

Weevil repellent constituents of

Chrysanthemum cinerariaefolium Vis. (Pyrethrum)

for Grain Protection against the

Sitophilus zeamais as a Substitute

for Methly Bromide

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Degree of Master of Environmental Science

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DECLARATION

DEDICATION

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

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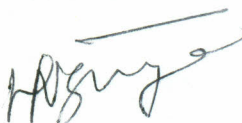
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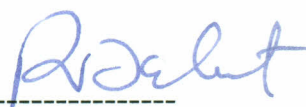
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DEDICATION

Prof. P. Noddin and Dr. W. Wangang for the guidance, encouragement and interest they accorded me in the course of my research work and in the ultimate ^{SP} complication of this research.

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LIST OF ABBREVIATIONS

GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
NBS	National Bureau of Standards
NIST	National Institute of Standards and Technology
WSN	White Spot Nitrogen

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ABSTRACT

The ozone layer that shields the Earth from damaging Sun's radiation is diminishing. This has been attributed to substances such as chloroflorohydrocarbons and halons etc. Phase-out dates for these substances have been laid out in the Montreal protocol.

Methyl bromide is one of these substances. In the Montreal Protocol, no date for its phase-out was set but annual consumption levels for signatory countries have been limited to 1991 levels. In Kenya, methyl bromide is used to protect maize from the maize weevil, *Sitophilus zeamais*. This work therefore, set out to look into the possibility of using the essential oil of pyrethrum as a substitute for methyl bromide to control maize weevil during storage.

The essential oil was extracted from the pyrethrum flowers by hydrodistillation. The constituents of the essential oil were examined by routine temperature programmed gas chromatography and then subjected to gas chromatography/mass spectrometry analysis utilising the electron impact ionization technique. The analysis of the mass spectra adduced, coupled with

the co-chromatography of the essential oil with the authentic samples of closely matching mass spectra, was the basis of the identification process. The oil consisted mainly sesquiterpenes. Those that were confirmed were confirmed were Isocaryophyllene, Germacrene-D, *trans*- β -farnesene, β -Cubebene, δ -Nerolidiol, (-)-Spathulenol, and α -Copaene. *Trans*-Chrysanthemic acid, β -Phenylethylisovalerate, Cadinene and Cadinol were identified using the mass spectrums only.

Repellency bioassay study of the essential oil and its constituents was performed on the maize weevil, *Sitophilus zeamais*. The Y-tube olfactometer method was used to study the repellency. The results showed that the essential oil of pyrethrum exhibited a higher repellency than DEET towards the *Sitophilus zeamais*. The oil was also very effective at low concentrations as 0.0001 μ l/disc.

Of the constituents identified in the essential oil, only Isocaryophyllene, δ -Nerolidiol and α -Copaene were bioassayed. The authentic samples for the other constituents were unavailable and thus not bioassayed.

CHAPTER 1

INTRODUCTION

1.1 Background of the Problem

Life on earth has been guarded for thousands of years because of a life-protecting layer in the atmosphere. This layer is composed of ozone. Ozone is found mainly in two regions of the Earth's atmosphere. Most ozone (about 90%) resides in a layer between approximately 10 and 50 kilometres above the Earth's surface, in a region of the atmosphere called the stratosphere which is commonly known as the 'ozone layer'. The remaining ozone (about 10%) is in the lower region of the atmosphere, the troposphere, which extends from the Earth's surface upto about 10 kilometres. While the ozone in these two regions is chemically identical (both consist of three oxygen atoms and have the chemical formula 'O₃') the ozone molecules have very different effects on humans and other living things depending upon their location.

The stratospheric ozone efficiently screens out almost all the harmful ultraviolet rays of the sun. The shorter the wavelength of the ultraviolet radiation, the greater the harm it can do to life and the better it is absorbed by the ozone layer. Ultraviolet radiation is divided into three types, according to wavelength. Ultraviolet radiation A (UV-A), emitted at wavelengths of 315-400nm, is unaffected by ozone reduction and is relatively harmless. Ultraviolet radiation B (UV-B), emitted at 280-315nm, is affected by decrease in atmospheric ozone.

It is UV-B that causes most of the damage to plants and animals. Ultraviolet radiation C (UV-C), which is lethal, is emitted at a wavelength of 200-280nm. Fortunately, UV-C is completely absorbed by atmospheric ozone and oxygen. Even with severe ozone reduction, UV-C radiation would still be absorbed by the remaining ozone.

Stratospheric ozone plays a beneficial role by absorbing most of the biologically damaging UV-B, allowing only a small amount to reach the Earth's surface. The absorption of UV radiation by ozone creates a source of heat, hence in this region temperature rises as one goes to higher altitudes. Ozone thus plays a key role in the temperature structure of the Earth's atmosphere. Furthermore, without the filtering action of the ozone layer, more of the sun's UV-B radiation would penetrate the atmosphere and would reach the Earth's surface in greater amounts. Many experimental studies of plants and animals, and clinical studies of humans have shown the harmful effects of excessive exposure to UV-radiation (UNEP/WMO, 1995).

