.12$	100^{k}	100^{k}	1.14×10^{-3}
Blend 7	$52.21^{b} \pm 0.22$	$65.01^{e} \pm 0.28$	$91.11^{i} \pm 0.33$	100^{k}	2.76×10^{-3}
Blend 8	$51.52^{b} \pm 0.41$	$70.36^{g} \pm 1.14$	$94.46^{j} \pm 1.14$	100^{k}	2.62×10^{-3}
Blend 9	$61.47^{h} \pm 0.48$	$73.96^{f} \pm 0.34$	$87.34^{l} \pm 0.48$	100^{k}	2.59×10^{-3}
Blend 10	$60.34^{h} \pm 0.87$	$71.56^{f} \pm 0.23$	$86.67^{l} \pm 0.73$	100^{k}	2.57×10^{-3}

Means with the same letter are not significantly different

The RD_{50} values of different blends were calculated from probit analysis and regression equation for each sample.

Blends containing limonene show less protective efficacy. For example at 0.01% concentration, blend 3 shows the lowest protective efficacy with an RD_{50} value 3.31×10^{-3} mg/cm^2, while blends 2, 4, 5 and 6 which contains no limonene exhibits the highest protective efficacy with RD_{50} values of 1.70×10^{-3} mg/cm^2, 1.72×10^{-3} mg/cm^2, 1.09×10^{-3} mg/cm^2 and 1.14×10^{-3} mg/cm^2 respectively. Note that blend 4 contains just peril alcohol and geraniol. This is also the case for the plant oil in which protective efficacy has a positive correlation with the level of peril alcohol and geraniol. From this observation it is evident that the protective efficacy of *C. newii* essential oil depends on the level of peril alcohol and geraniol. The level of peril aldehyde also plays an important role in the

protective efficacy of *C. newii* oil. When the protective efficacy of blend 2 which contains peril aldehyde, peril alcohol and geraniol is compared to that of blend 5 which contains peril aldehyde and geraniol, it is revealed that there is higher protective efficacy in the later. For example at 0.01% concentration, blend 2 exhibits a high protective efficacy with an RD_{50} value of 1.70×10^{-3} mg/cm^2 while blend 5 exhibits a higher protective efficacy with an RD_{50} value of 1.09×10^{-3} mg/cm^2. Comparing the protective efficacy of blend 5 and 8, it is shown that peril aldehyde significantly enhances the activity of geraniol while limonene lowers the activity of geraniol. For example, at 0.01%, the activity of blend 5 (containing geraniol and peril aldehyde) is high with an RD_{50} value of 1.09×10^{-3} mg/cm^2 while that of blend 8 (which contains geraniol and limonene is low with an RD_{50} value of 2.62×10^{-3} mg/cm^2 showing a significant difference in the activity. This fact is also confirmed when the protective efficacy of blend 6 is compared to that of blend 9. It is revealed that peril aldehyde enhances the activity of peril alcohol while limonene reduces the activity of peril alcohol. At the same concentration (0.01%), the activity of blend 6 (containing peril aldehyde and peril alcohol) is high with an RD_{50} value of 1.14×10^{-3} mg/cm^2 while that of blend 9 (containing limonene and peril alcohol) is low with an RD_{50} value of 2.59×10^{-3} mg/cm^2. The trend is repeated at all the four concentrations. It can be concluded from this observations that peril aldehyde enhances the activity of the two compounds (geraniol and peril alcohol) in determining the mosquito repellency of *C. newii* oil, while limonene reduces the activity of these compounds.

4.5 Chemical composition of thermally expelled *Conyza newii* oil

From GC-MS analysis, it was observed that chemical composition of thermally expelled *C. newii* oil (Table 10) is closely related to that of the steam distilled oil (Table 8).

Table 10: Chemical composition (%) of thermally expelled essential oil from *Conyza newii*

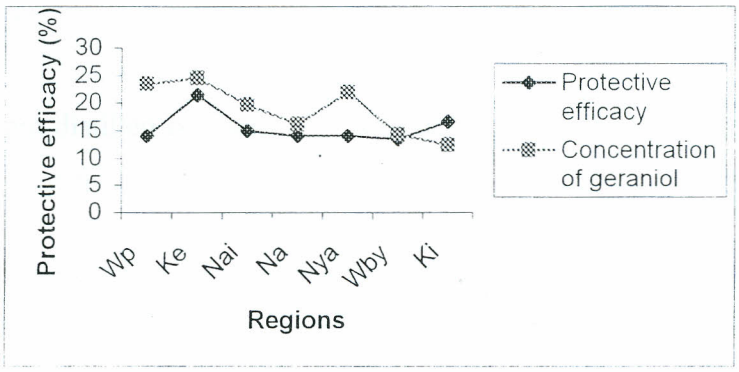
		Relative abundance (%)						
Peak	Compound	West Pokot	Webuye	Kilome	Nairobi	Naivasha	Kericho	Nyakach
1	δ-4-carene	t	t	t	t	t	t	t
2	α-pinene	0.2	0.11	0.17	0.15	0.13	0.14	0.88
3	limonene	6.23	6.69	5.11	5.48	4.43	4.39	7.08
4	α-caryophylene	0.21	0.19	0.24	0.29	0.17	0.25	0.24
5	trans-β-ocimene	1.82	1.08	0.95	0.99	1.78	1.74	1.77
6	geraniol	23.41	14.27	12.41	19.67	16.19	24.48	21.91
7	geranyl acetate	t	t	t	t	t	t	t
8	β-myrcene	t	t	t	t	t	t	t
9	peril aldehyde	21.71	25.81	26.7	24.48	19.49	26.72	19.88
10	carvone	t	t	t	t	t	t	t
11	germacrene B	0.14	0.11	0.12	t	t	0.18	t
12	α-amorphene	0.67	0.54	0.42	0.47	0.38	0.45	0.47
13	1,8 cineole	3.14	4.41	3.22	3.18	4.12	3.11	4.12
14	α-terpeneol	t	t	t	t	t	t	t
15	fenchyl alcohol	0.65	0.55	0.47	0.46	t	t	t
16	peril alcohol	t	t	t	t	t	t	t
17	germacrene D	t	t	t	t	t	t	t
18	camphor	t	t	t	t	t	t	t
19	Isocaryophyle	t	t	t	t	t	t	t

t = trace

The relative abundance of constituent compounds was lower in the case of thermally expelled samples. For example, the relative abundance of peril alcohol in steam-distilled essential oil from West Pokot is 5.21% while it is present in trace amounts in thermally expelled *C. newii* oil. The protective efficacy of thermally expelled *C. newii* oil was positively correlated to the relative abundance of geraniol (fig. 9).

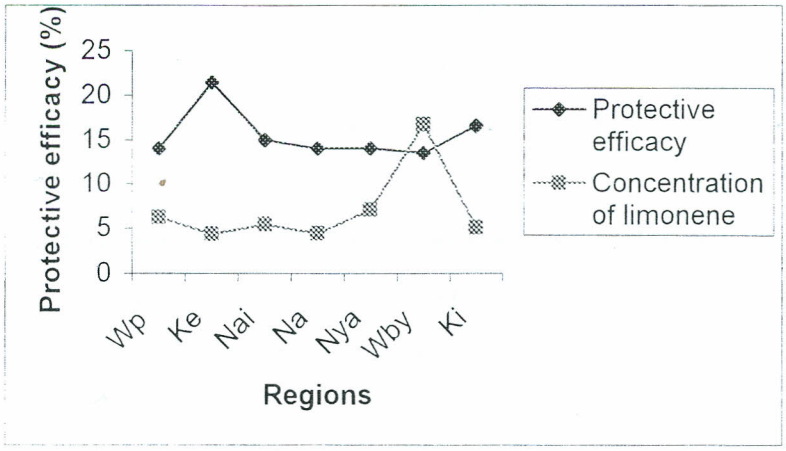
Thermally expelled oil from regions with high relative abundance of geraniol showed high protective efficacy. For example at 0.01% concentration, the trend in protective efficacy is: Kericho (24.48%) > West Pokot (23.41%) > Nyakach (21.91%) > Nairobi (19.67%) > Naivasha (16.19%) > Webuye (14.27%) > Kilome (12.41%) with corresponding relative abundances of geraniol indicated in parentheses (Fig. 9).

Figure 9: Correlation of relative abundance of geraniol in thermal expelled *Conyza newii* to mean % PE



Although the relative abundance of peril alcohol is trace in thermally expelled oil the trend in protective efficacy of plants from different regions is same as that of steam distilled oil. This indicates that geraniol plays an equally important role in the mosquito repellency of *C. newii* oil. As in steam distilled essential oil, thermally expelled *C. newii* oil with low concentration of limonene exhibits high protective efficacy (Fig. 10).

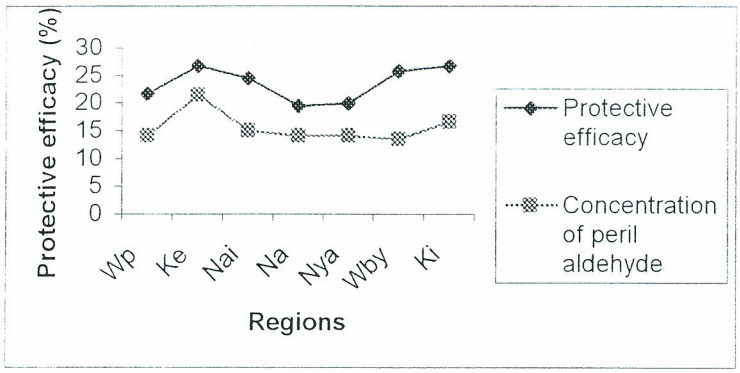
Figure 10: Correlation of relative abundance of limonene in thermally expelled *Conyza newii* oil to mean % PE



For example at 0.01% concentration, the trend in protective efficacy is as follows: Kericho (4.39%) > Naivasha (4.43%) > Nairobi (5.48%) > Kilome (5.11%) > West Pokot (6.23%) > Webuye (6.69%) > Nyakach (7.08%). This confirms that, there is an inverse relationship between the relative abundance of limonene and protective efficacy of essential oil of *C. newii*.

Like the case of steam-distilled oil, peril aldehyde did not have direct relationship with the protective efficacy of thermally expelled plant oil (Fig. 11).

Figure 11: Correlation of relative abundance of peril aldehyde in thermally expelled *C. newii* oil to % mean PE



For example, thermally expelled oil from Kericho exhibits the highest protective efficacy of 21.46 ± 5.44, with the highest relative abundance of peril aldehyde of 26.72% (at 0.01% concentration). This trend in protective efficacy in relation to peril aldehyde for thermally expelled oil is same at all the four concentrations and the seven regions.

4.3 Chemical composition of directly burnt *Conyza newii* material

Like in the case of thermally expelled *C. newii*, the oil from directly burned leaves closely relate to the steam distilled one in chemical composition (Table 11).

Table 11: Chemical composition of directly burned essential oil from *Conyza newii*

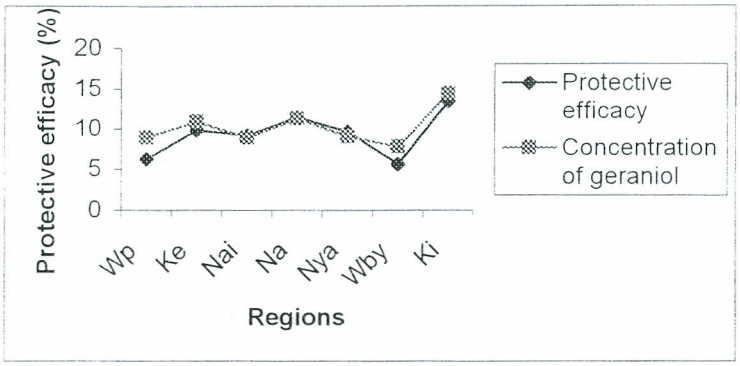
		Relative abundance (%)						
Peak	Compound	West pkokt	Webuye	Kilome	Nairobi	Naivasha	Kericho	Nyakach
1	δ-4-carene	t	t	t	t	t	t	t
2	α-pinene	t	t	t	0.12	0.11	t	t
3	limonene	3.08	3.33	2.27	5	4.41	4.3	3.21
4	α-caryophylene	0.18	t	t	t	t	t	0.13
5	trans-β-ocimene	1.34	0.89	1.28	1.35	1.21	0.87	1.3
6	geraniol	8.89	7.78	14.48	8.87	11.34	10.91	9.07
7	geraniol acetate	t	t	t	t	t	t	t
8	β-myrcene	t	t	t	t	t	t	t
9	peril aldehyde	18.19	22.74	19.49	20.22	27.48	23.48	19.48
10	carvone	t	t	t	t	t	t	t
11	germacrene B	0.11	t	t	0.11	t	t	t
12	α-amorphene	0.23	0.14	0.18	0.1	0.13	0.11	0.14
13	1, 8-cineole	2.21	2.49	2.11	2.84	3.08	2.46	3.11
14	α-terpeneol	t	t	t	t	t	t	t
15	fenchyl alcohol	0.37	0.41	0.42	0.47	0.39	0.33	0.34
16	peril alcohol	t	t	t	t	t	t	t
17	germacrene D	t	t	0.11	0.12	t	t	t
18	camphor	t	t	0.14	t	t	t	t
19	Isocaryophylene	t	t	0.18	t	t		t
20	β-phillandrene	t	t	0.18	t	t	t	t
21	spathulenol	t	t	0.14	t	t	t	t
22	Isopropyl benzaldehyde	t	t	0.11	t	t	t	t

t = trace

Comparison of composition of essential oil from direct burning and thermal expulsion revealed that relative abundance amounts of constituent compounds was higher in the latter. The relative abundance of compounds identified in vapors from directly burnt *C. newii* material was much lower than steam distilled and thermally expelled essential oils. Since the relative amounts of major repellent compounds were higher in thermally expelled oil, this explains the fact that thermally expelled oil exhibit higher protective efficacy than directly burned oil. The high relative abundance of geraniol and low relative abundance of limonene in directly burned *C. newii* oil explains the irregularity observed in their protective efficacy.

As in the case of steam-distilled oil, the protective efficacy of directly burned oil correlates positively with the relative abundance of geraniol (Fig. 12).

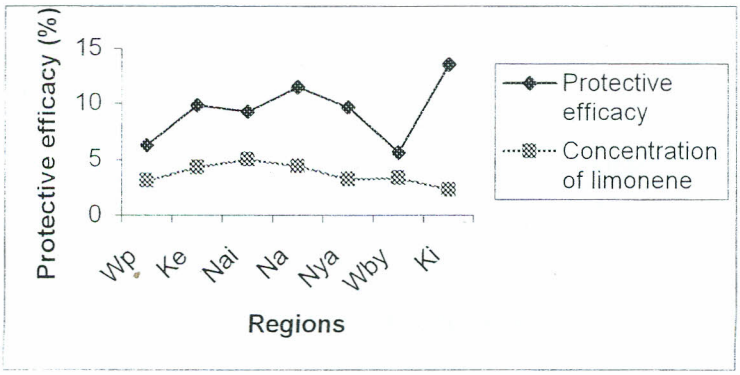
Figure 12: Correlation of relative abundance of geraniol in directly burned oil to % mean PE



Regions with high relative abundance of geraniol show high protective efficacy confirming the trend observed in the case of steam distilled and directly burned oil.

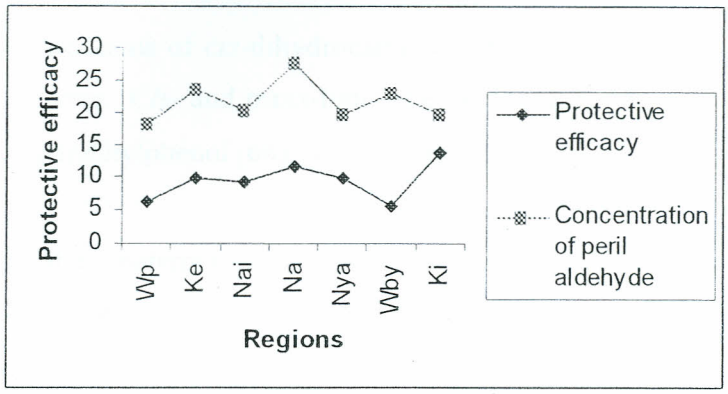
The inverse relationship between protective efficacy and the relative abundance of limonene observed in the case of steam distilled and thermally expelled oil was also observed in the case of directly burned oil (Fig. 13).

Figure 13: Correlation of relative abundance of limonene in directly burned *Conyza newii* oil to % mean PE



There was no direct relationship between the relative abundance of peril aldehyde and the protective efficacy, just as was the case with steam distilled and thermally expelled oil (Fig. 14).

Figure 14: Correlation of relative abundance of peril aldehyde in directly burned *Conyza newii* oil to % mean PE



4.4 Headspace trapping of *Conyza newii* essential oil

Due to unfavorable weather conditions (heavy rains), headspace trapping was successfully done in two regions (West Pokot and Kericho) only. Porapak S was used as an adsorbent. Major compounds identified in the two regions were *cis*-limonene oxide (**62**), *trans*-limonene oxide (**63**), *cis*-dihydrocarvone (**64**), 4-methoxyphenol (**65**) and 3-ethoxy-2-methylphenol (**66**) Table (12).

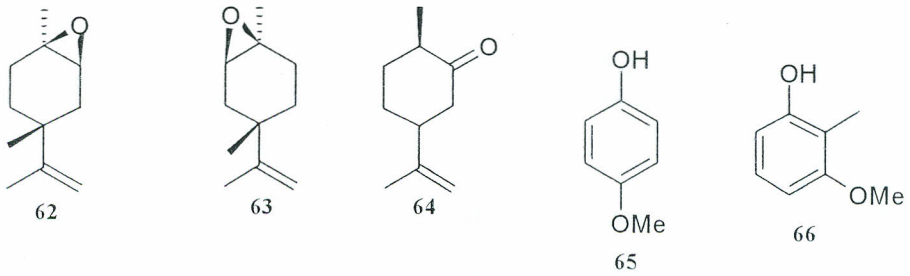
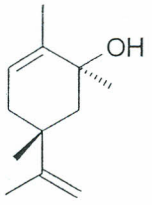


Table 12: The chemical composition of vapors obtained from *Conyza newii* leaves by headspace trapping.

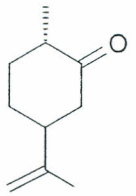
		Relative abundance(%)	
Peak	Compound	Nyakach	Kericho
1	*cis*-limonene oxide	18	20
2	*trans*-limone 0xide	12	14
3	*cis*-dihydrocarvone	41	41
4	4-methoxyphenol	28	24
5	3-methoxy-2-methylphenol	t	t

The relative concentrations of *cis*-dihydrocarvone (**64**) and 4-methoxyphenol (**65**) were high in the two regions. *Cis*- and *trans*-limonene oxide (**62** & **63**) occur in low amounts while 3-methoxy-2-methylphenol (**66**) occurs in trace amounts.

The biosynthesis of monoterpenes *cis*-limonene oxide (**62**), *trans*-limonene oxide (**63**) and dihydrocarvone (**64**) proceeds from geranyl pyrophosphate via a three-step pathway (Ivan, 1998). In the first step geranyl pyrophosphate is cyclized to (+)-limonene (**55**) by an enzyme (cyclase). The cyclized intermediate is stored in the essential oil ducts without further metabolism. *Trans*- and *cis*- limonene oxide (**62** & **63**) are synthesized from (+)-limonene (**55**) catalyzed by limonene oxygenase. Limonene-6-hydroxylase catalyzes the conversion of (+)-limonene (**55**) to carveol (**67**). Carveole (**67**) is transformed to *cis*-dihydrocarvone (**64**) and *trans*-dihydrocarvone (**68**) by carvone dehydrogenase (Ivan, 1998).