At the planet's surface, ozone comes into direct contact with life-forms and displays its destructive side. Because ozone reacts strongly with other molecules, high levels are toxic to living systems and can severely damage the tissues of plants and animals. Many studies have documented the harmful effects of ozone on crop production, forest growth and human health (UNEP/WMO, 1995). The substantial negative effects of surface-level troposphere ozone from this direct toxicity contrast with the benefits of additional filtering of UV-B radiation that it provides (UNEP/WMO, 1995).

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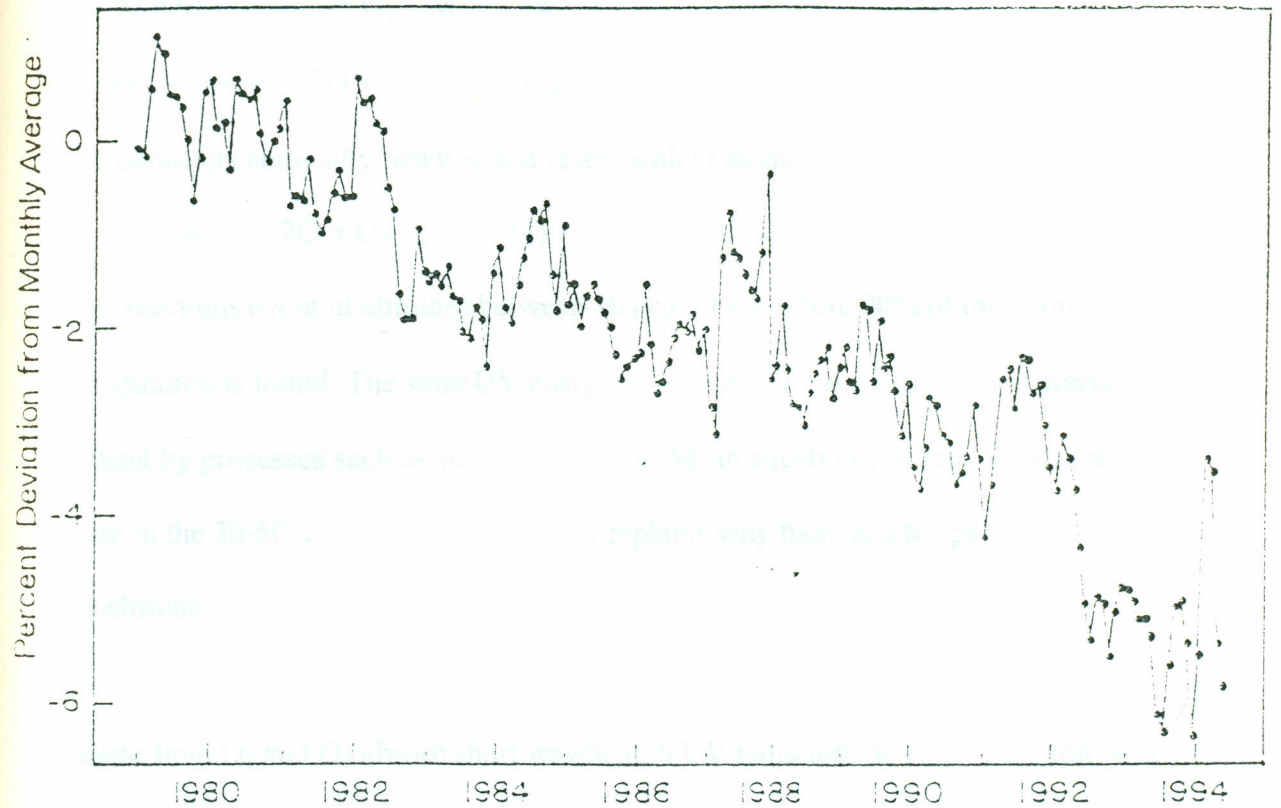
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With these dual aspects of ozone there are also two separate environmental issues, controlled by different forms in the atmosphere. In the troposphere there is concern about increase in ozone. Low-lying ozone is a key component of smog, a familiar problem in the atmosphere of many industrialised cities around the world. Higher than usual amounts of surface-level ozone are now increasingly being observed in rural areas as well. However, the ground-level ozone concentrations even in the heavily polluted cities are very much smaller than the concentration routinely found in the stratosphere (UNEP/WMO, 1995).

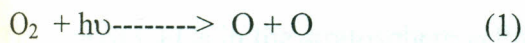
There is widespread scientific and public interest and concern about loss of stratospheric ozone. Ground-based and satellite instruments have measured decreases in the amount of stratospheric ozone in our atmosphere. Figure 1 shows the trend in ozone losses observed over a period of 15 years. Over some parts of Antarctica upto 60% of the total overhead amount of ozone is depleted during September and October resulting in a phenomenon now known as the Antarctic 'Ozone hole'. Smaller, but still significant stratospheric decreases have been seen at other, more polluted regions of the Earth. Increases in surface UV-B regions have been observed in association with decreases in stratospheric ozone (UNEP/WMO, 1995).