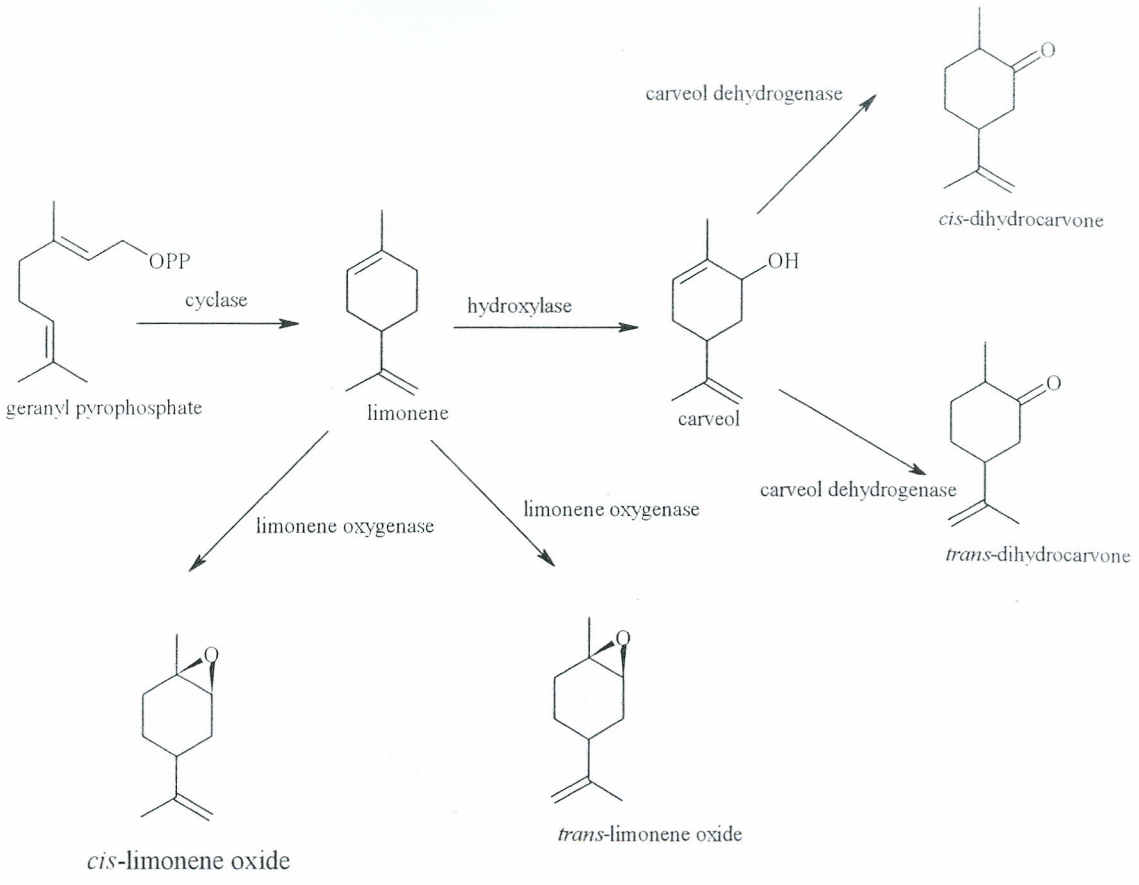


67



68

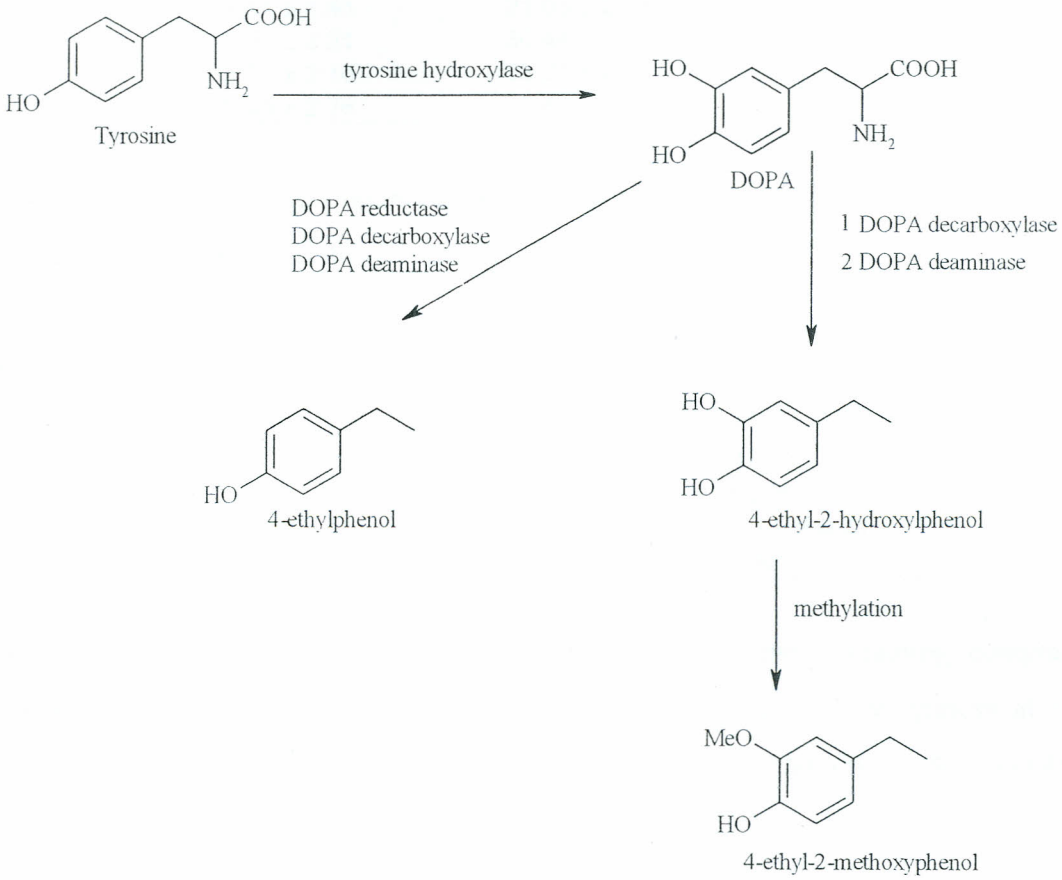
In plants monoterpenes like limonene oxide and dihydrocarvone exhibit high anti-microbial activity and hence secretions containing such compounds protect plants from microbial attack (Ivan, 1998). *trans*-Limonene oxide, *cis*-limonene oxide, *cis*-dihydrocarove and *trans*-dihydrocarvone are synthesized in plants as shown in scheme 1.



Scheme 1: Biosynthetic scheme of monoterpenes.

Phenols are secondary plant metabolites widely distributed in the plant kingdom (Ivan, 1998). 3-Methoxyphenol and 3-methyl-2-methoxylphenols are synthesized from tyrosine by the shikimic acid pathway (Ivan, 1998). Phenols play an important role in plants as anti-oxidants and regulate reproductive cycle in plants (Ivan, 1998).

In plants, biosynthesis of most phenols takes places through shikimate pathway and they are characterized by at least one hydroxylated aromatic ring. A general scheme of shikimate pathway is shown (Scheme 2)



Scheme 2: Shikimate pathway of biosynthesis of phenols (Catlin *et al.*, 1998)

4.4.1 Bioassay of standards from headspace trapping

Due to very low amounts of headspace trapping essential oils, standards of compounds obtained by headspace trapping were bioassayed. The individual compounds from headspace trapping (*trans*-limonene oxide, dihydrocarvone and 4-methoxyphenol) were assayed (Table 13). These compounds exhibit appreciable repellent activity but 100% protective efficacy was not achieved even at 10% concentration. *trans*-Limonene oxide had the highest protective efficacy followed by dihydrocarvone and 4- methoxyphenol.

Table 13: Protective efficacy of some compounds isolated by headspace trapping

	Mean % protective efficacy		
Concentration	*trans*-Limonene oxide	*cis* - Dihydrocarvone	4-Methoxyphenol
0.01	28.61 ± 1.41	21.03 ± 2.08	14.4 ± 1.81
0.1	41.59 ± 2.21	30.44 ± 1.41	23.15 ± 2.11
1	63.85 ± 2.18	53.21 ± 2.21	31.32 ± 1.29
10	85.40 ± 2.78	62.88 ± 1.71	53.83 ± 1.41

A mixture containing *trans*-limonene oxide, *cis*-dihydrocarvone and 4-methoxyphenol in their natural ratios 1:1:1, respectively, was prepared and assayed (Table 14).

Table 14: Bioassay of blends of volatiles obtained by headspace trapping

Concentration	Mean % PE
0.01	32.58 ± 0.42
0.1	54.09 ± 0.21
1	67.9 ± 0.34
10	100

Whereas no compound achieved 100% repellency, the blend containing compounds present in essential oil exhibited 100% protective efficacy against mosquitoes at 10%. However, the blend exhibited a lower protective efficacy compared to the steam distilled essential oil.

Extensive studies of volatiles of plants from the family compositae have been reported. For example, volatiles from *Melaleuca quinquenervia* have been analysed and their anti-weevil activity assessed (Purcell & Bulcinus, 1994). From chemical analysis of the volatiles extracted from the plant, it has been established that the main compounds in the volatiles are 1, 8-cineole, viridiflorol, terpineol, α-pinene, *trans*-nerolidol, limonene, β-pinene, terpene-4-ol, γ-terpinene and α-terpinene (Purcell & Bulicinus, 1994). Studies on other plants in this family have reported similar compounds from plants. From the results of the present study, the compounds reported in the volatiles of *C. newii* obtained by headspace trapping are slightly different. Apart from limonene oxide and dihydrocarvone, there are also aromatic compounds including 4-methoxyphenol and 3-methoxy-2-methylphenol.

The difference in chemical composition can be explained by the different methods used in obtaining volatiles in previous studies and the present study. In previous studies, leaf volatiles were obtained by a modified microwave technique. In addition, in previous studies volatiles were obtained from potted plants. In the present study, volatiles were obtained from plants in their natural ecological niches by headspace trapping technique using porapak S adsorbent. Interestingly, the volatile composition from headspace is completely different from the 3 other methods that involve cell wall breakage. The destruction of the cell wall may lead to the release of enzymes responsible for volatile production.

4.5 Solvent extraction

From TLC analysis, it was established that the chemical composition of the hexane extracts of the plants from the seven regions were the same. Consequently, they were combined and a representative sample assayed. One compound (oil) was isolated by column chromatography from the solvent extraction and characterized using NMR. The methyl group protons appeared as a singlet at δ 1.58 (3H) in the ^{1}H NMR (Table 15) suggesting a quaternary carbon next to the methyl. The singlet at δ 3.99 (2H) in the ^{1}H NMR was associated with protons attached to the same carbon as oxygen. The olefinic protons were associated with the singlet at δ 4.72 (2H) and δ 5.70 (2H)

Table 15: ^{1}H NMR values of the isolated compound in $CDCl_3$

Position	Chemical shift (δ)	Integrals
2	5.70	1H
3	2.13	2H
4	2.09	1H
5	2.11	2H
6	2.13	2H
7	3.99	2H
9	4.72	2H
10	1.58	3H

From the ^{13}C NMR spectroscopy (Table 16), the isolated compound was suspected to be a monoterpene (10 carbon atoms). The DEPT spectrum of the compound revealed the presence of 1 methyl group (δ 20.8), 5 methylenes (δ 30.4, 27.5, 29.7, 67.7 and 108.7), 2 methines (δ 41.1 and 122.3) and 2 quartenary carbons (δ 137.2 and 149.8). The presence

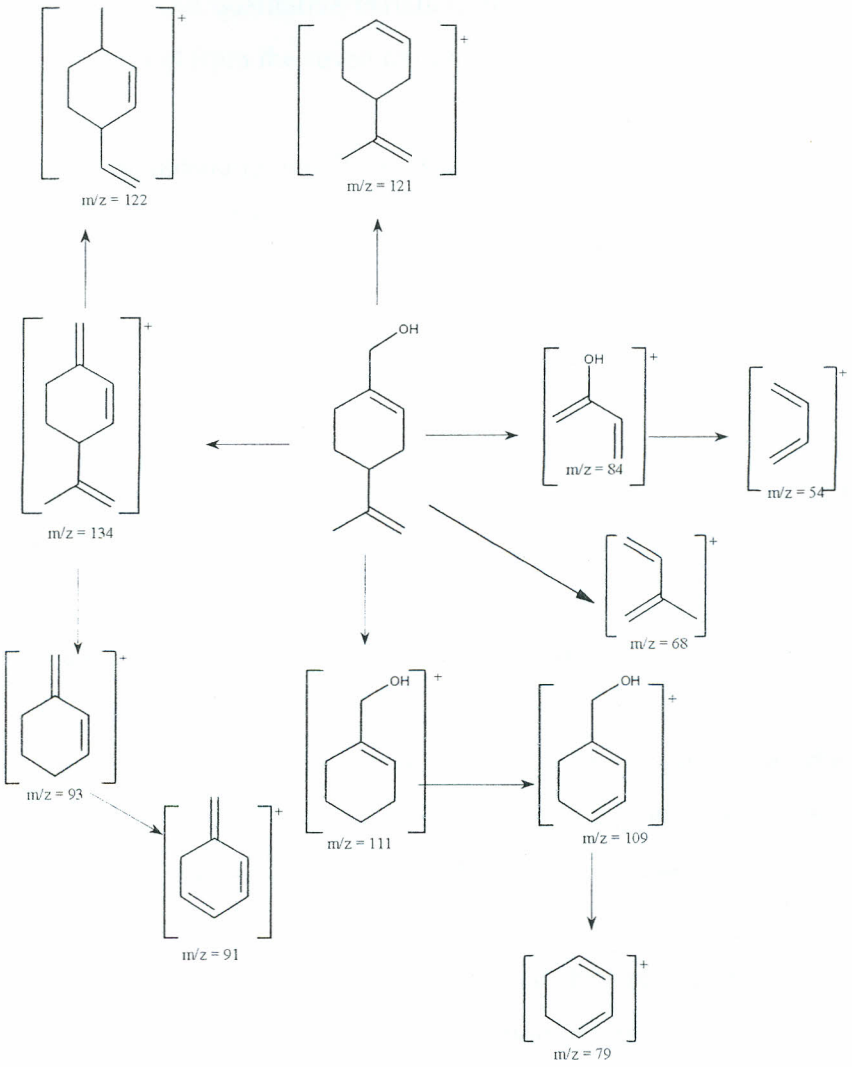
of two olefinic quaternary carbons was suggested by the absence peaks at δ 137.2 and 149.8 in the DEPT spectrum. The presence of an oxygenated carbon was suggested by the peak at δ 67.1 in the ^{13}C NMR, that was confirmed as CH_2 by DEPT analysis. An olefinic CH_2 group was suggested by the peak at δ 108.7 in the ^{13}C NMR and DEPT. Three other non-olefinic CH_2 groups were associated with peaks at δ 30.4, 27.5 and 29.7. An olefinic CH group was confirmed by the peak at δ 122.3 and DEPT while the remaining methine was associated with the peak at δ 41.1.

Table 16: ^{13}C NMR values of the isolated compound in $CDCl_3$

Position	chemical shift (δ)	DEPT
1	137.2	C
2	122.3	CH
3	30.4	CH_2
4	41.1	CH
5	29.7	CH_2
6	27.5	CH_2
7	67.1	CH_2
8	149.8	C
9	108.7	CH_2
10	20.8	CH_3

From mass spectroscopy analysis of the compound (Scheme 3), a molecular ion peak was observed at *m/z* 152. A stable peak at *m/z* 134 suggested loss of water and further the presence of a hydroxyl group.

Scheme 3: The mass fragmentation pattern of the isolated compound



From ^{1}H, ^{13}C, DEPT, HETCOR NMR and MS, the compound was proposed as peril alcohol. Comparison of the spectral data of the isolated compound and existing literature information confirmed that the isolated compound is peril alcohol (**61**) (Shell, 1997).

Conclusions and future directions

There was quantitative and qualitative variation in chemical composition of essential oil of *Conyza newii* collected from the seven regions within Kenya.

There is variation in mosquito repellent efficacy of *Conyza newii* essential oil with geographical location of the plant.

The chemical basis of variation in protective efficacy of essential oil from different regions is the concentration of peril alcohol and geraniol in the oil.

Peril aldehyde enhances the protective efficacy of peril alcohol and geraniol against *An. gambiae s.s* mosquitoes.

Limonene reduces the activity of geraniol and peril alcohol.

The efficacy of *Conyza newii* as a mosquito repellent depends on the mode of application. For instance steam-distilled oil has the highest protective efficacy compared to thermally expel and directly direct burning oil has the lowest protective efficacy.

The protective efficacy of *Conyza newii* essential oil depends on the concentration of peril alcohol therein. The higher the concentration of peril alcohol the higher the protective efficacy of the oil

Artificial mixtures of the components of essential oil that are more repellent than the natural blend have been discovered.

Since smoke emanations show appreciable repellency, candles and coils made of plant material could be produced for mosquito control.

The chemistry of the essential oil of intact *C. newii* plant has been shown to be different from that of steam distilled, thermally expelled or directly burnt one.

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APPENDIX 1: Repellency assay data of essential oil by steam distillation

West Pokot

	% Protective efficacy						Mean % P.E
Concentration	RO	MA	VW	MAA	RK	JK	
0.01	13.39	30	10	25	29.17	36.36	23.99
0.1	89.29	82.34	77.22	46.67	50	60	67.59
1	100	82.35	91.67	100	100	100	95.67
10	100	100	100	100	100	100	100

Kilome

	% Protective efficacy						Mean % P.E
Concentration	RO	MA	VW	MAA	RK	JK	
0.01	7.69	5.88	11.76	10.71	22.22	3.7	10.33
0.1	17.78	10.71	23.08	15.79	28.57	8.33	17.38
1	100	82.5	88.89	33.33	84	88.46	79.53
10	100	100	100	100	100	100	100

Naivasha

	% Protective efficacy						Mean % P.E
Concentration	RO	MA	VW	MAA	RK	JK	
0.01	20	25	25	8.33	16.67	8.33	17.22
0.1	61.67	36.84	37.93	54.54	64.71	68.75	54.07
1	94.44	100	100	90.91	66.67	100	92
10	100	100	100	100	100	100	100

Webuye

	% Protective efficacy						Mean % P.E
Concentration	RO	MA	VW	MAA	RK	JK	
0.01	14.84	11.71	13.74	9.5	9.5	4.2	10.58
0.1	68.2	36.7	29.6	23.8	46.4	43.8	41.42
1	100	57.6	66.7	94.4	92.5	93.9	84.18
10	100	100	100	100	100	100	100

Nyakach

	% Protective efficacy						Mean % P.E
Concentration	RO	MA	VW	MAA	RK	JK	
0.01	31.25	11.76	12.5	20	33.33	6.67	19.25
0.1	50	36.36	60	37.5	38.57	43.33	44.29
1	100	95.7	100	70.6	100	100	94.38
10	100	100	100	100	100	100	100

Kericho

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	56.2	57	34.76	35.71	33.33	15.38	32.77
0.1	81.1	77.3	71.43	50	60	68.97	68.13
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100

Nairobi

	% Protective efficacy						
Comncentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	27.8	10	4.3	11.1	13.3	33.3	16.63
0.1	50	36.36	60	37.5	38.57	43.3	44.29
1	100	93.33	87.5	86.67	100	80	91.25
10	100	100	100	100	100	100	100

Appendix 2: Repellency assay data of essential oil by thermal expulsion

Nairobi

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	7.14	11.76	10	33.8	7.14	20	14.97
0.1	25	16.67	16.67	44.62	11.76	15.79	21.75
1	78.57	56.67	39.13	37.5	23.08	30	44.16
10	95.83	66.67	48.46	50	40.54	55	59.42

Kilome

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	4.25	12.5	6.67	5.56	4.76	5.88	6.6
0.1	10.71	24	25	17.41	25	9.09	18.86
1	20	38.71	43.08	30.43	38.79	14.29	30.88
10	62.86	79.92	88.24	64.71	60	56.15	68.15

Naivasha

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	15.65	7.14	16.67	9.09	12.9	40	16.9
0.1	17.39	41.18	22.58	28.57	28.57	44.44	30.45
1	22.92	60.98	28.57	53.66	57.56	46.67	45.06
10	69.57	68.75	36	63.89	78.95	54.35	61.92

Webuye

	% Protective efficacy						
Comncentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	16.67	12.5	13.33	12.5	16.67	12.5	14.02
0.1	17.65	14.29	25	25	22.22	23.08	21.21
1	23.53	19.09	31.05	35	36.67	28.57	28.99
10	60	61.54	84.62	42	44.44	50	57.1

Kericho

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	35.29	5.88	39.39	12.5	14.29	21.43	21.46
0.1	40	16.67	38.88	18.18	43.75	33.33	31.8
1	56.82	75	50	53.85	75	53.49	60.69
10	92.86	93.33	92	94.74	93.33	93.41	93.28

Nyakach

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	10	12.5	7.69	16.67	23.08	11.76	13.51
0.1	22.22	22.22	35.48	37.03	28.57	16.67	27.96
1	30	33.21	55.55	47.18	40	50	60.69
10	83.33	55.56	60	57.5	61.81	74.19	63.65