Figure 1: Global Ozone Trends between the Latitudes 60°S-60°N (UNEP/WMO, 1995).



The scientific evidence accumulated over more than two decades of study has shown that human-made chemicals are responsible for the observed depletions. These Ozone-depleting compounds contain various combinations of the chemical elements chlorine, fluorine, bromine, carbon and hydrogen and are often described by the general term halocarbons (UNEP/WMO, 1995).

In the atmosphere, for wavelengths shorter than 290nm the stratospheric chemical process for the formation and re-formation of ozone balance is maintained.

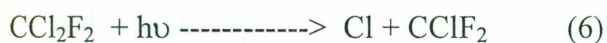


Also, ozone is chemically reactive and reacts with O atoms.



These reactions occur at altitudes between 10 and 50 km where 90% of the ozone concentration is found. The solar UV energy absorbed ($h\nu$) in reaction (3) is converted into heat by processes such as energy transfer to M^+ in equation (2), providing a heat source in the 30-50 km altitude range. This explains why there is a temperature increase with altitude.

Because both O_2 and O_3 absorb short wavelength UV radiation, no solar radiation with wavelengths shorter than 290nm penetrates below the stratosphere. The 3-chloro-fluoromethane (CCl_3F) molecule absorbs UV radiation below 220nm as shown in equations 5 and 6. For it to be decomposed, it has to be transported upward (30-50km altitude). At this rarefied atmosphere (low concentrations of O_2 and O_3) the chlorofluorocarbons (CFCs) are exposed to very short wavelengths UV radiation and decomposes with the release of chlorine (Cl) atoms.



Because at any one time, only a small fraction of CFC molecules are found at altitudes above 30 km, the average molecule survives for so many decades before it is decomposed by solar UV radiation.

The fate of CFCs in the stratosphere is thus the release of chlorine atoms. These in turn react with ozone as illustrated in the equation 7 below.



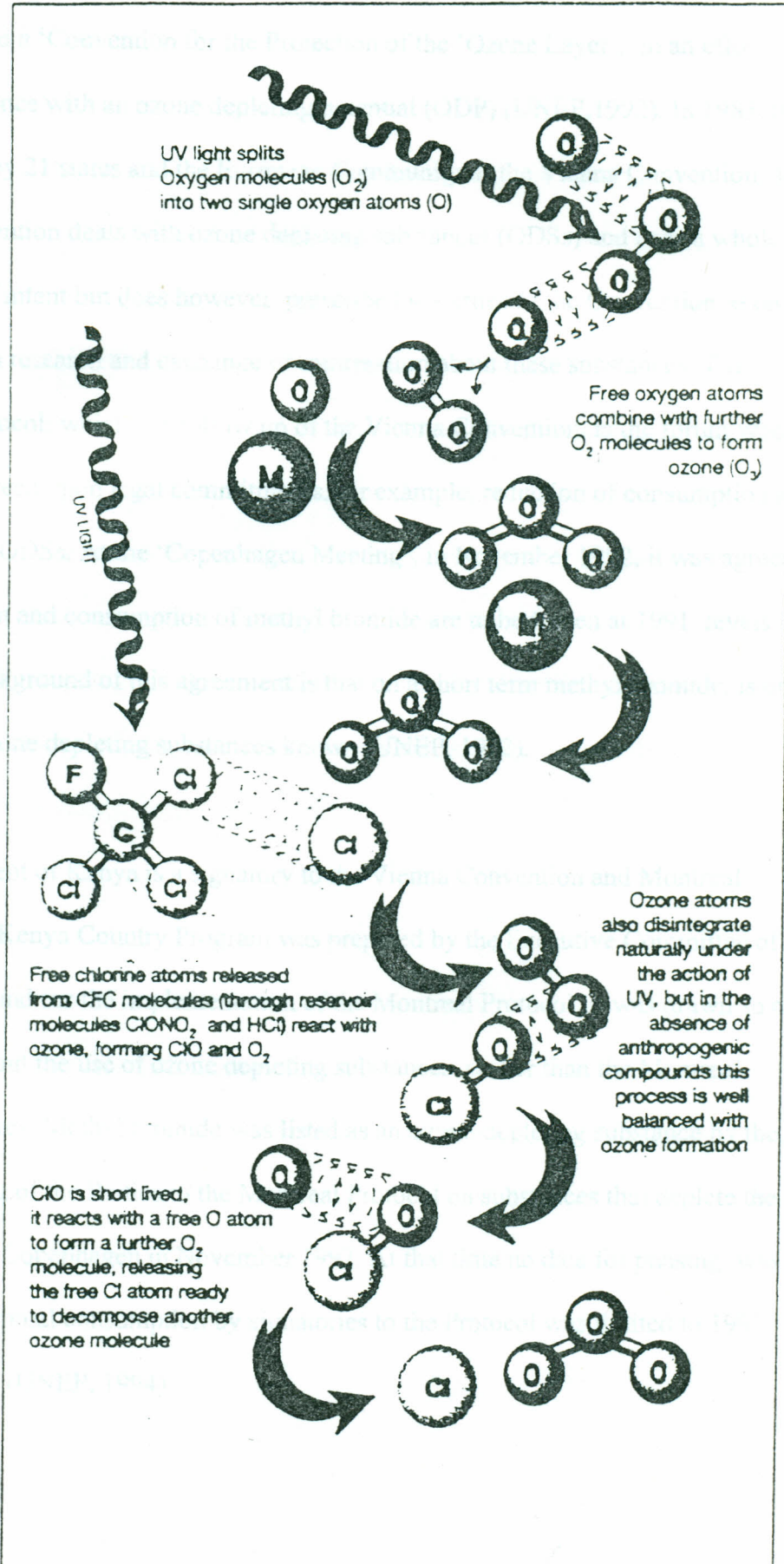
The next question is the fate of ClO at 30km.