West Pokot

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	7.14	3.7	4.35	5.88	7.54	5.26	5.65
0.1	19.09	12.5	12.5	31.58	20	11.76	17.9
1	25.63	27.27	42.31	43.33	34.24	20	25.63
10	50	48	56.55	58	61.29	41.03	52.48

Appendix 3: Repellency assay data of oil by direct burning

Naivasha

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	4.25	12.5	6.67	5.56	4.76	5.88	6.6
0.1	10.71	24	25	17.41	25	9.09	18.86
1	20	38.71	43.08	30.43	38.79	14.29	30.88
10	62.86	79.92	88.24	64.71	60	56.15	68.15

Webuye

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	7.14	3.7	4.35	5.88	7.54	5.26	5.65
0.1	19.09	12.5	12.5	31.58	20	11.76	17.9
1	25.63	27.27	42.31	43.33	34.24	20	25.63
10	50	48	56.55	58	61.29	41.03	52.48

Nyakach

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	11.33	5.55	16.67	5	11.76	7.69	9.67
0.1	16.67	9.69	20	15.79	19.23	9.09	15.08
1	25	15.38	25	20	22.22	20	21.27
10	63.64	53.85	55	68.75	62.5	23.53	54.55

Kericho

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	11.76	13.33	9.09	6.67	10	8.33	9.86
0.1	14.29	15.76	14.29	9.09	22.5	17.14	15.51
1	35.71	28.57	21.05	14.18	31.21	23.53	25.71
10	40	38.19	33.08	36.67	42.07	33.33	37.22

Nairobi

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	13.33	6.25	8.33	9.52	11.76	6.25	9.24
0.1	22.22	37.5	19.52	21.11	26.77	16.67	23.97
1	30	44.44	33.33	26.67	31.21	20	30.94
10	50	50	52	42.31	42.86	50	47.86

Appendix 4: Repellency assay data of blends

Limonene, peril aldehyde, peril alcohol and geraniol (blend 1)

	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	51.8	58.29	44.28	41.47	53.29	51.22	50.1
0.1	77.29	77.33	70.42	68.71	65.66	69.89	71.6
1	91.22	96.58	97.22	98.41	93.55	94.46	95.2
10	100	100	100	100	100	100	100

Peril aldehyde, peril alcohol and geraniol (blend 2)

	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	62.22	65.17	71.22	62.86	76.7	78.1	64.4
0.1	88.22	89.41	91.22	92.22	93.44	94.22	91.5
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100

Limonene, peril alcohol and geraniol (blend 3)

	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	48.22	41.08	43.13	51.22	40.71	44.77	44.9
0.1	61.22	64.33	68.19	61.18	58.07	64.17	62.49
1	81.22	82.41	85.19	88.22	89.18	88.42	85.8
10	100	100	100	100	100	100	100

Peril alcohol and geraniol (blend 4)

	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	67.88	69.2	70.24	65.28	60.19	69.22	67
0.1	92.44	90.41	88.21	91.44	96.88	91.41	91.8
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100

Geraniol and peril aldehyde (Blend 5)

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	71.48	74.22	71.42	70.48	74.11	72.17	72.31 ± 0.26
0.1	99.44	98.69	96.22	98.17	97.29	99.54	98.22 ± 0.38
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100

Peril alcohol and peril aldehyde (blend 6)

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	69.28	67.44	69.11	68.28	69.24	68.35	68.35 ± 1.04
0.1	95.28	94.22	95.22	96.11	94.44	94.44	94.99 ± 1.12
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100

Peril aldehyde and limonene (blend 7)

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	51.44	52.49	53.28	54.29	53.44	51.29	52.21 ± 0.41
0.1	66.78	65.44	65.28	65.91	62.22	64.45	65.01 ± 0.28
1	100	100	100	100	100	100	91.11 ± 0.33
10	100	100	100	100	100	100	100

Geraniol and limonene (blend 8)

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	53.22	52.58	51.44	50.22	50.19	50.44	51.52 ± 0.22
0.1	70.19	68.22	71.14	69.22	71.18	72.22	70.36 ± 1.14
1	94.22	95.33	95.29	94.18	95.41	94.33	94.46 ± 1.14
10	100	100	100	100	100	100	100

Peril alcohol and limonene (blend 9)

	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	60.48	61.18	62.22	61.48	63.31	62.22	61.47 ± 0.48
0.1	73.22	74.28	75.11	73.49	73.48	74.18	73.96 ± 0.34
1	87.42	88.19	86.44	87.19	87.33	88.44	87.34 ± 0.48
10	100	100	100	100	100	100	100

Appendix 5: Repellency assay data of standards

cis-limonene oxide

	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	28.41	26.79	28.23	29.42	31.46	27.32	28.61
0.1	41.31	38.29	40.42	43.17	42.22	44.14	41.59
1	62.22	68.11	60.41	64.56	62.77	65.22	63.88
10	84.19	89.2	87.45	86.81	81.29	83.44	85.4

cis- dihydrocarvove

	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	19.48	17.29	22.46	21.22	23.44	22.28	21.03
0.1	38.29	23.44	24.17	27.32	32.41	36.42	30.44
1	51.46	54.89	50.19	53.22	54.19	55.28	53.21
10	61.88	63.22	60.08	61.22	64.19	66.71	62.88

4-methoxyphenol

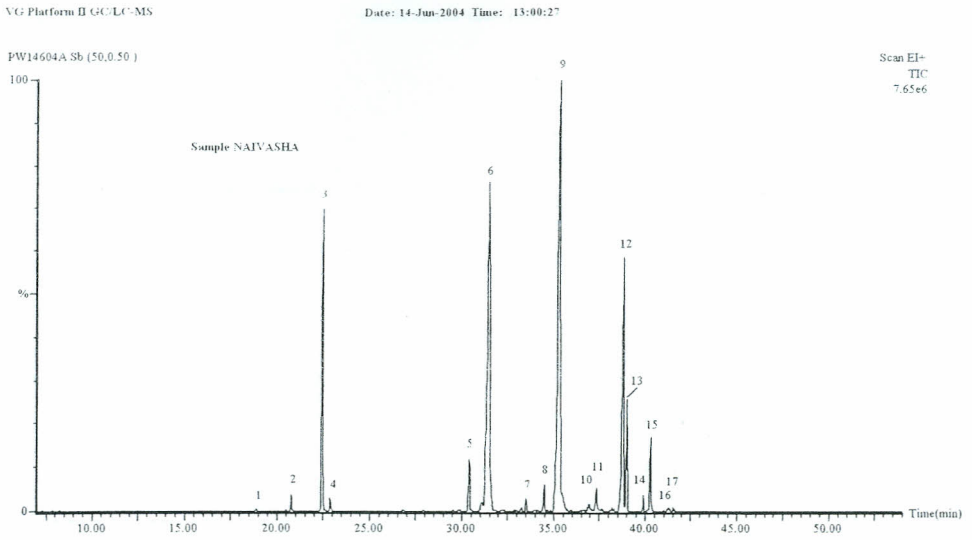
	% protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	14.27	15.22	18.17	13.48	13.22	12.01	14.4
0.1	21.42	22.14	23.22	29.48	22.43	20.19	23.15
1	32.48	31.22	30.14	31.42	33.18	29.48	31.32
10	56.43	49.88	51.28	52.29	53.44	59.64	53.83

Bioassay data of the crude hexane extract

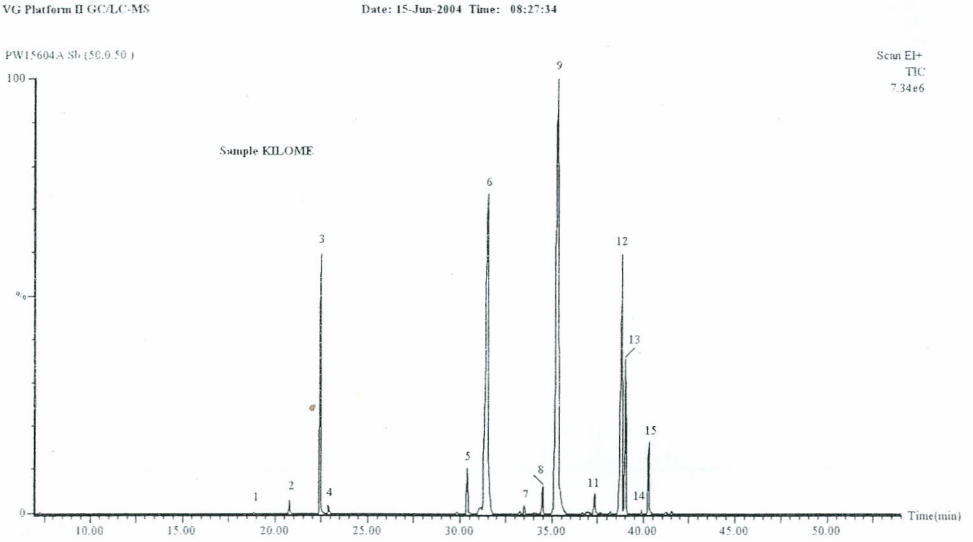
	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	44.29	58.11	59.24	48.24	47.28	52.44	51.6 ± 0.21
0.1	100	100	96.28	100	100	100	99.38 ± 0.4
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100

Bioassay data of the isolated compound

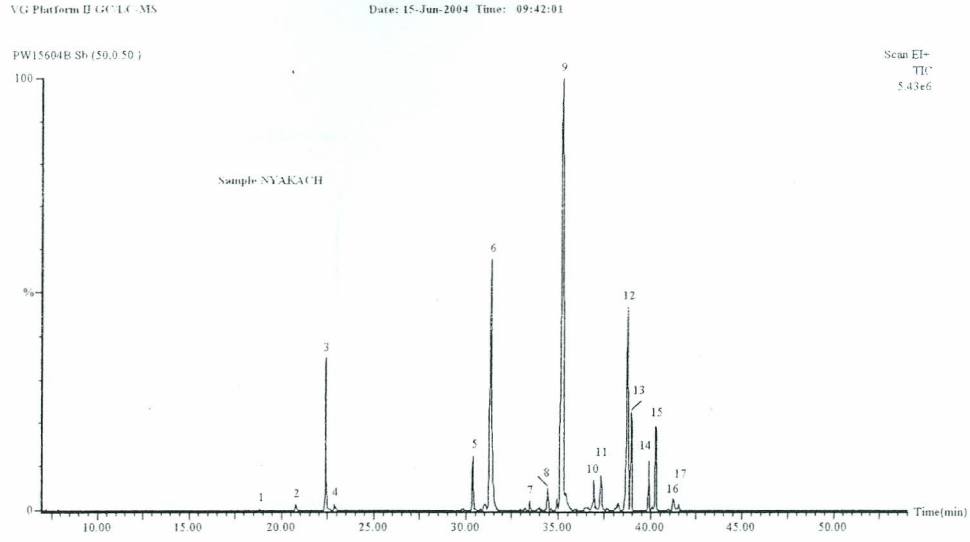
	% Protective efficacy						
Concentration	RO	MA	VW	MAA	RK	JK	Mean % PE
0.01	78.24	76.22	77.48	75.44	74.22	77.41	76.5 ± 0.11
0.1	100	100	100	100	100	100	100
1	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100



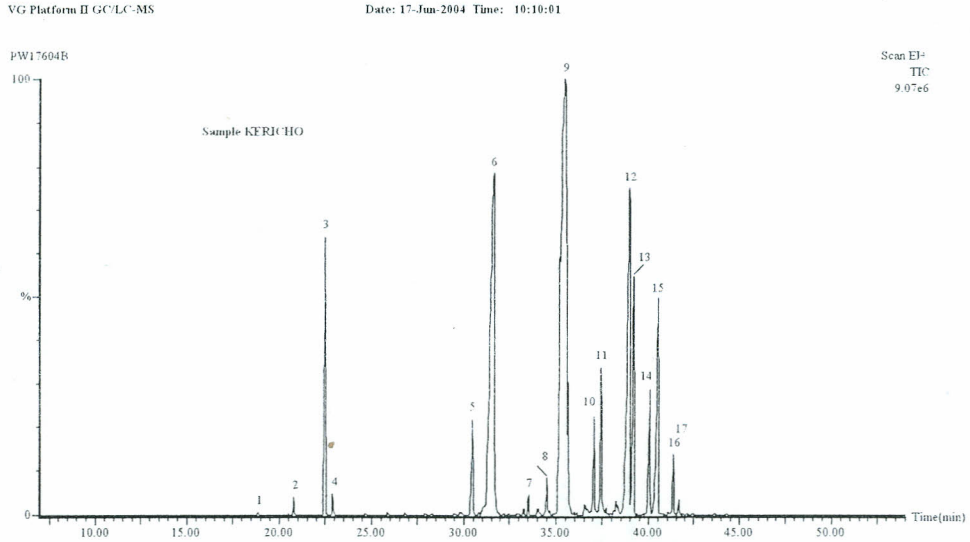
GC-MS spectrum of Naivasha essential oil



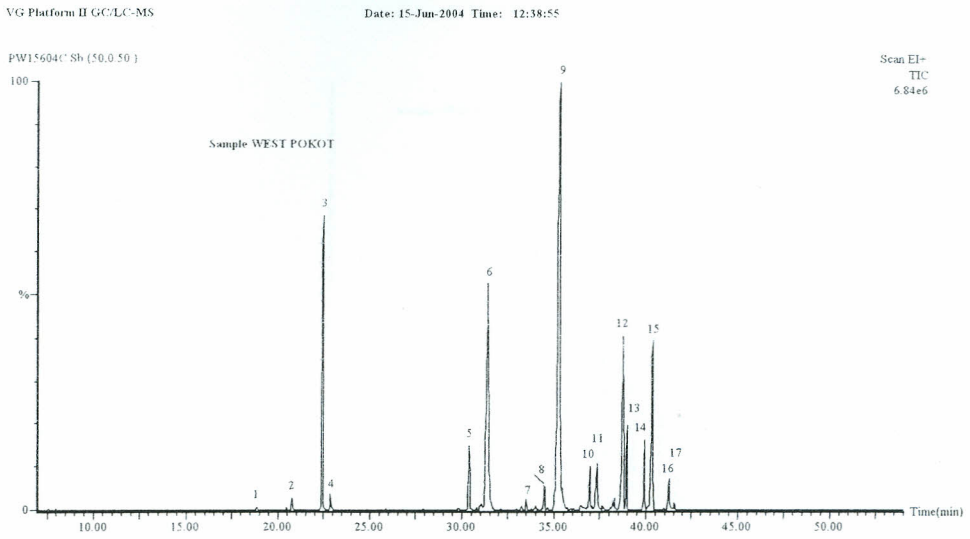
GC-MS spectrum of Kilome essential oil



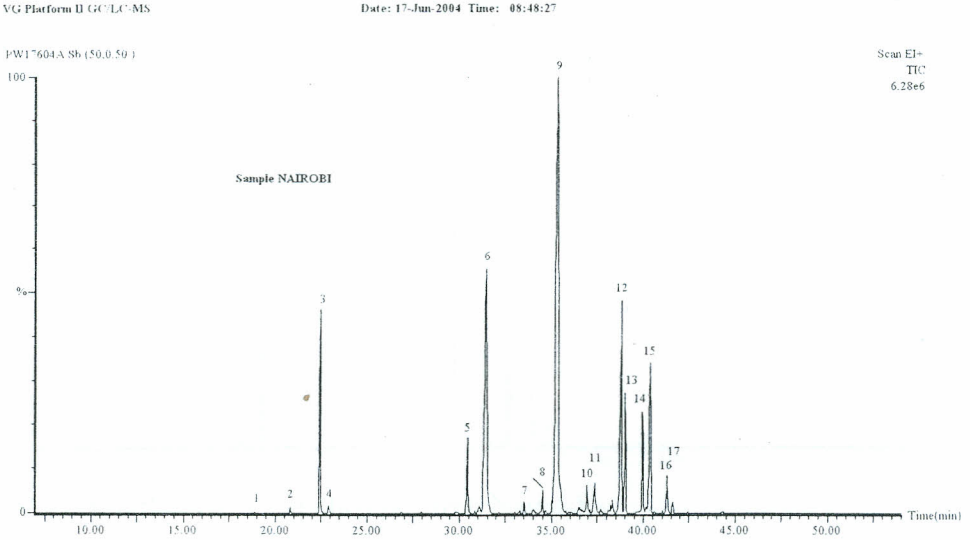
GC-MS spectrum of Nyakach essential oil



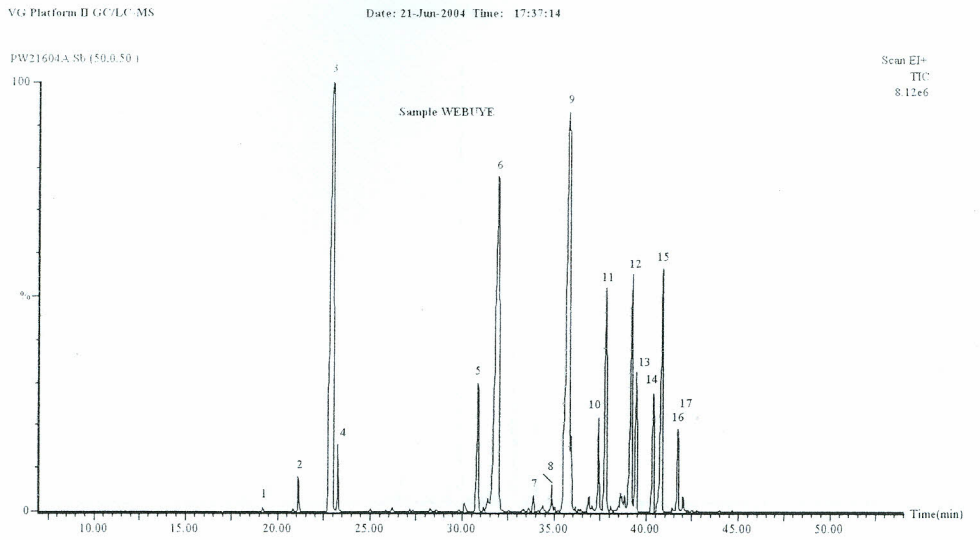
GC-MS spectrum of Kericho essential oil



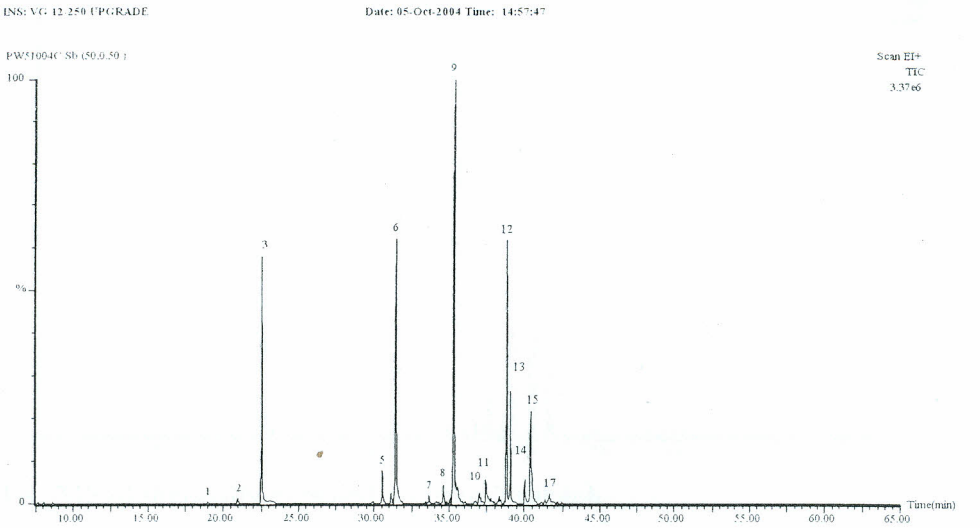
GC-MS spectrum of West pokot essential oil