Combination of equations(7) and (8) yield equation (4) and constitute a free radical catalytic chain reaction in which the chlorine atoms alternates between the chemical species Cl and ClO. The first step removes one O₃ molecule while the second intercepts an O atom which could have become O₃ by reaction (2), but instead is converted into O₂. The chlorine atom is only a catalyst and remains to initiate the process once more.

Figure 2 shows how the Cl/ClO cycle can be repeated a thousand times converting molecular O₂ into one ozone molecule and one oxygen atom. With this catalytic efficiency about 100,000 molecules released per chlorine atom is coupled with yearly release to the atmosphere of about one million tons of CFCs, then it becomes obvious why these reactions are of immense environmental concern- the depletion of stratospheric ozone by chlorine containing CFCs. Bromine atoms in the halons and methyl bromide is fifty times more efficient in its reaction with the ozone (UNEP/WMO,1995).

Figure 2: Schematic sequence of the destruction of ozone by active Cl released from a CFC-12 molecule (UNEP/WMO,1995).



In 1981, in accordance with a United Nations Environmental Programme (UNEP) Governing Council decision, a working group of government, legal and technical experts drafted a 'Convention for the Protection of the 'Ozone Layer', in an effort to control substance with an ozone depleting potential (ODP) (UNEP,1992). In 1985, this was adopted by 21 states and the European Community as the Vienna Convention. The Vienna Convention deals with ozone depleting substances (ODSs) and is as a whole a declaration of intent but does however, prescribe the Parties of the Convention to cooperate within research and exchange of information about these substances. The Montreal Protocol, which is a follow up of the Vienna Convention, is the forum where the Parties agreed upon legal commitments, for example, reduction of consumption and production of ODSs. At the 'Copenhagen Meeting', in November 1992, it was agreed that production and consumption of methyl bromide are to be frozen at 1991 levels in 1995. The background of this agreement is that on a short term methyl bromide, is one of the most ozone depleting substances known (UNEP, 1992).

4.1 Introduction from 1986-1995.

The Government of Kenya is a signatory to the Vienna Convention and Montreal Protocol. The Kenya Country Program was prepared by the Executive Committee of Multilateral Fund for the implementation of the Montreal Protocol. It was drawn so as to try and phase out the use of ozone depleting substances earlier than the Montreal Protocol requires. Methyl bromide was listed as an ozone-depleting substance by the Fourth meeting of the Parties to the Montreal Protocol on substances that deplete the ozone layer in Copenhagen in November 1992. At that time no date for phasing was set although the annual consumption by signatories to the Protocol was limited to 1991 levels by 1995 (UNEP, 1994).

1.2 Significance of the Study

The maize weevil, *Sitophilus zeamais*, is a serious pest of stored grains, especially maize. Its distribution ranges from tropical to temperate zones (Hill and Waller, 1990).

The larvae bore thin tunnels from the surface towards the inside of the grain kernel. The only effective control measure for the weevil is a range of synthetic pesticides in the form of gaseous fumigants and residual insecticides (Hill and Waller, 1990).

In Kenya, about 10-20% of the annual maize production of 1.5 million tons is lost during storage reflecting an economic loss of more than K£ 1 million (UNEP, 1994).

Kenya has been using methyl bromide since the 1960s. Currently methyl bromide is being used to protect maize from the *S. zeamais*. Other sectors in Kenya where methyl bromide is also used is in the horticultural industry, soil fumigation, fumigation of cereals and for quarantine, with the horticultural industry being the largest consumer, as it is a major growing industry. The table 1 shows the amounts of methyl bromide imported into the country from 1986 -1995.

Table 1: Annual imports of methyl bromide since 1986 in metric tons (MT).