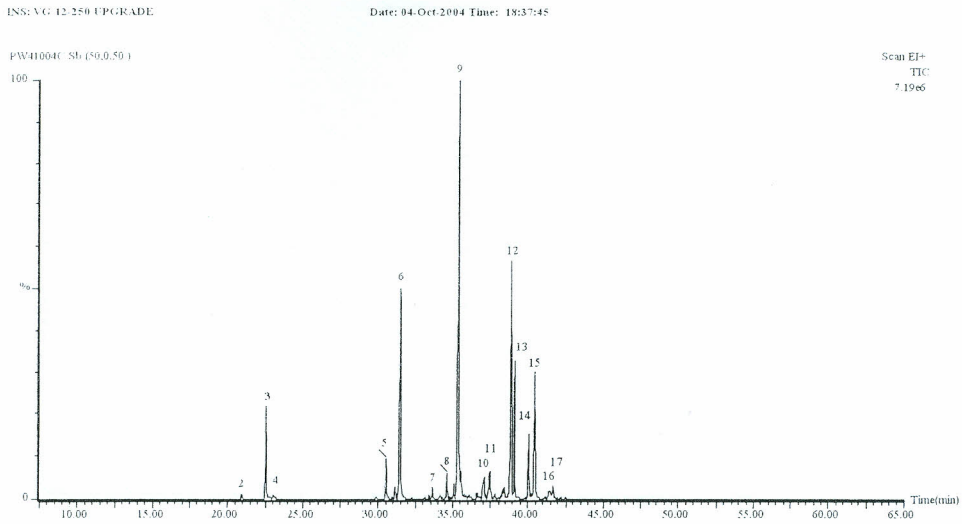
GC-MS spectrum of Nairobi essential oil



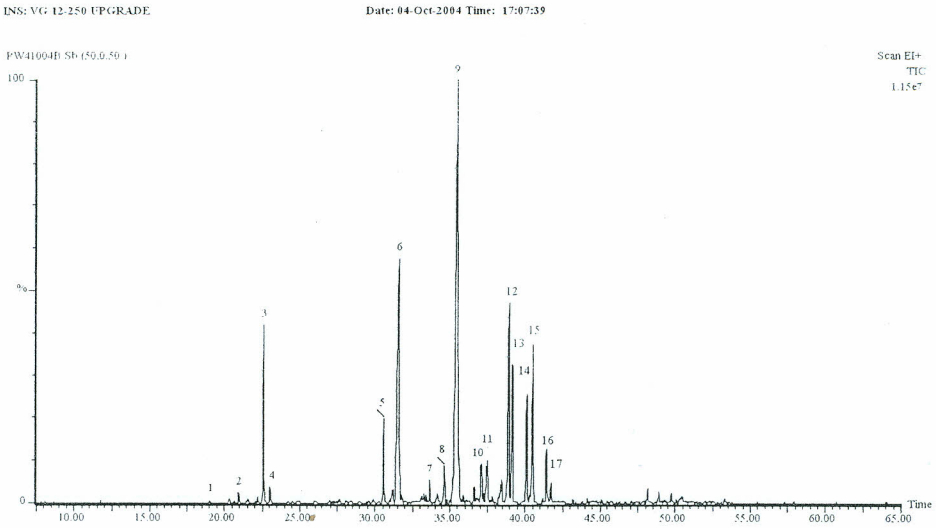
GC-MS spectrum of Webuye essential oil



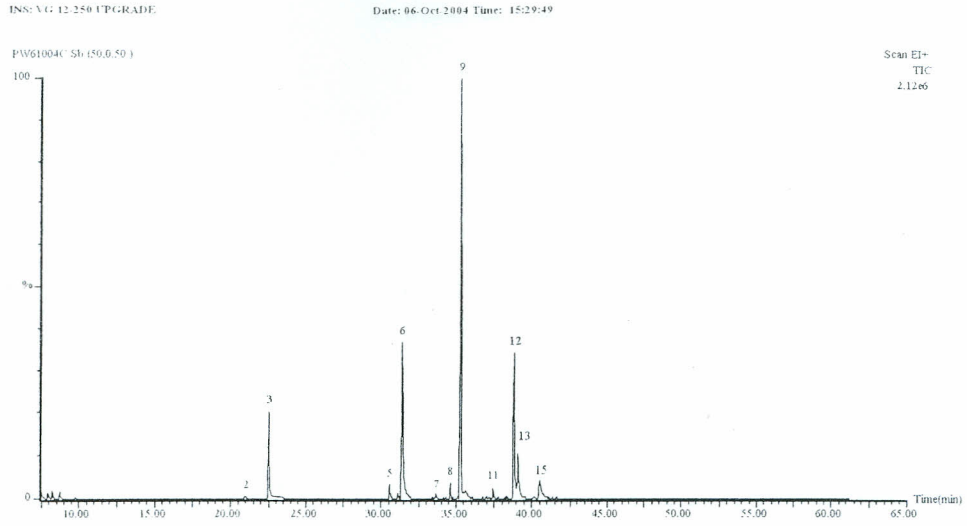
GC-MS of thermally expelled oil from Naivasha



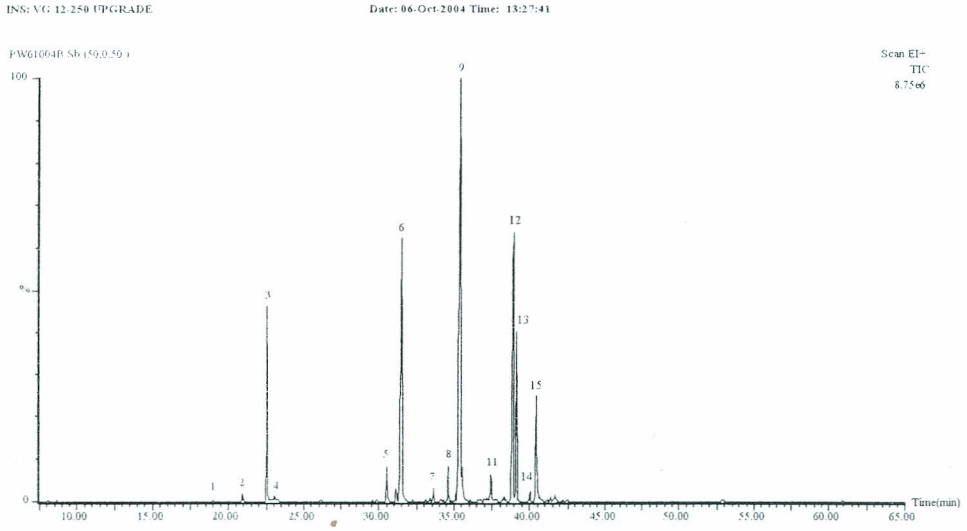
GC-MS spectrum of thermally expelled oil from Kilome



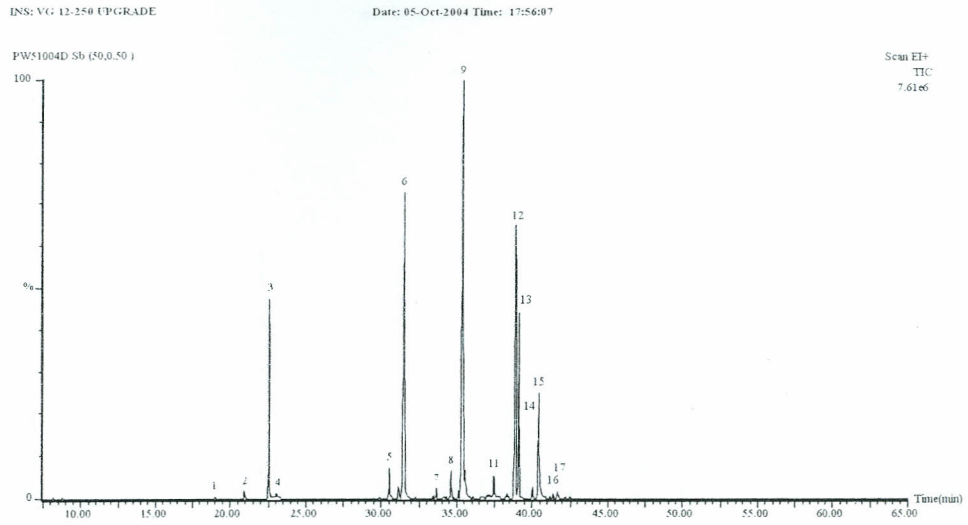
GC-MS of thermally expelled oil from Nyakach



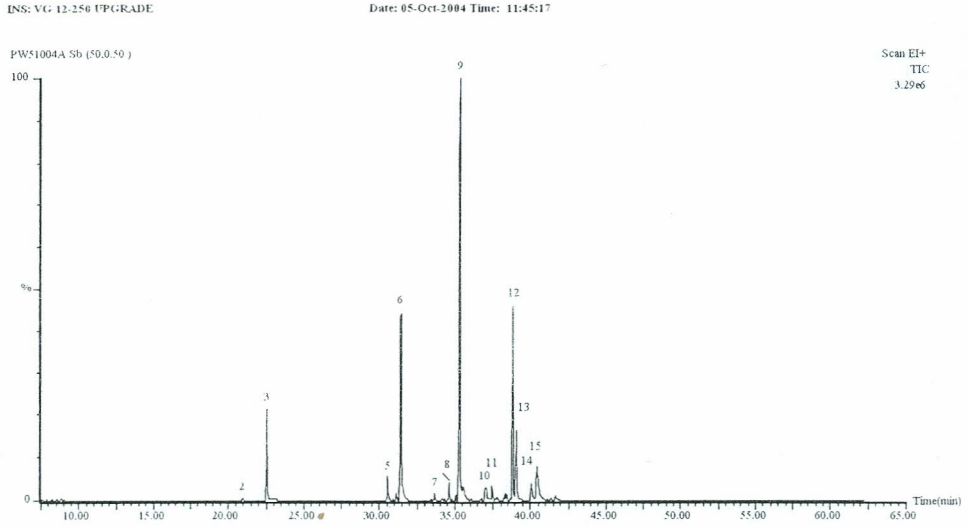
GC-MS spectrum of thermally expelled oil from Kericho



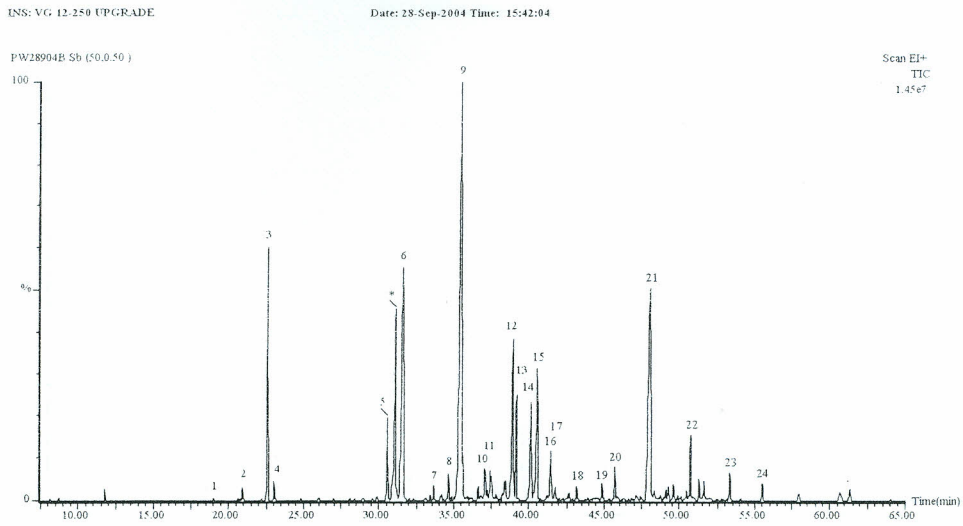
GC-MS spectrum of thermally expelled oil from West Pokot



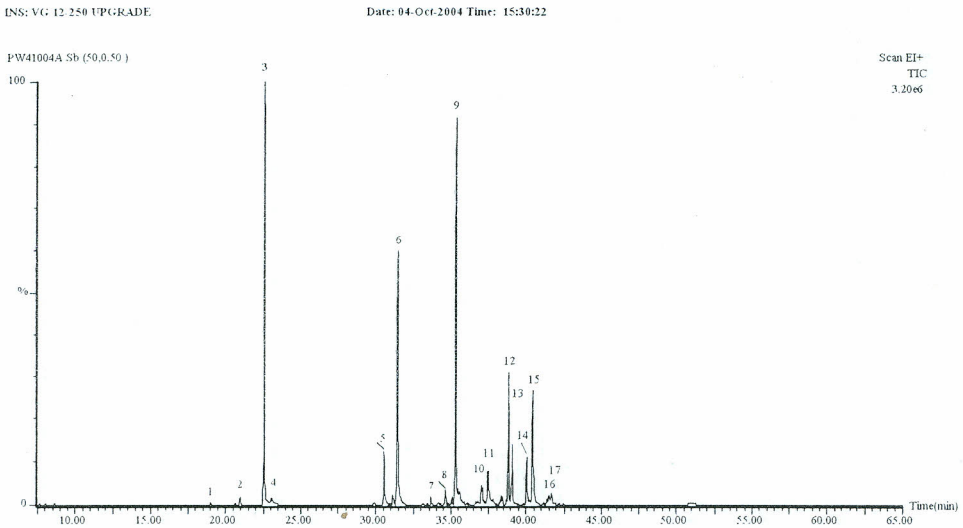
GC-MS of thermally expelled oil from Nairobi



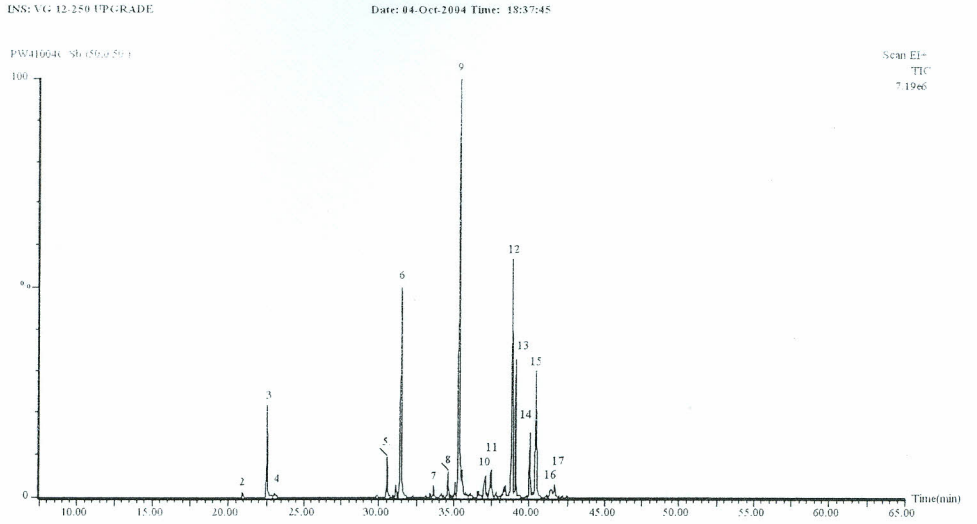
GC-MS of thermally expelled oil from Webuye



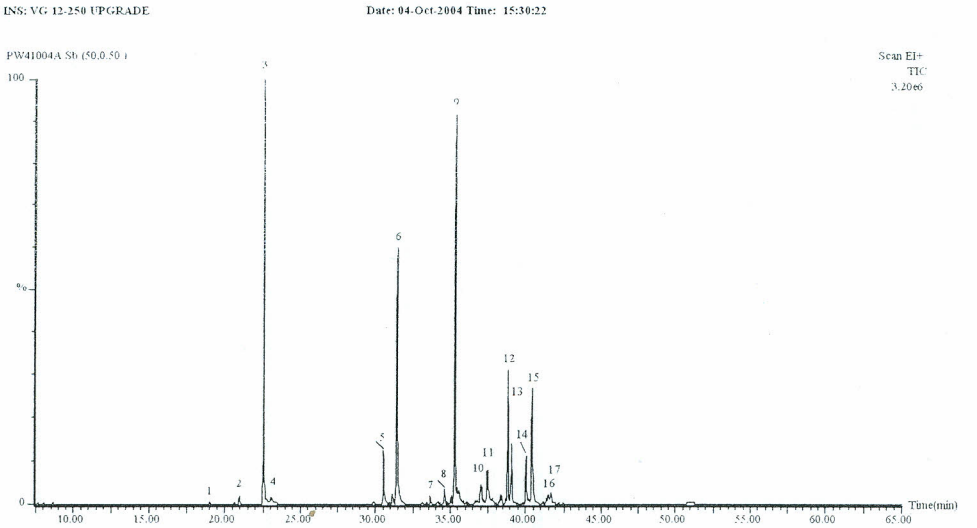
GC-MS spectrum of directly burned oil from Naivasha



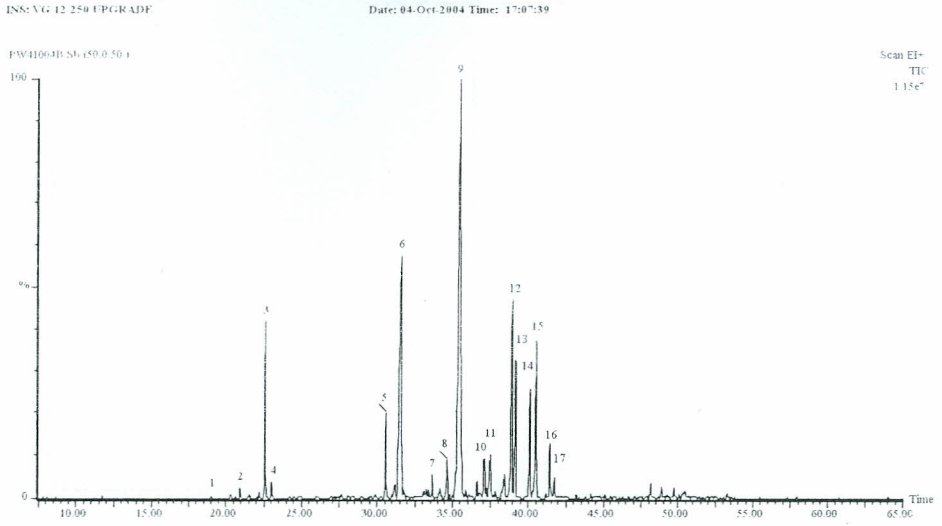
GC-MS spectrum of directly burned oil from Kilome



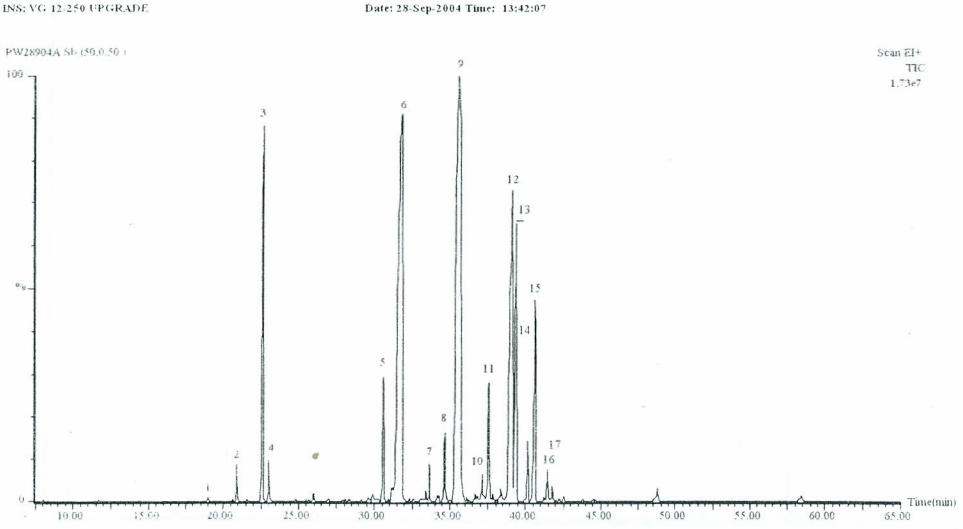
GC-MS of directly burned oil from Nyakach



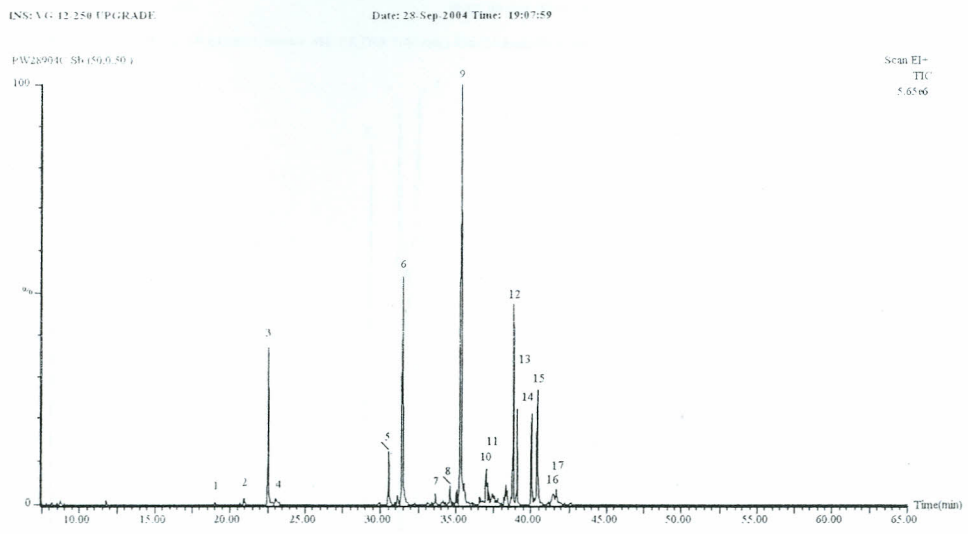
GC-MS spectrum of directly burned oil from Kericho



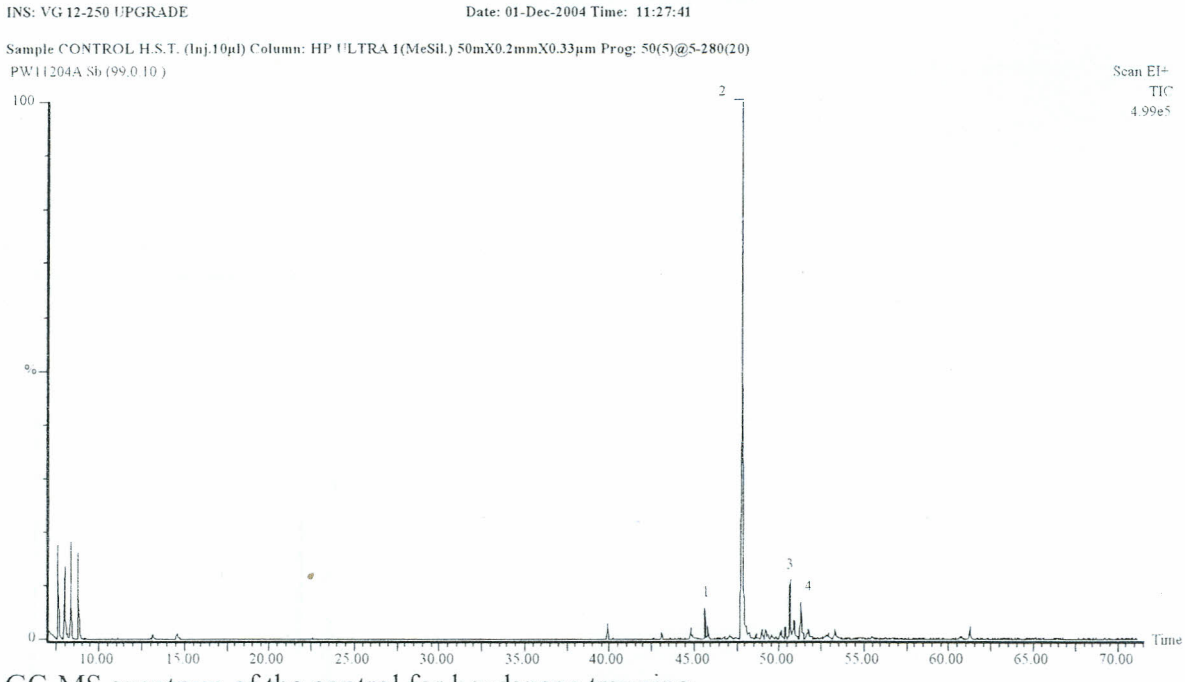
GC-MS spectrum of directly burned oil from West Pokot



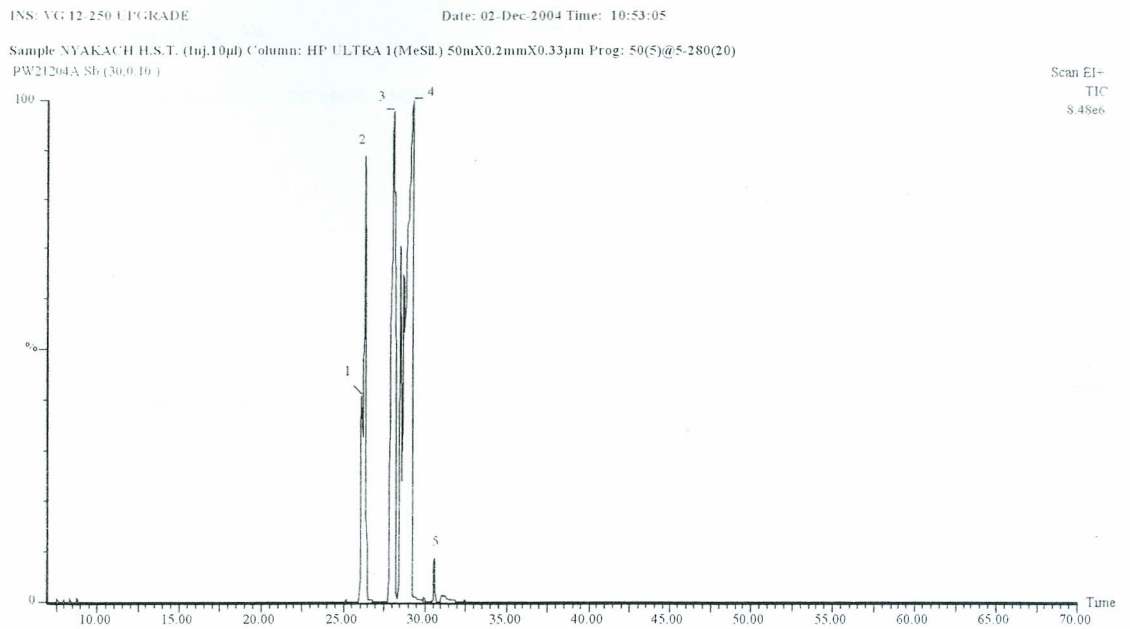
GC-MS spectrum of Directly burned oil from Nairobi



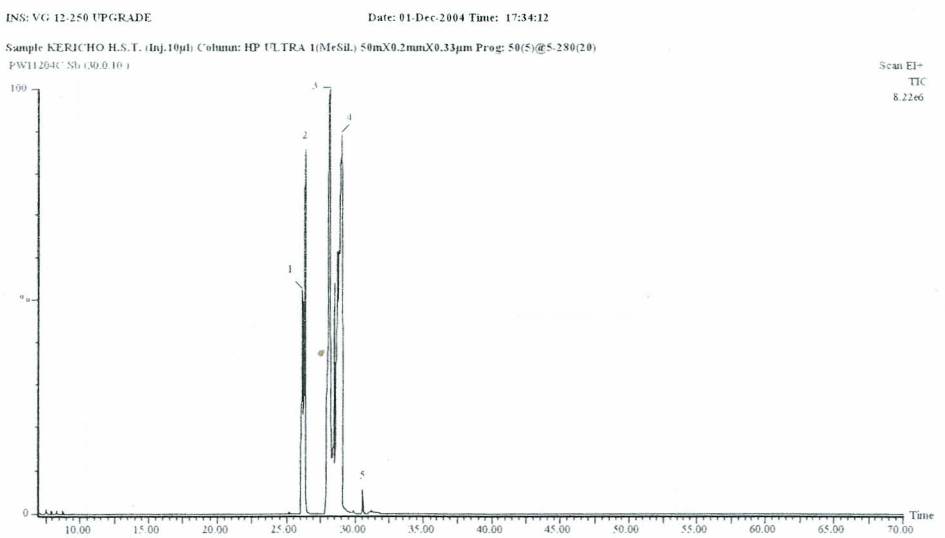
GC-MS spectrum of directly burned oil from Webuye



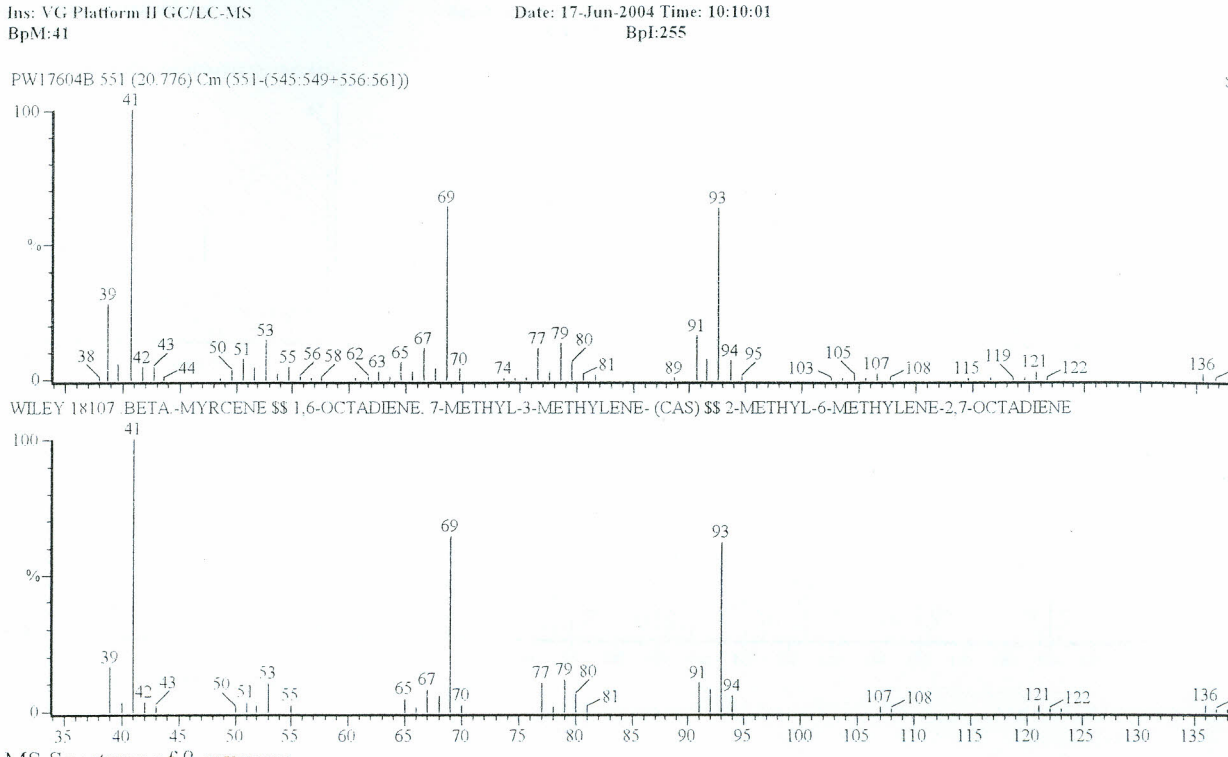
GC-MS spectrum of the control for headspace trapping



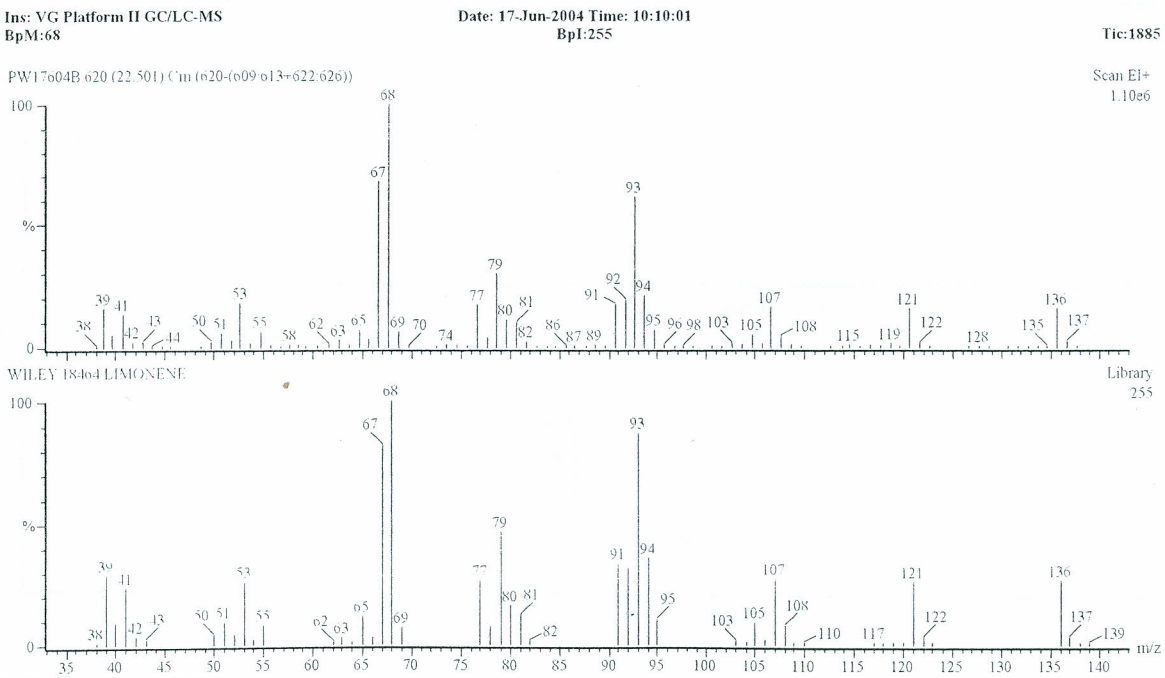
GC-MS of oil obtained by headspace trapping in west pokot



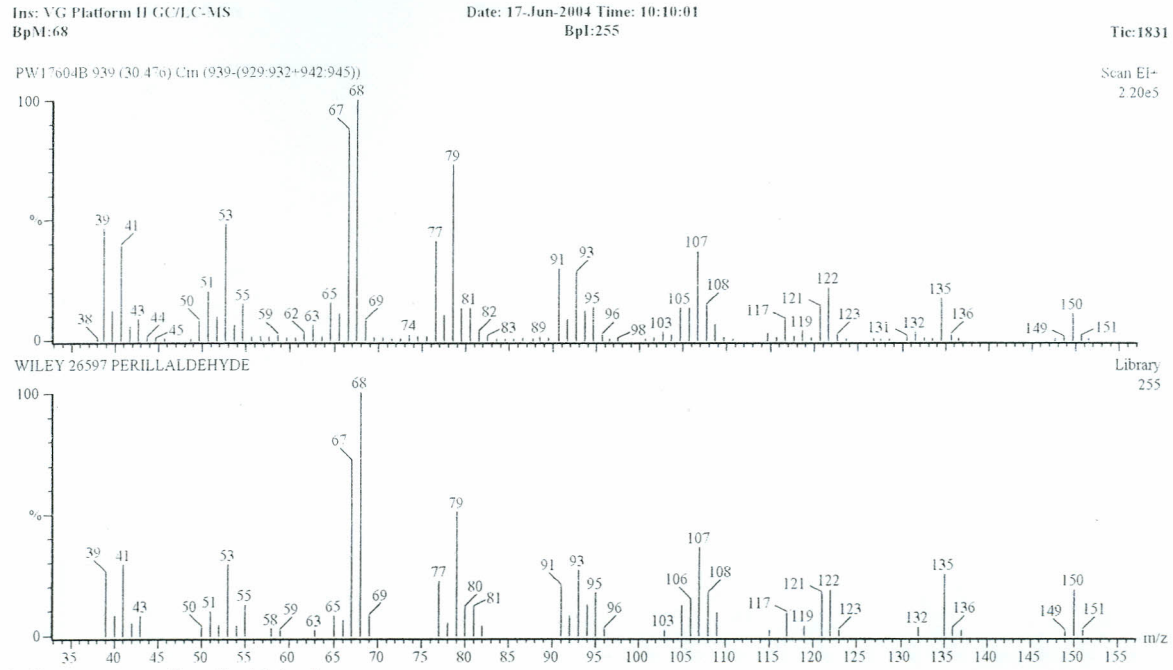
GC-MS of oil obtained by headspace trapping in Kericho