Year	Total imported into the country	Quantity used to treat stored grains
1986	140 MT	46.8 MT
1987	170 MT	61.1 MT
1988	295 MT	48.4 MT
1989	361 MT	52.8 MT
1990	340 MT	52.2 MT
1991	331 MT	30.8 MT
1992	204 MT	10.6 MT
1993	68 MT	10.6 MT
1994	N/A	7.5 MT
1995	--	15.0 MT

The above figures have been supplied by the NCPB , 1995.

The decrease in the amount of methyl bromide used from 1990 to 1994 was because there was a shortage of grain in the country due to drought. Considering that methyl bromide has an ozone depleting potential (ODP) of 0.6 and in 1993, Kenya imported 68MT, giving an ODP of 40.8MT. This figure shows the urgency of finding an appropriate and viable alternative.

Methyl bromide can have adverse effects on a number of commodities, causing a brown taint and odours. It is an effective weedicide in soil treatments, but its usefulness is limited in the treatment of growing plants and perishables against pest's (UNEP, 1995). Treatment of durables with methyl bromide also results in the production of bromide ion residues. These may accumulate to excessive levels in commodities that are fumigated several times and have been a cause for concern in ground water in some European countries (UNEP, 1995).

1.2.1 Alternatives for Methyl Bromide for the Treatment of Durables as

Suggested by Methyl Bromide Technical Options Committee (MBTOC)

Durable commodities are of low moisture content hence they are stable in storage over a long period of time. Durable commodities currently treated with methyl bromide include a wide variety of foodstuff; principally cereal grain, oilseeds, legumes, grain products, dried fruits and nuts. Insect and mite pests can breed on these materials during storage if not protected. Pests may also be present at time of harvest and persist in storage or during transport.

Control of pests infesting durables is essential to keep commodity losses to a minimum, to maintain quality, to prevent damage and to prevent the spread of pests to other countries. Fumigation is a very effective method of controlling pests. Methyl bromide and phosphine are two fumigants which are currently being used for the control of storage pests. Methyl bromide is able to provide complete control of pests, mostly within 24 hours of exposure, at a minimum temperature of 25°C. Phosphine requires long exposure periods (5-15 days) and usually temperatures greater than 15° C, for effective action (UNEP/ WMO, 1995).

Kenya is using phosphine to treat only small quantities of grain (NCPB, 1995). With further investigation it may be possible to extend its use into some other areas where methyl bromide is currently used, but complete substitution by phosphine is unlikely. For example, methyl bromide is currently being used to control phosphine-resistant pests.

There are several other fumigants which may have some restricted potential as alternatives for methyl bromide. Hydrogen cyanide was widely used at one time for treatment of durable commodities, but was superseded by methyl bromide because it was safe and more effective. Ethyl formate is in use in some countries for disinfection of packed dried foods. Carbon bisulphate was once commonly used but has been discontinued in most countries. Ethylene oxide as a fumigant for food has been withdrawn from most countries because of the production of carcinogenic residues (UNEP/WMO, 1995).

Controlled and modified atmospheres, such as nitrogen and carbondioxide, provide a potential alternative to conventional fumigation if time (2-6 weeks) and temperature (10-30°C) permit (UNEP/WMO, 1995). This technique is most likely to be used selectively in countries with warmer climate. The cost capital to implement a controlled or modified atmosphere disinfection treatment in addition to operational costs in some cases renders this alternative uneconomical.

Physical methods of insect control, including cold, heat and irradiation treatment, offer further potential as non-chemical alternatives, although difficulties exist in the practical implementation of these methods. Biological method of insect control, include use of

pheromone, and microbiological control agents are at an early stage of investigation.

Their widespread use as control measures are not expected in the near future.

Contact insecticides are used extensively in certain situations to protect raw durable commodities. Contact insecticides include synthetics, inert dust, insect growth regulators and plant extracts or their analogues. Various compounds are selectively effective in controlling different insect species. One of the main constraints associated with insecticides is the presence of chemical residues in treated commodities which normally prevents their use on processed products.

Out of the above alternatives as presented by UNEP (1995), Kenya has tried using phosphine and carbondioxide. One of the major limitations encountered in carrying out further research into other alternatives has been lack of funds and technical know-how. Thus finding and developing new compounds from plants which have the potential to replace the toxic fumigants and eliminate insecticide residue while being less harmful and more convenient to use, would be of significant benefit. To that end, this study was undertaken to screen pyrethrum, which is locally grown, for repellency against the maize weevil and also to identify the repellent constituents of its essential oils.

1.3 Objectives of the Study

This study was designed with the following objectives:

- a) To extract the essential oil from the *Chrysanthemum cinerariaefolium* Vis. by steam distillation of the dried flowers using a Clevenger-type apparatus.
- b) To determine repellency of the essential oil of pyrethrum to the maize weevil, *S. zeamais* using the Y-tube olfactometer and to compare the repellency of the essential oil of *C. cinerariaefolium* against pyrethrin extract and DEET (a commercial repellent).
- c) To identify the chemical constituents of the essential oil using gas chromatography and mass spectroscopy.
- d) To carry out the same bioassay tests as (b) above, but this time using the authentic compounds identified from the active oil.