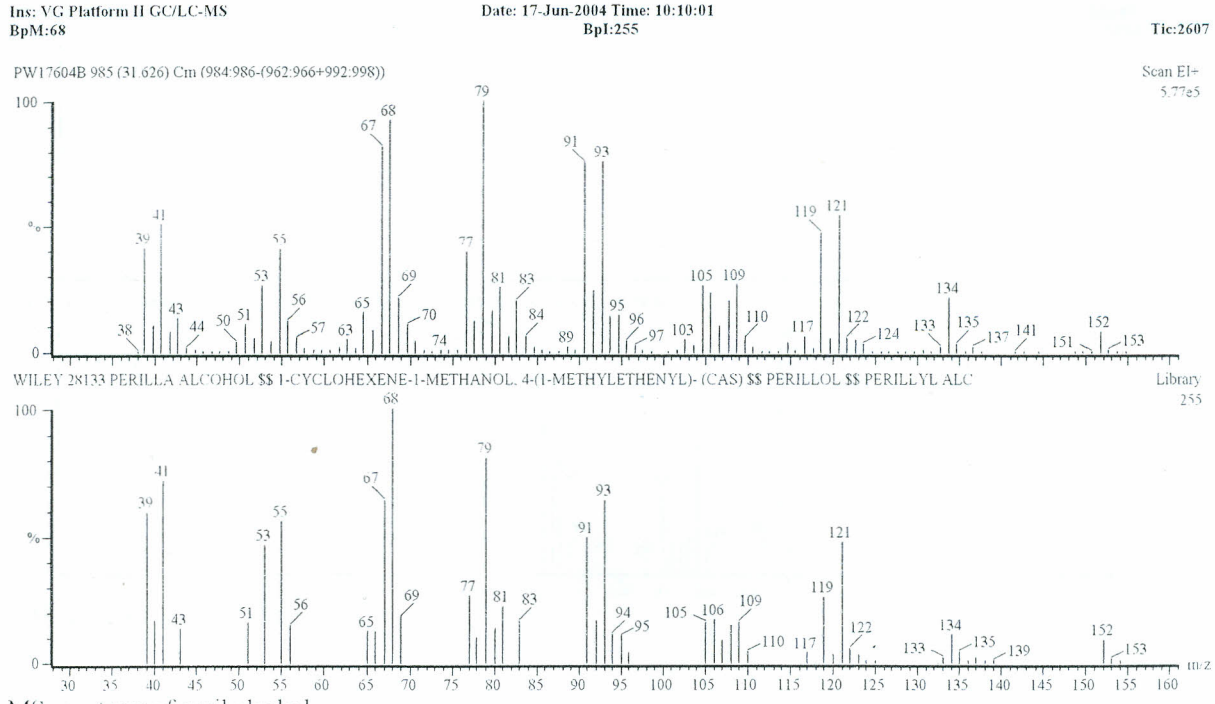
MS Spectrum of β-myrcene



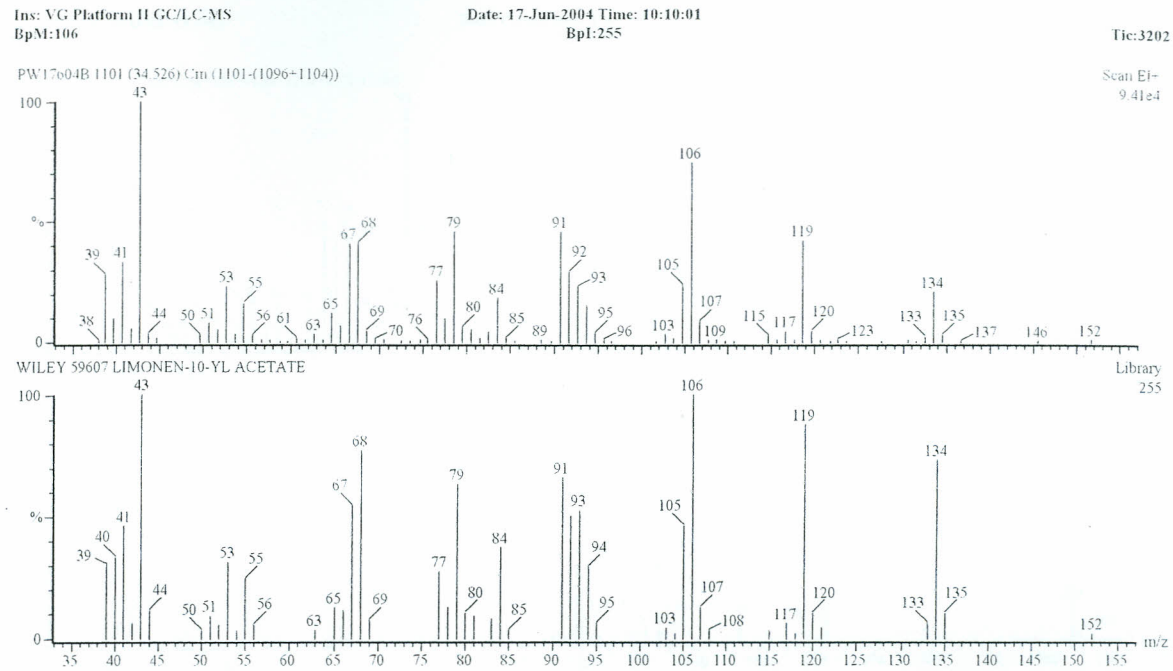
MS spectrum of (s)-limonene



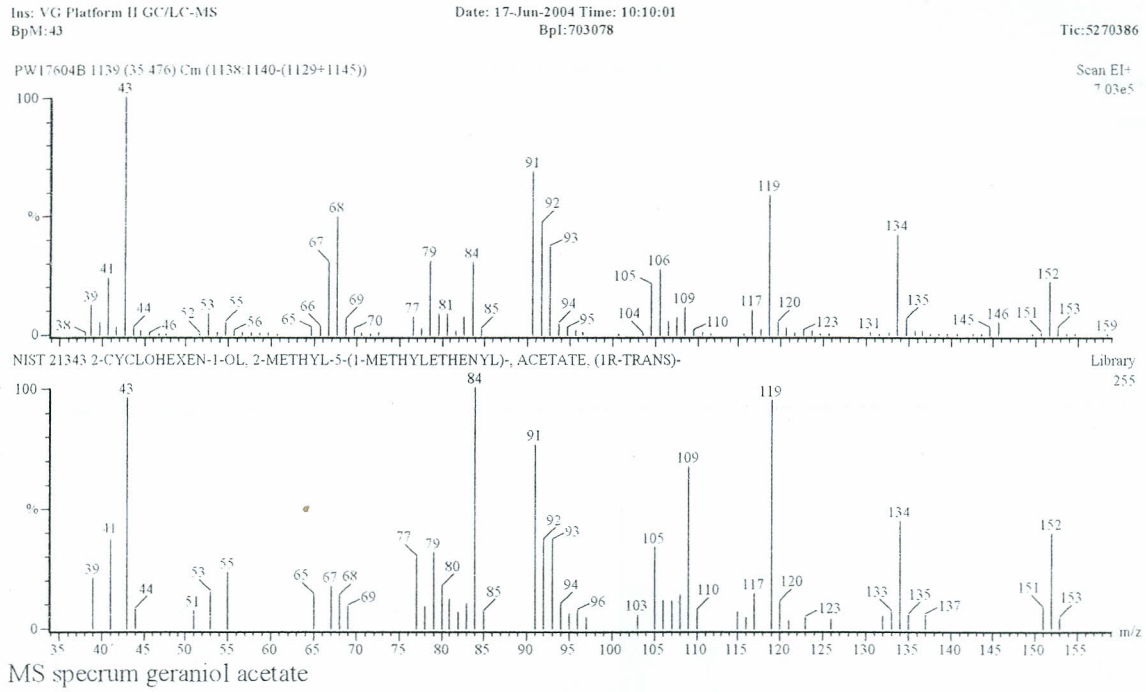
MS spectrum of peril aldehyde



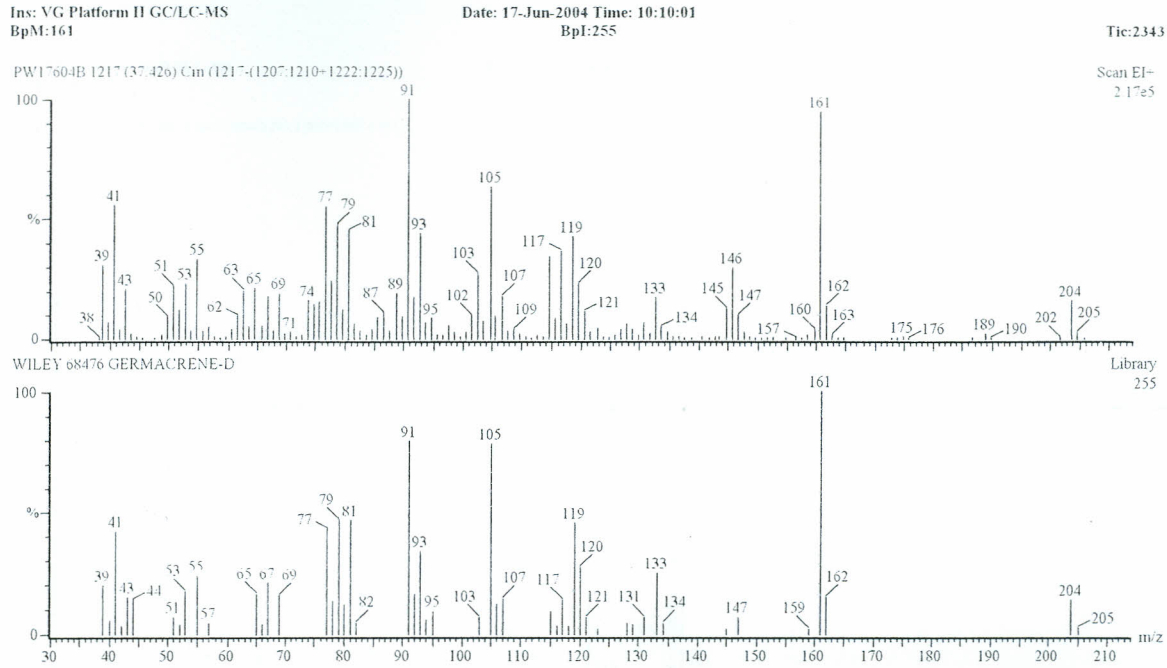
MS spectrum of peril alcohol



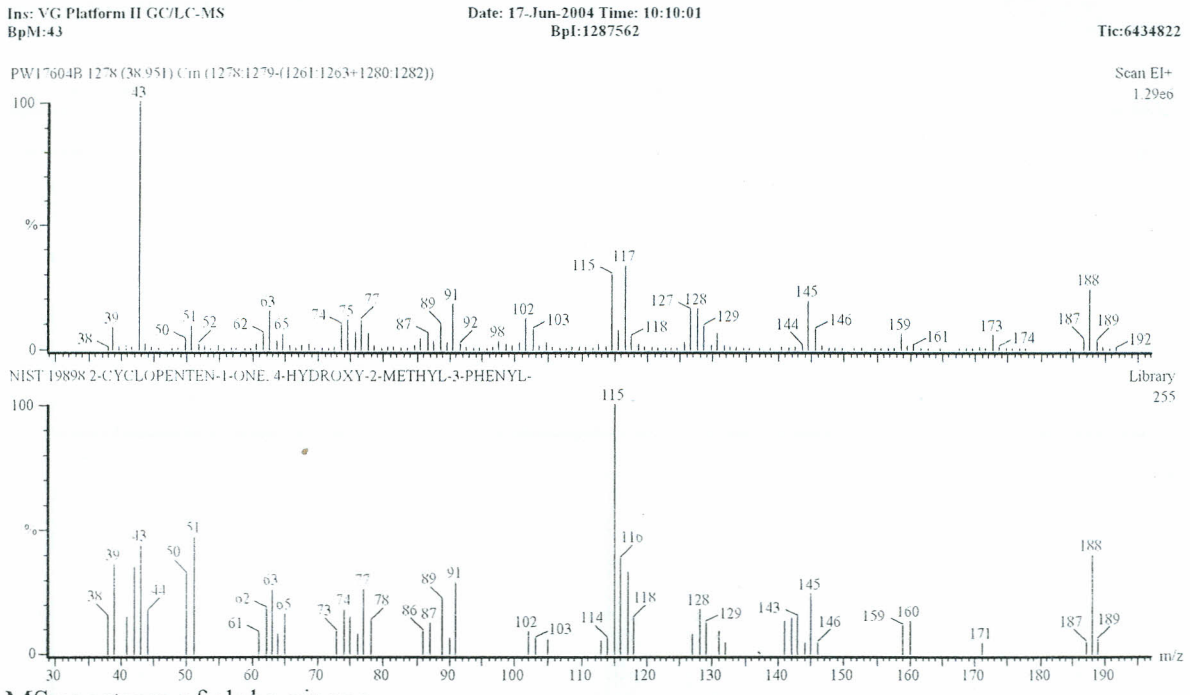
MS spectrum of geraniol



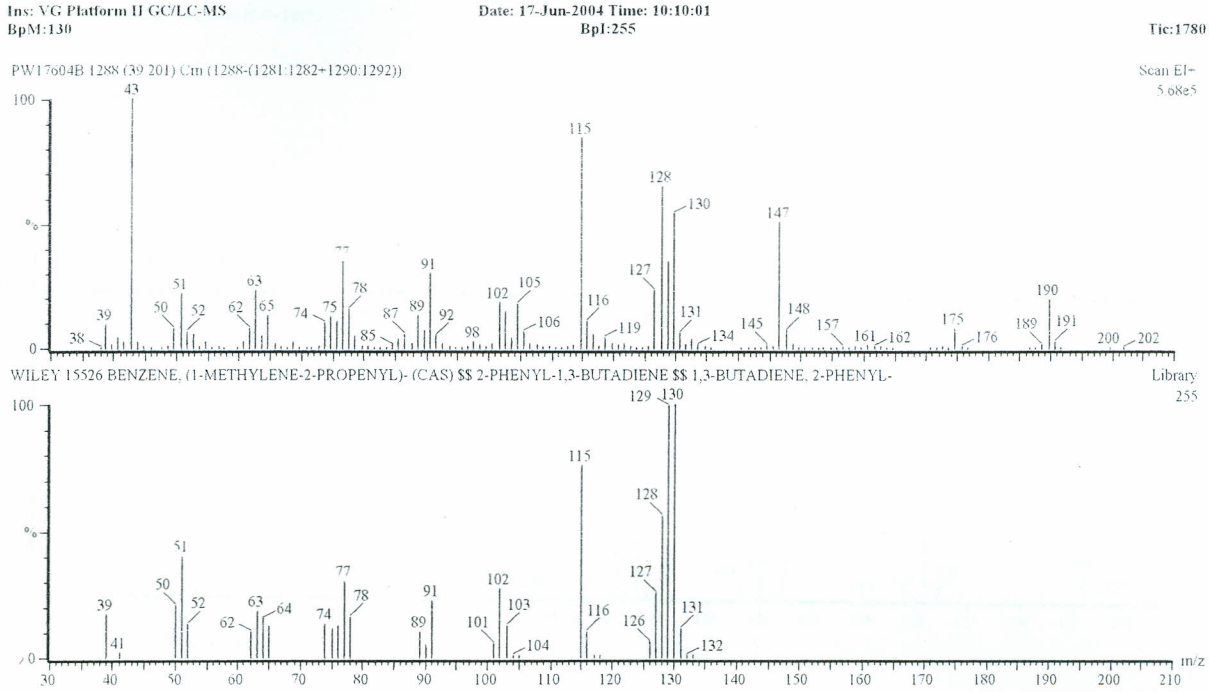
MS specrum geraniol acetate



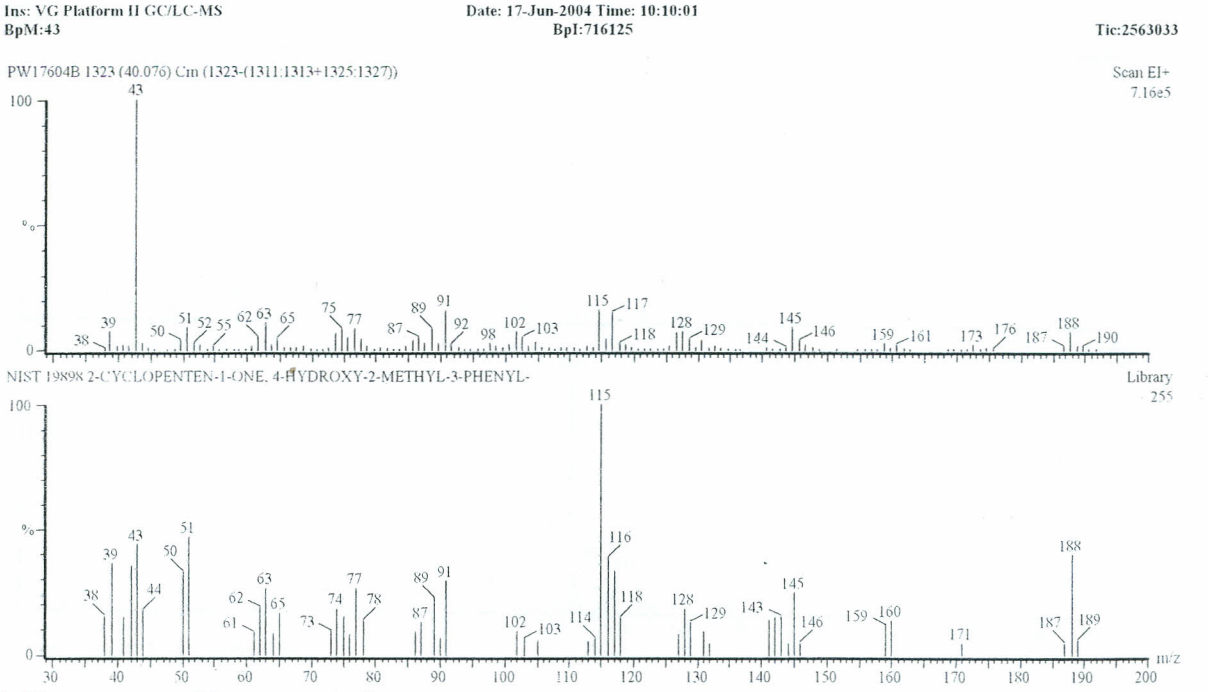
MS spectrum of Germacrene-D



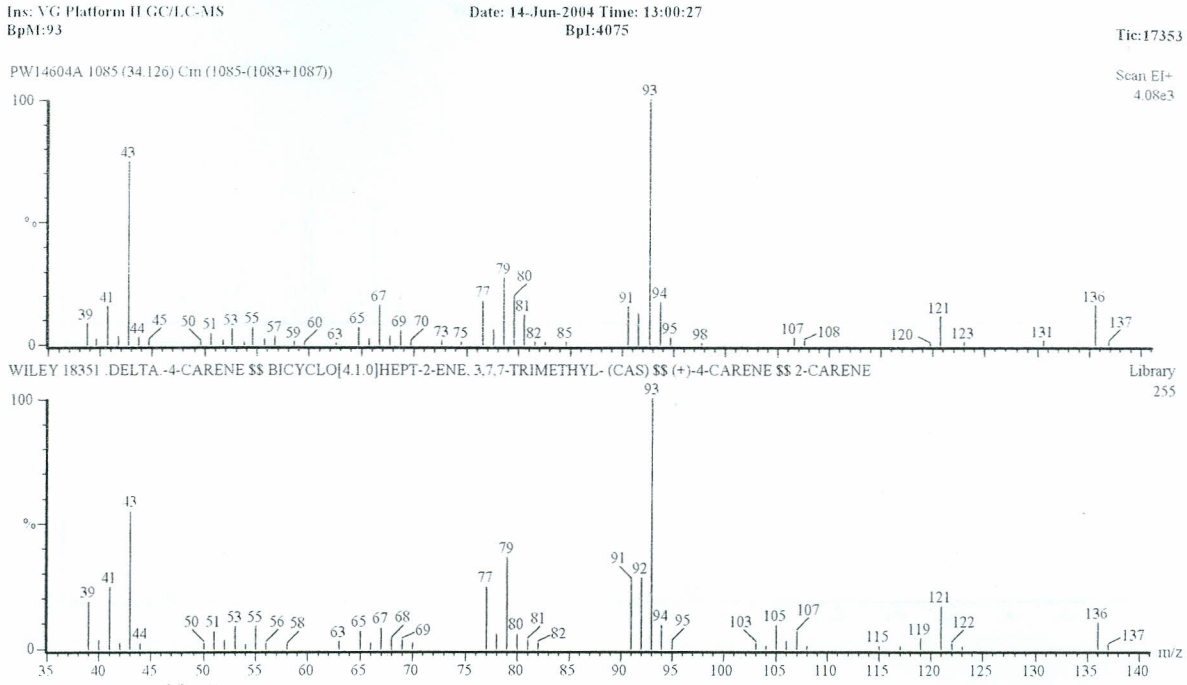
MS spectrum of alpha pinene



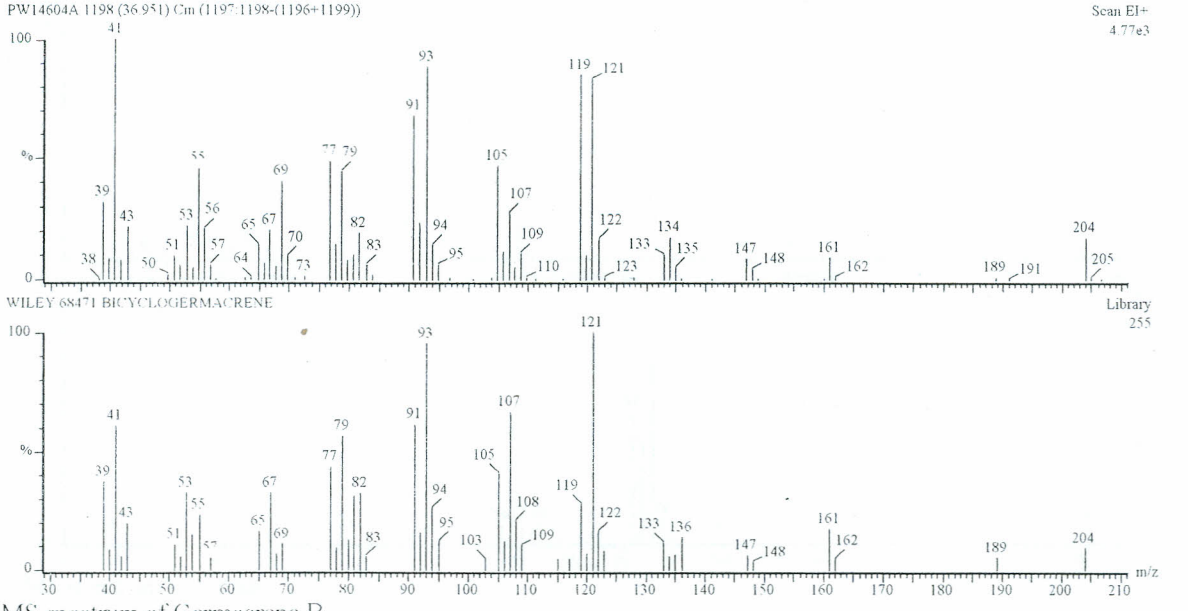
MS spectrum of Camphor



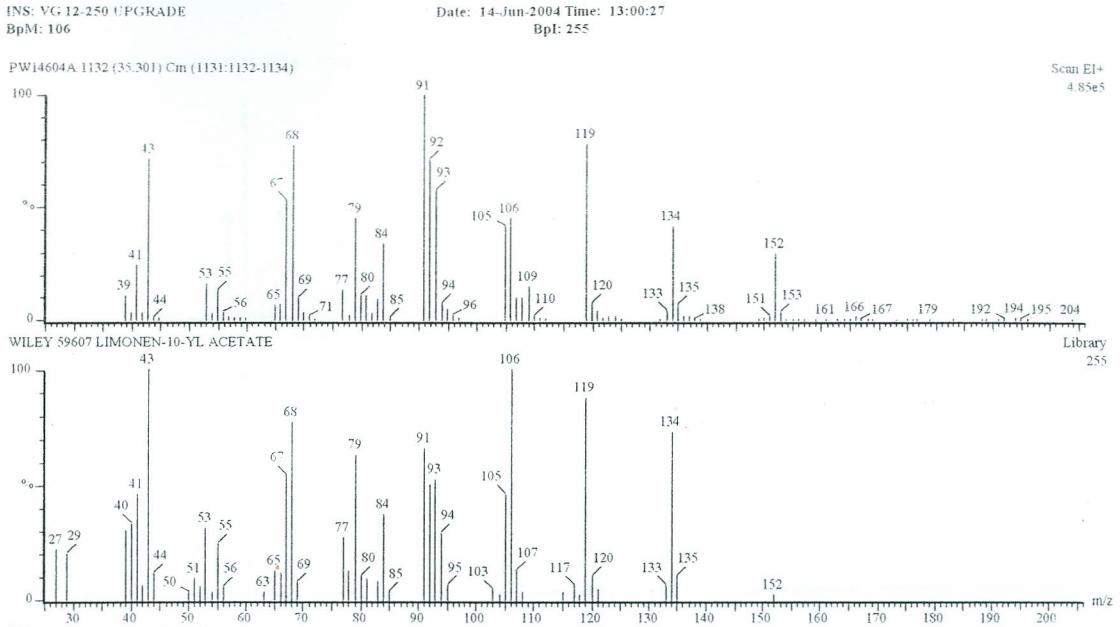
MS spectrum of Isocaryophylene



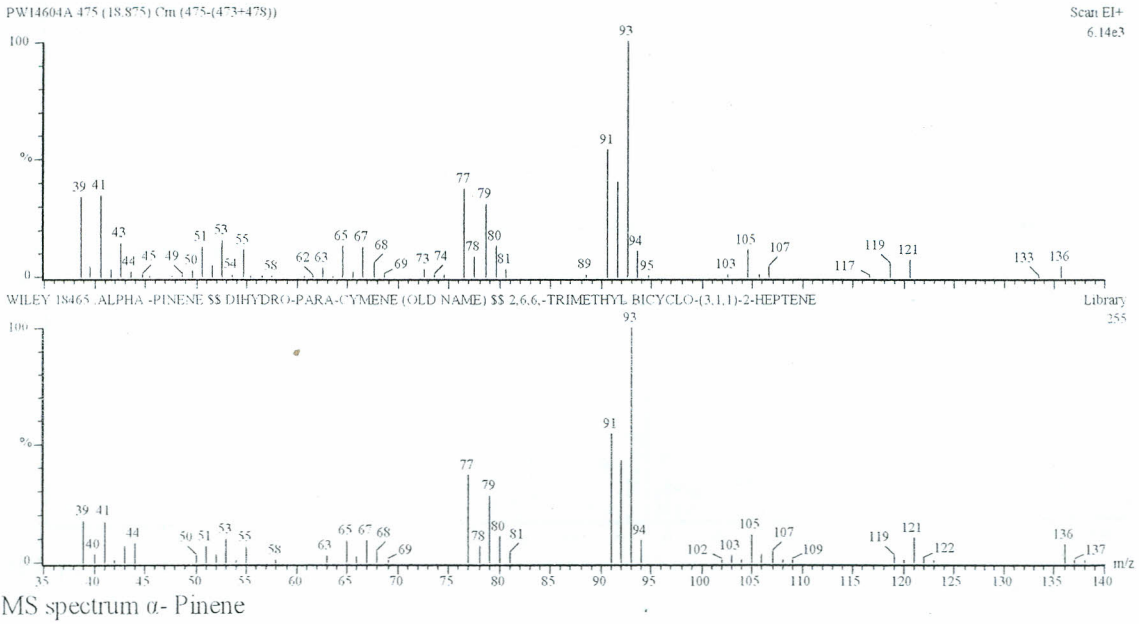
MS spectrum of δ- Carene



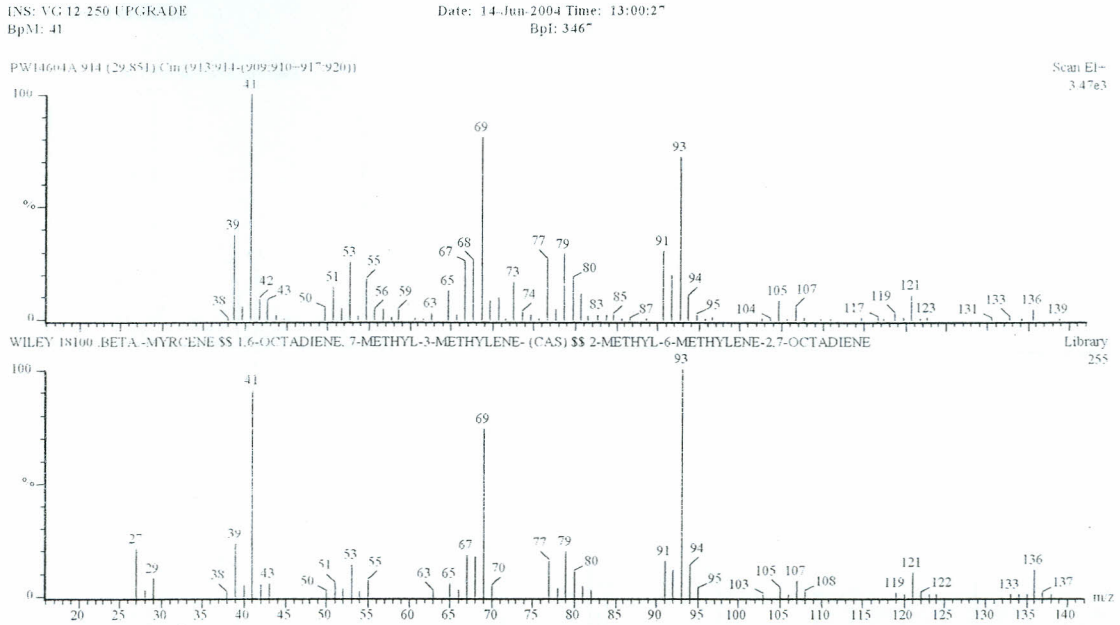
MS spectrum of Germacrene B



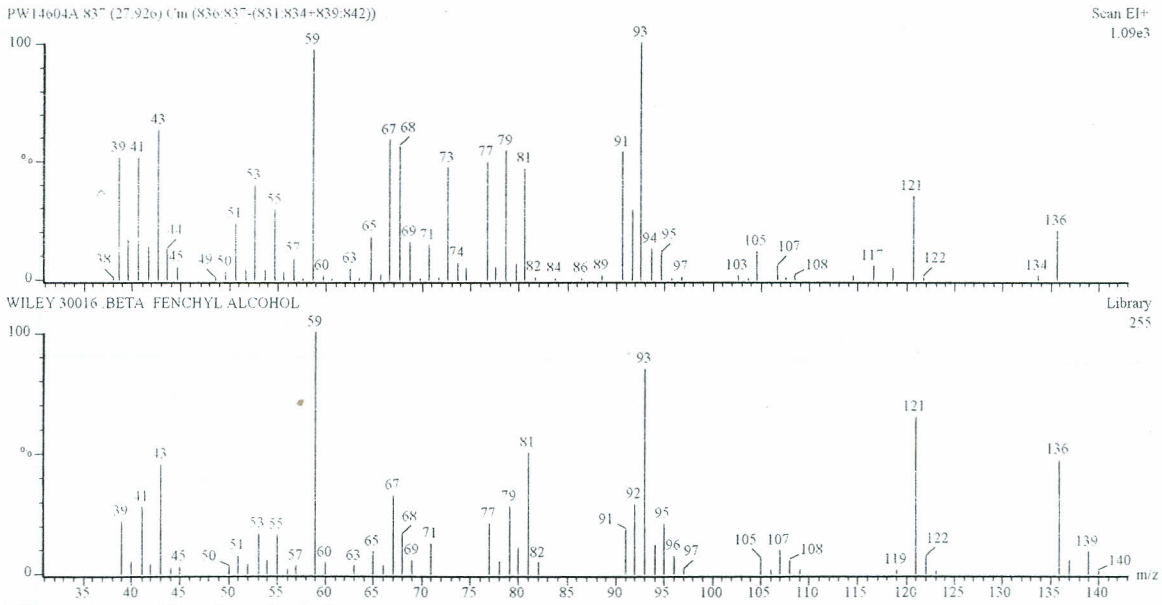
MS spectrum 1.8 cineole



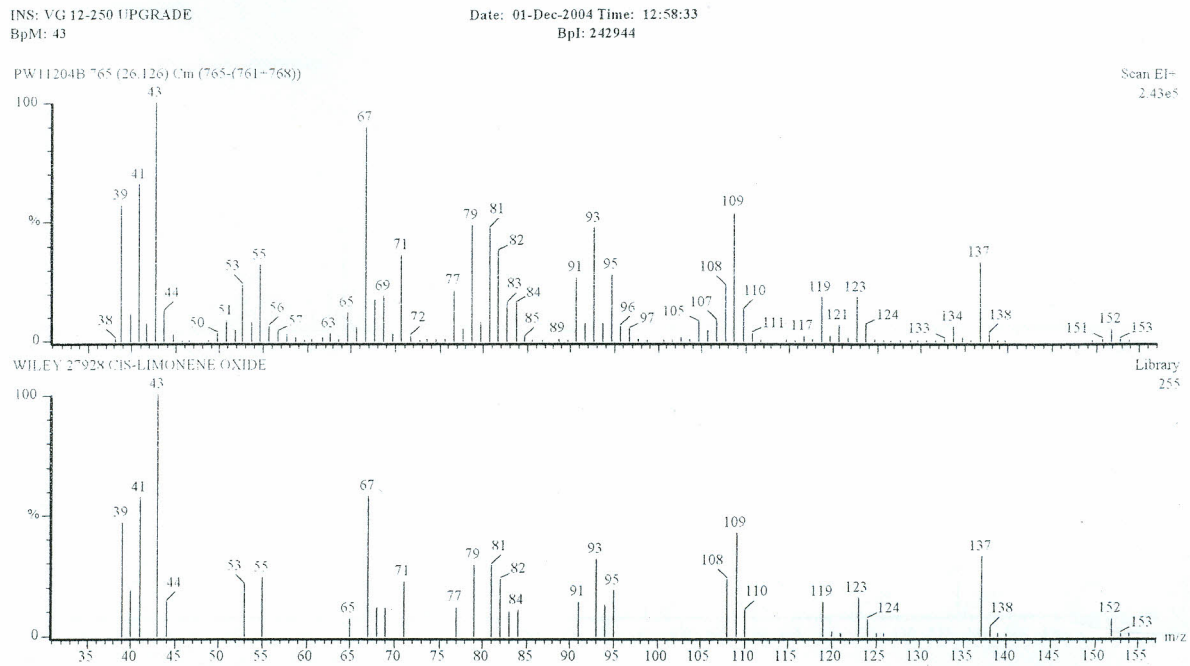
MS spectrum α- Pinene



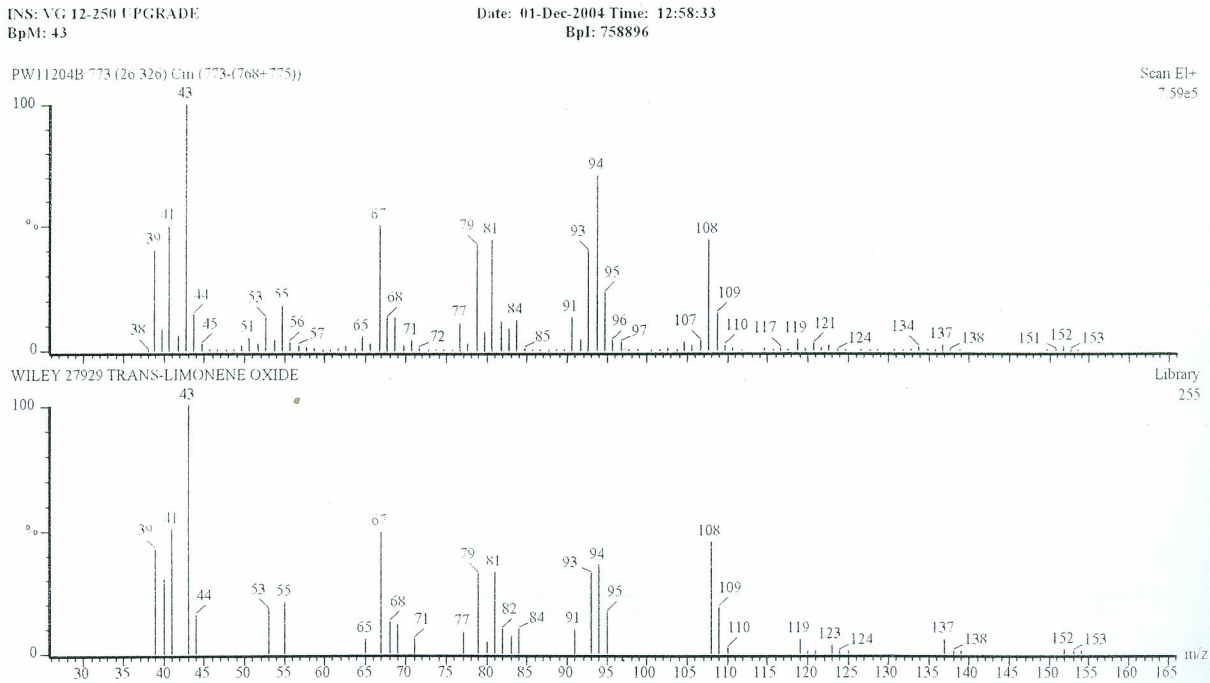
MS spectrum of β- Myrcene



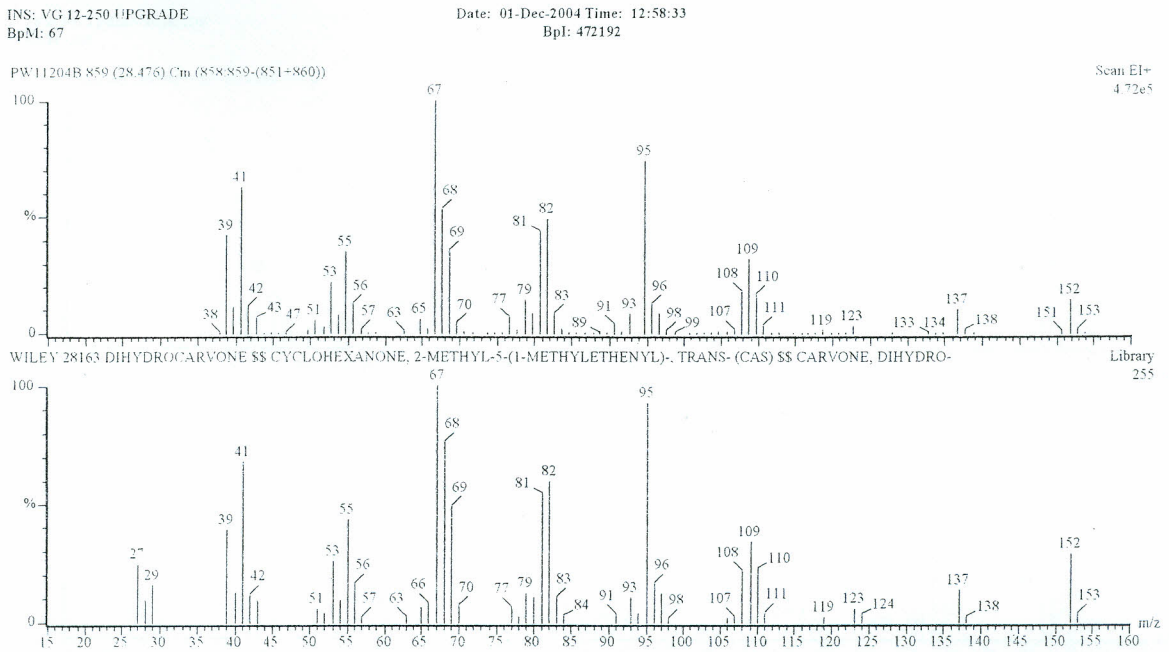
MS spectrum ofFenchyl alcohol



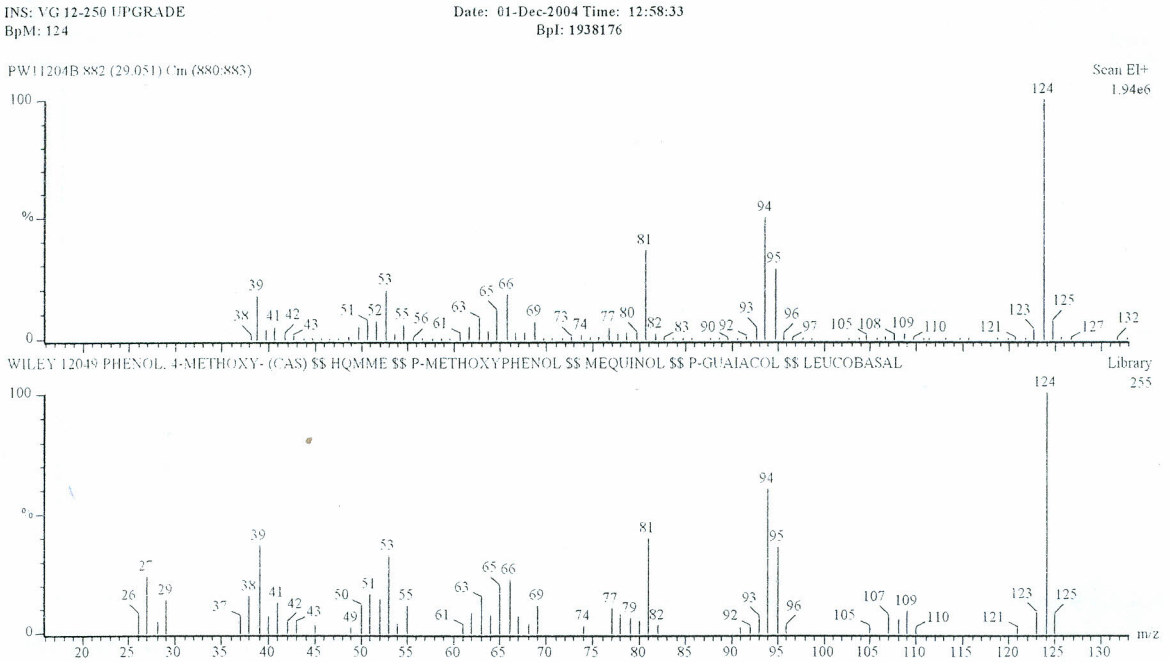
MS spectrum of *cis*- limonene oxide



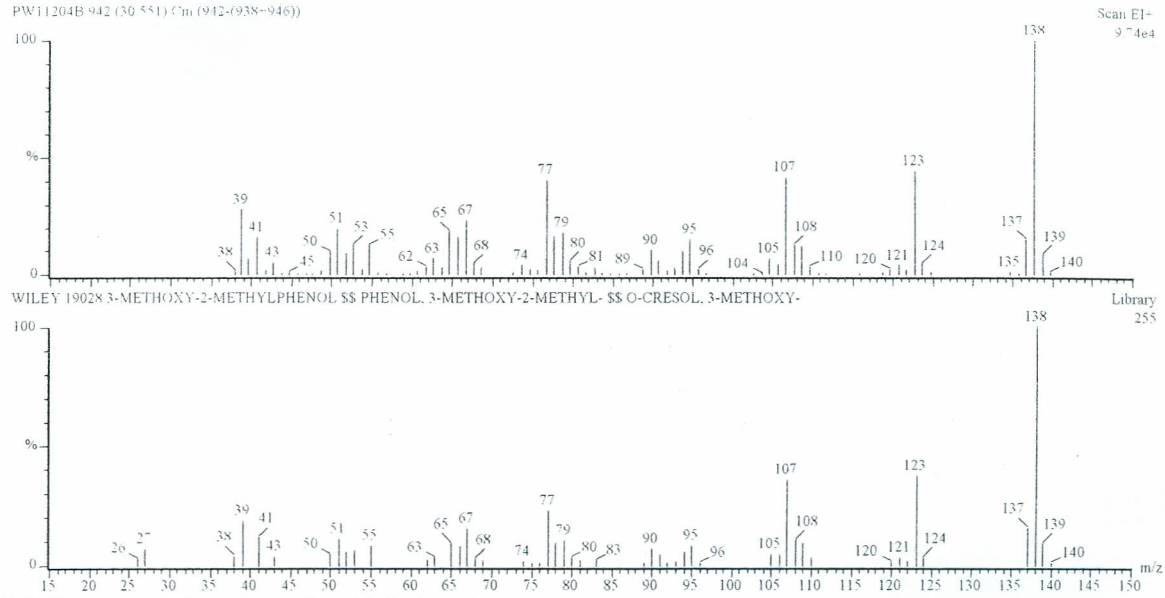