1.4 Statement of the Problem

In 1992, methyl bromide was listed as an ozone-depleting substance in the Montreal Protocol. Its annual consumption by signatory countries was limited to the 1991 levels by 1995. Kenya signed the Montreal Protocol on the 9 November 1988 and therefore has to follow its requirements. In Kenya, methyl bromide is used for quarantine purposes, in the horticultural industry and as a fumigant for stored grain. Since the consumption levels for methyl bromide have been frozen, Kenya is frantically looking for a substitute.

The reason for choosing pyrethrum is that it has many properties necessary for insect control. It has a rapid 'knock-down' and killing action, a good flushing out effect (for example forcing insects out into the open), strong repellency action (detering the insect from entering the area), effective against a wide spectrum of insects and it is virtually non-toxic to human and mammalian life.

Repellent is a chemical or a mixture of chemicals that acts on the sense organs of the insect, causes the organisms to behave in a way which tends to prevent them from becoming a source of the matter or pest or as a repellent from seeking a host or from being attracted to a particular place in respect to this precise behaviour. In the past, workers involved in the development of repellent substances use the term 'repellent' to describe a chemical that elicits a combination of behavioural responses, the most important result is simply the prevention of biting by an organism. It is now generally accepted that topical repellents were used for the protection against biting arthropods and the pathogens that they transmit (Davis, 1985).

Recently, the most critical evidence suggests that repellents act directly on the sensory systems (Hague and Dool, 1967). The practical problem associated with repellency is essentially a behavioural one in that a candidate compound must first be shown to have a repellent effect, that is, be capable of stimulating overt sensory reactions that mediate a response. Secondly, since the response of the organism depends on which sensory system has been stimulated and which reflex arcs are placed in operation, the repellent must act upon a system which has some influence on locomotion or feeding. The mode of the response elicited by a repellent also depends upon a variety of other biological factors, such as age and state of nutrition, the concentration of the

CHAPTER 2

LITERATURE REVIEW

2.1 Repellents

A repellent is a chemical or a mixture of chemicals that, acting in the vapour phase or otherwise, cause the organism to behave in ways which result in its movement away from the source of the matter or prevents an organism from reaching a target it would otherwise be attracted to (Dethier, 1956). In contrast to this precise behavioural definition, workers involved in the development of repellent substances use the term 'repellent' to describe a chemical that elicits a combination of behavioural response whose net result is simply the prevention of biting by an organism. This may accrue from the fact that tropical repellents were used for the protection against biting organisms and the pathogens that they transmit (Davis, 1985).

Currently, the most critical evidence suggests that repellents act directly on the chemosensory systems (Kilgore and Doult, 1967). The practical problem associated with repellency is essentially a behavioural one in that a candidate compound must first possess inherent repellency, that is, be capable of stimulating some sensory system other than that mediates attraction. Secondly, since the response of the organism depends upon which sensory system has been stimulated and which reflex arcs are placed in operation, the repellent must act upon a system which has some influence on locomotion or feeding. The nature of the response elicited by a repellent also depends upon a variety of intrinsic biological factors such as age and state of nutrition, the concentration of the

repellent and which sensory systems are being acted upon simultaneously by other stimuli (Dethier, 1956).

Repellents may be divided into two classes depending on their mode of affording protection (Sankaria and Brown, 1951). Those materials such as N,N-diethyltoluamide (DEET), which are sufficiently volatile to keep an organism at a distance are designated as vapour or olfactory repellents. The majority of synthetic chemicals found to be effective are repellents in the vapour phase. Those that are slightly volatile such as indoline, that the insect must approach and touch before being repelled are designated as contact or gustatory repellents. Such repellents act upon specialized chemoreceptors not normally sensitive to vapours. Such receptors, located on the mouth parts and tarsi are concerned with monitoring some aspects of feeding. One would expect that repellent compounds acting on these receptors would prevent feeding (Dethier, 1956, Kilgore and Doult, 1967). Since these compounds are usually detectable by other animals, including man, one of the most important criterion for an economically feasible chemical is that it be repellent to insects while still be acceptable to the animal species to whose protection it is intended (Kilgore and Doult, 1967).

2.1.1 Chemical properties of a repellent

Several investigators have sought to gain some insight into what constitutes a good repellent, that is, what are the chemical, physical, and structural properties that a repellent must possess. Skinner and Johnson (1980) commented that the vapour pressure or boiling point is the only parameter that anyone has been able to correlate with repellent activity. This is a rather obvious property, in that if a chemical is going to

repell, it will most likely act in the vapour phase. In addition, it should not evaporate too fast lest it loses its ability to protect. Thus, it has been possible to define a range of boiling point temperatures into which most repellents should fall in. Other properties, such as partition coefficients, melting points, molecular weights, infrared absorption, viscosity, surface tension and molecular polarizability, have been shown to have no correlation with repellency.