MS spectrum of *trans*-limonene oxide



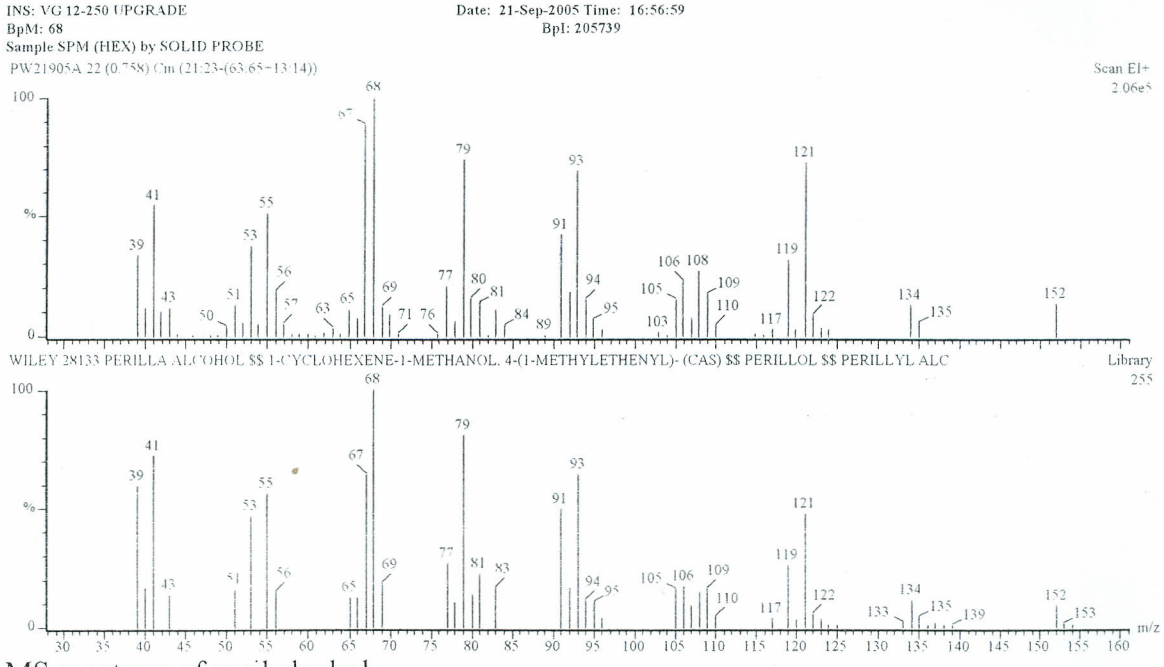
Mass Spectrum of cis dihydrocarvone



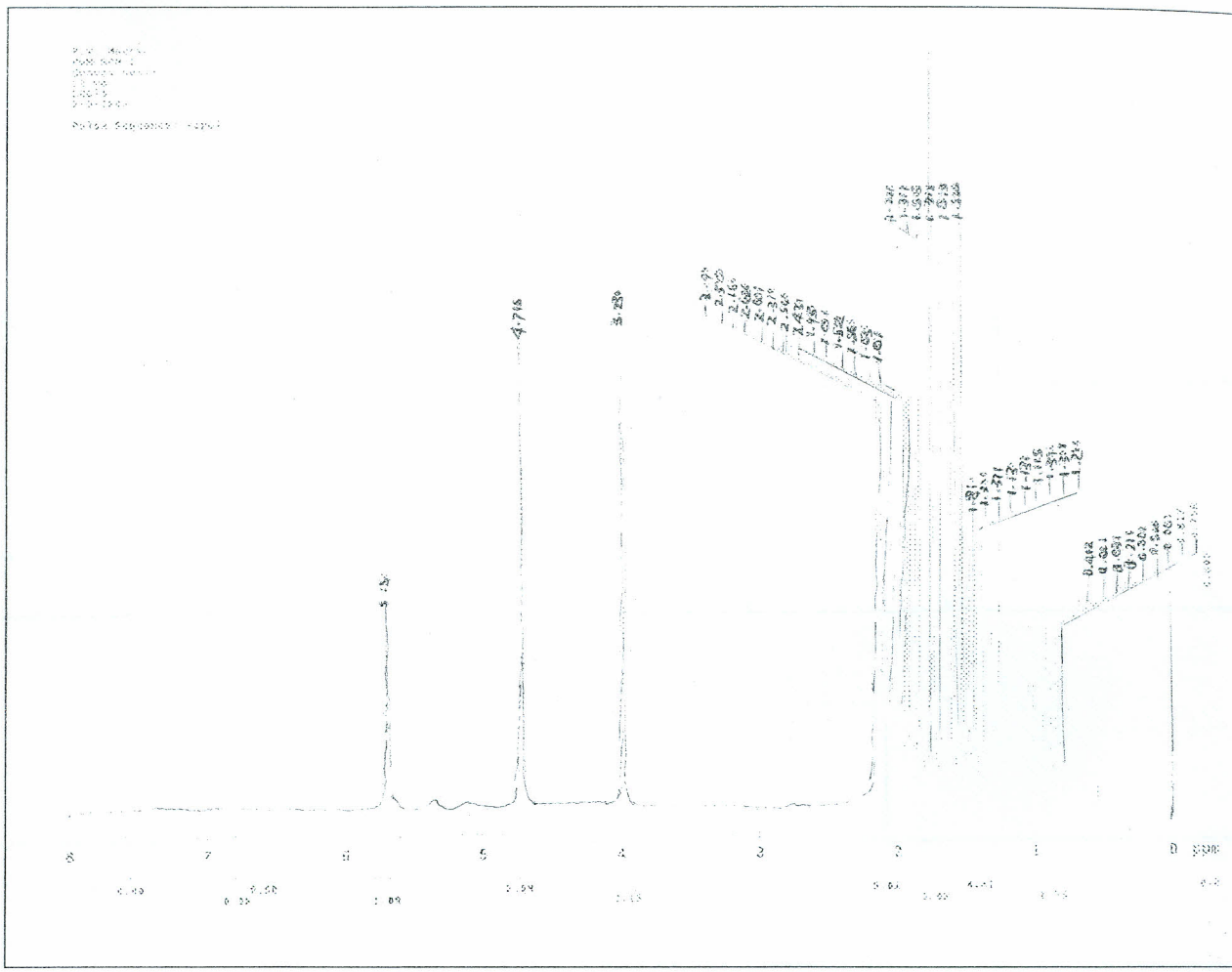
MS Spectrum o 4-methoxy phenol



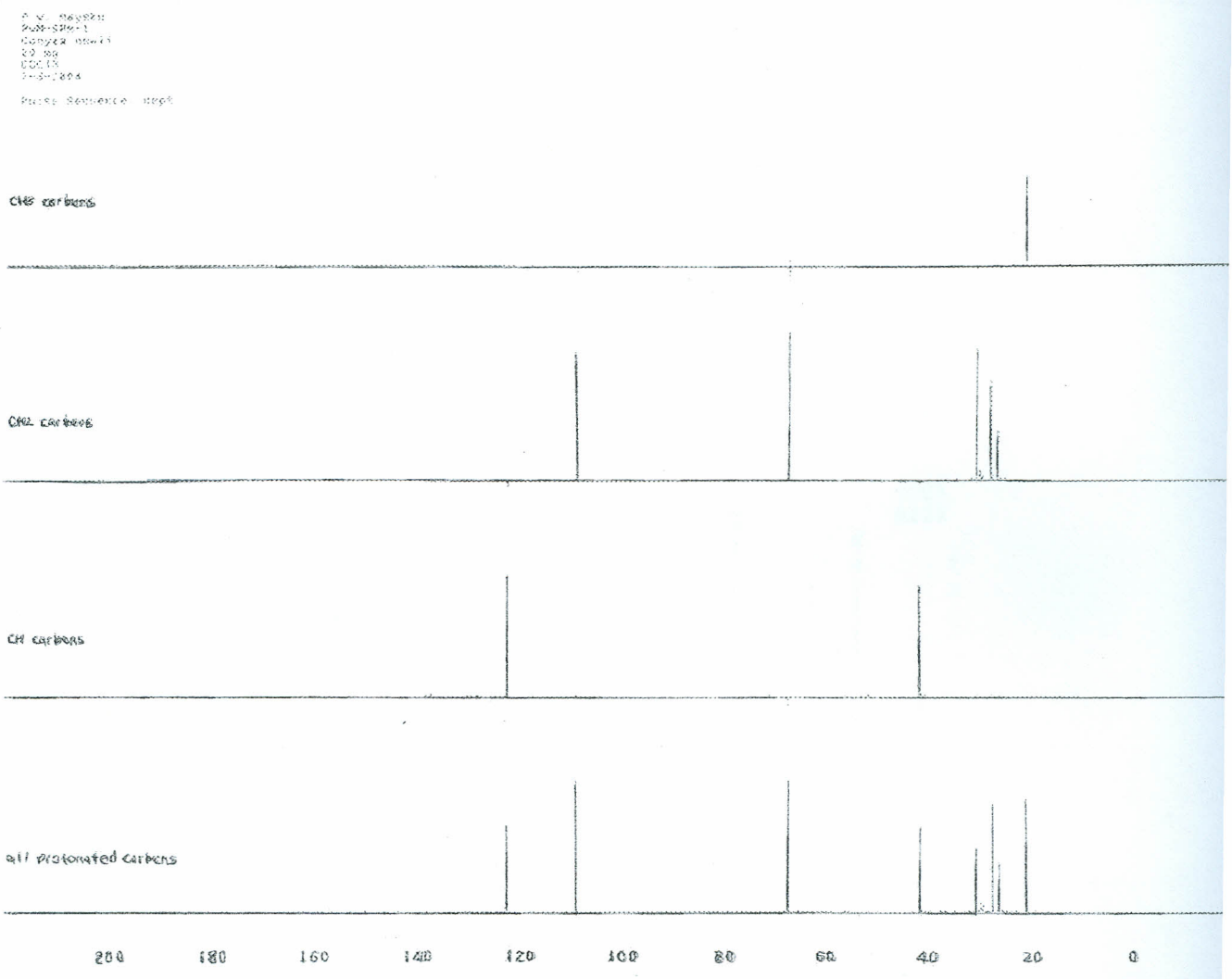
MS Spectrum of 3-methoxy-2-methyl phenol



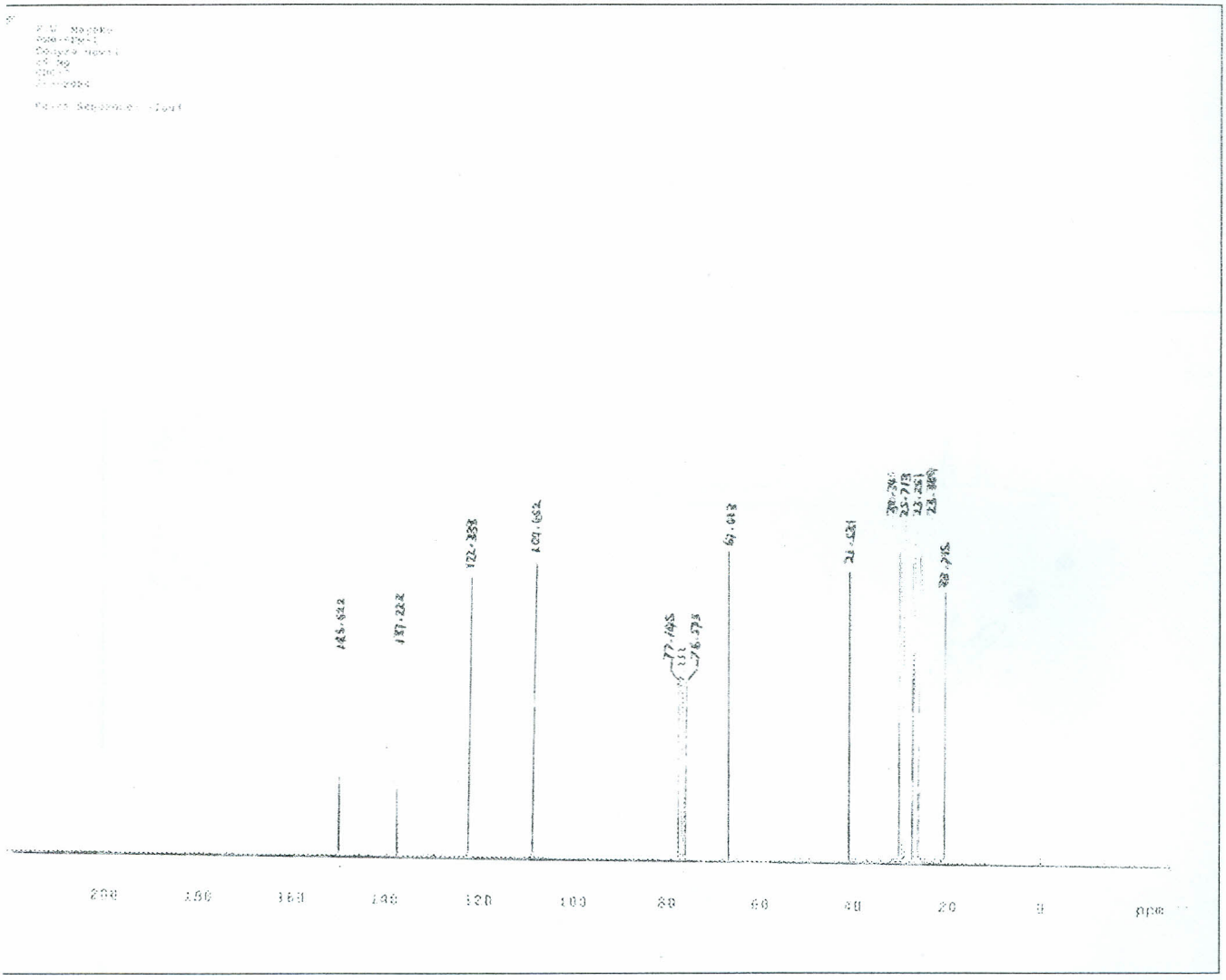
MS spectrum of peril alcohol



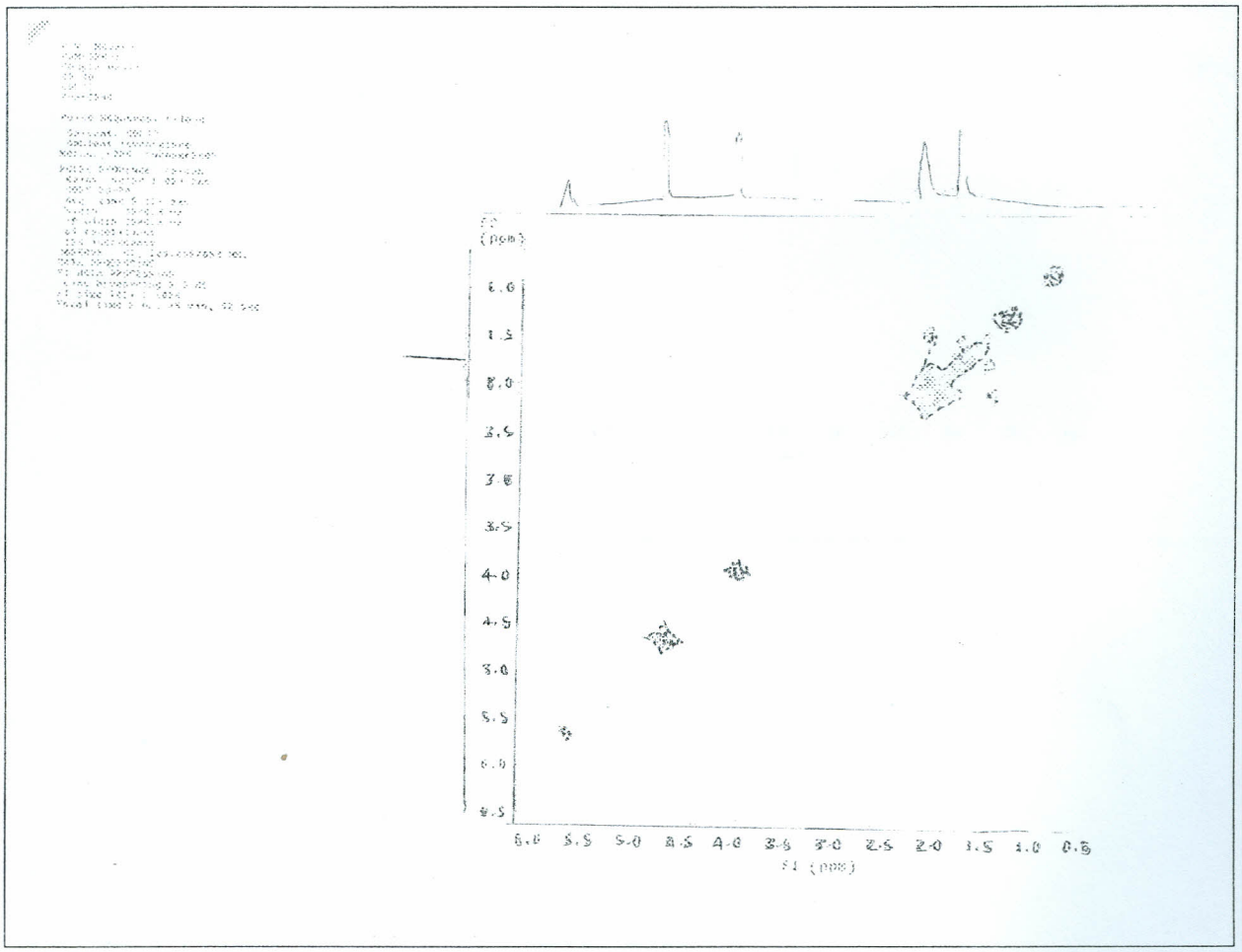
^{1}H NMR spectrum of peril alcohol



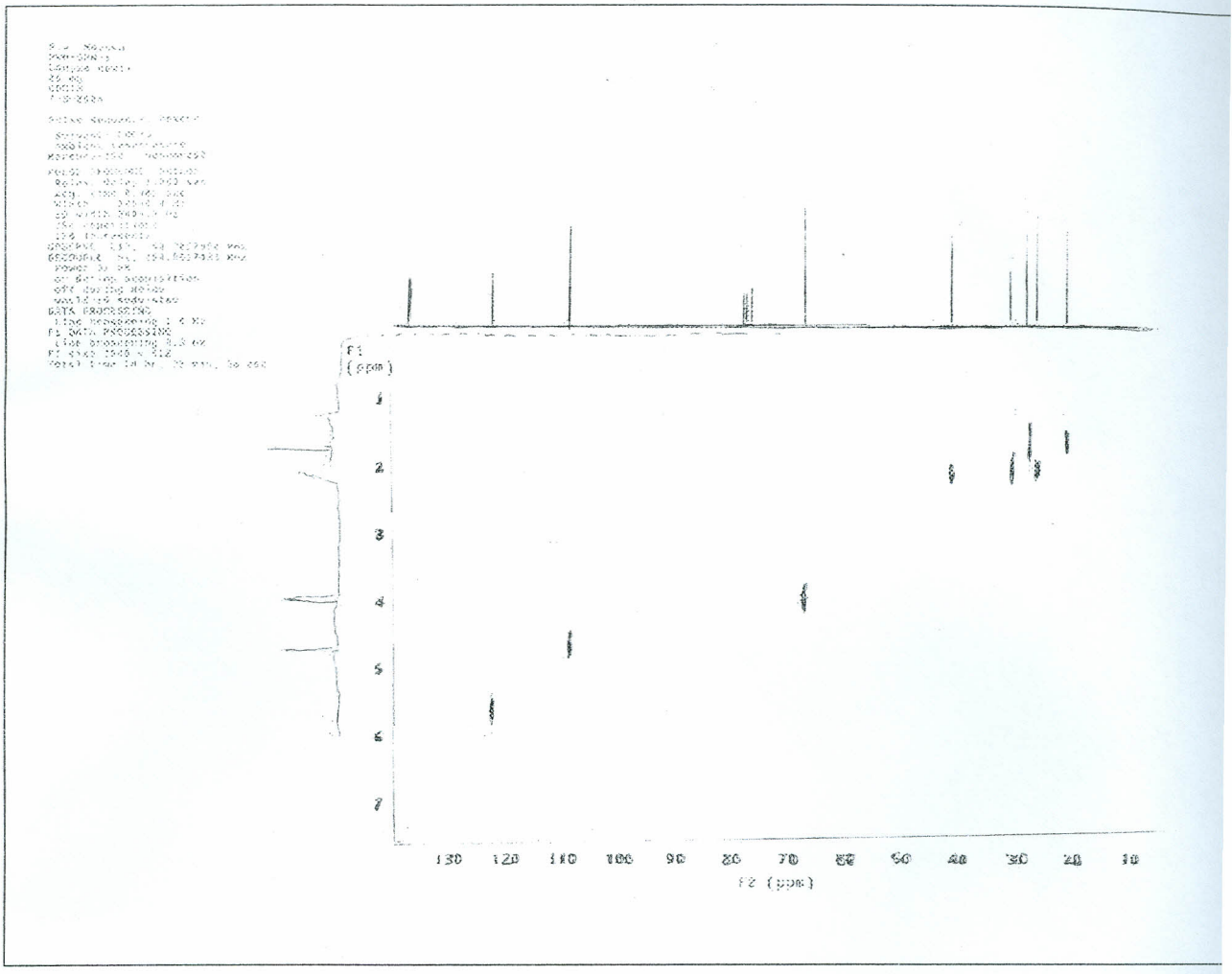
DEPT NMR spectrum of peril alcohol



^{13}C NMR spectrum of peril alcohol



^{1}H COSY NMR spectrum peril alcohol



HETCOR NMR peril alcohol