Regarding functional group and structural correlation's, Garson and Winnike (1968) stated that of the 4308 compounds examined for repellent activity, those found to be most effective were amides, imides, alcohols, and phenols.

2.1.2 Synthetic Commercial repellent : DEET

Chemical repellents are used for the protection against pathogen transmitting organisms such as mosquitoes, ticks and flies. N,N-Diethyl toluamide (DEET) was the first synthetic repellent that was found broadly effective against a wide range of biting pests (Gilbert et al., 1955, 1957). It is noted for its essential odourlessness, water-like texture and long lasting effectiveness. Although DEET is a very good repellent (Beuscher et al., 1983), its adverse effects are known (Anonymous, 1981). It causes a burning sensation in the eyes, cuts, membranous areas and will damage some plastics and synthetic fibres. Consequently, investigators have been seeking new effective repellents and new formulations of repellent compounds though without much success. One important reason for the lack of success is that it is not yet known how repellents act on the target organism (Davis , 1985).

2.1.3 Need for Screening Plants for Repellency

There is good reason to believe that plants have survived largely because of defensive mechanisms that have evolved in time in relation to attacks by various pathogens such as viruses, bacteria and protozoa. This is particularly so for tropical plants that are confronted with much harsher conditions of survival, such as unfavourable climate and soil conditions, than their temperate counterparts. This necessarily leads to efficient built-in defence mechanisms. It is presumably for this reason that tropical flora offer a rich and intriguing source for isolating natural products possessing attractive entomological or medicinal properties (Kubo and Nakanishi, 1977; Rice, 1983; Guenther, 1972).

As repellents, plant odours have shown a great potential. These odours can be isolated either as exudates from glandular trichomes of certain plants or commonly as essential oils (Levin, 1973).

2.2 Essential Oils

Essential oils are odoriferous principles of plant origin, which are mainly present in the leaves, blossoms, fruits, twigs, roots and occasionally in the wood (Simonsen, 1953; McGraw-Hill, 1987). The oils are stored as micro-droplets in glands of plants. After diffusing through the walls of the glands, the droplets spread over the surface of the plant before evaporating and filling the air with the perfume. The most odoriferous plants are found in the tropics where the solar energy is the greatest. Younger plants produce more oil than the older ones, but the latter produce oil which is more resinous and darker because of the continuing evaporation of the lighter fraction of the oil (Encyclopaedia Britannica, 1991).

Commercially, essential oils are used as (i) odourants in cosmetics, perfumes, soaps, detergents and other industrial products ranging from animal feeds to insecticides, (ii) as flavours in bakery goods, confections and soft drinks, and (iii) as pharmaceuticals in dental products and a wide, but diminishing, group of medicines (Encyclopaedia Britannica, 1991).

2.2.1 Isolation of Essential Oils

The initial step in the isolation of essential oils involves crushing or grinding the plant material to reduce the particle size and to rupture some of the cell-walls of the oil-bearing glands. After crushing, diverse methods have been used in the isolation of essential oils of which steam distillation is the most common method of production. Three different methods of steam distillation are practised. In the simplest method, a vessel containing water and the chopped or crushed plant material is heated by a direct flame and water vapour and the volatile oil is recovered by a water-cooled condenser or if in small quantities the oil is extracted in a light solvent such as petroleum ether or hexane (Langenau, 1948). This method is being replaced by a process in which the plant material is suspended on a grid above the water level and steam from a second vessel introduced under the grid. The volatiles are condensed and the oil is separated. In the third process, the vessel containing the plant material on a grid is heated to prevent condensation of the steam so that dry distillation is attained (Encyclopaedia Britannica, 1991).

Essential oils are also extracted with cold fat. This process is applied to flowers that do not yield an appreciable amount of oil by steam distillation or whose odour is changed by boiling water or steam. Volatile solvents are also used for the recovery of such

essential oils (Encyclopaedia Britannia, 1991). Such extractions remove not only most of the perfume material but also waxes, colouring material and resinous materials. The solvents are removed and the material re-extracted with alcohol to obtain oils of higher purity (McGraw-Hill, 1987). Where thermally induced changes are detrimental, then other methods of extraction must be used. For example, some *Citrus* oils are prepared by the expulsion of the oil from the glands in the fruit skin by mechanical techniques (Hay and Waterman, 1993).

Comparison of these extraction techniques reveal that steam distillation of foliage for 8 hours in a circular still is a more reproducible technique for essential oil isolation than either solvent extraction by a lighter solvent such as pentane and petroleum ether or even steam distillation for longer or shorter periods of time (Muzika et al.,1990).

However, recently the use of rapid microwave techniques for the extraction of plant oils has been shown to afford products almost identical to those obtained *via* conventional steam distillation (Hay and Waterman, 1993).

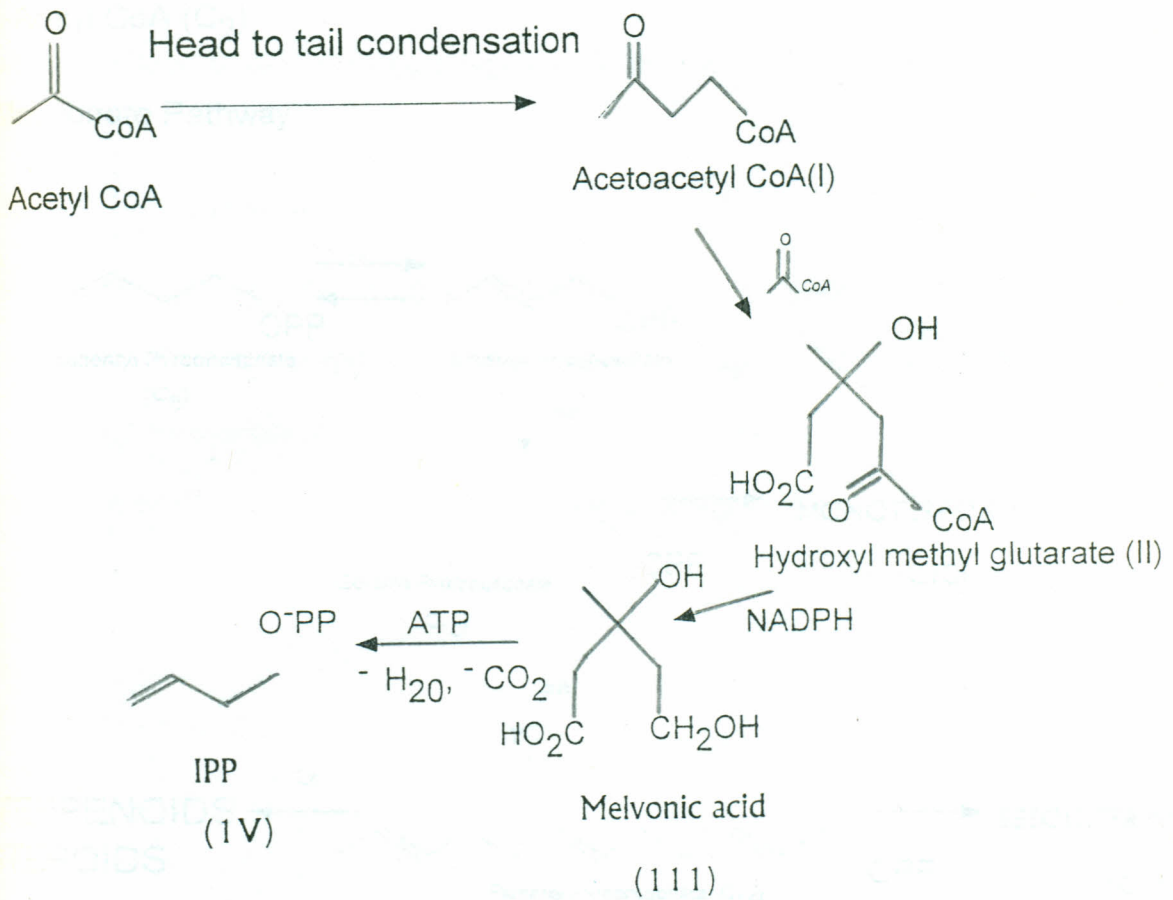
2.2.2 Chemical Composition of Essential Oils

Essential oils are chemically complex mixtures, often containing in excess of 100 individual components. Most oils have one to several major constituents which imparts the characteristic odour/taste but the minor constituents also play their part in producing the final product. Essential oils are composed entirely of two classes of compounds namely, terpenoids and phenylpropenes. (Hay and Waterman, 1993).

acid) and shikimate pathways. The terpenes are wholly mevalonate derived (Hay and Waterman, 1993).

Mevalonic acid is a chemical intermediate containing six carbons that is formed in the plant by the combination of three molecules of acetate (which have, in turn, been derived from acetyl coenzyme A). This is a universal process in all higher plants and produces compounds vital to the life processes of the cell as well as secondary metabolites. The initial modification of mevalonic acid converts it from a 6-carbon structure to a 5-carbon skeleton, with the isoprene arrangement that is typical of mono- and sesquiterpenes. In fact, two different forms of this 5-carbon intermediate are produced: isopentyl pyrophosphate (IPP) and dimethyl pyrophosphate (DMAPP) (Scheme 1).

One molecule of IPP and one molecule of DMAPP can combine under the influence of another enzyme, geranyl pyrophosphatesynthase, to give geranyl pyrophosphate (GPP), the first recognizable monoterpene. This process can then be continued by the addition of another IPP to GPP, through the mediation of a further synthase enzyme, resulting in the production of the first 15-carbon sesquiterpene compound, farnesyl pyrophosphate (FPP) (Scheme 2).

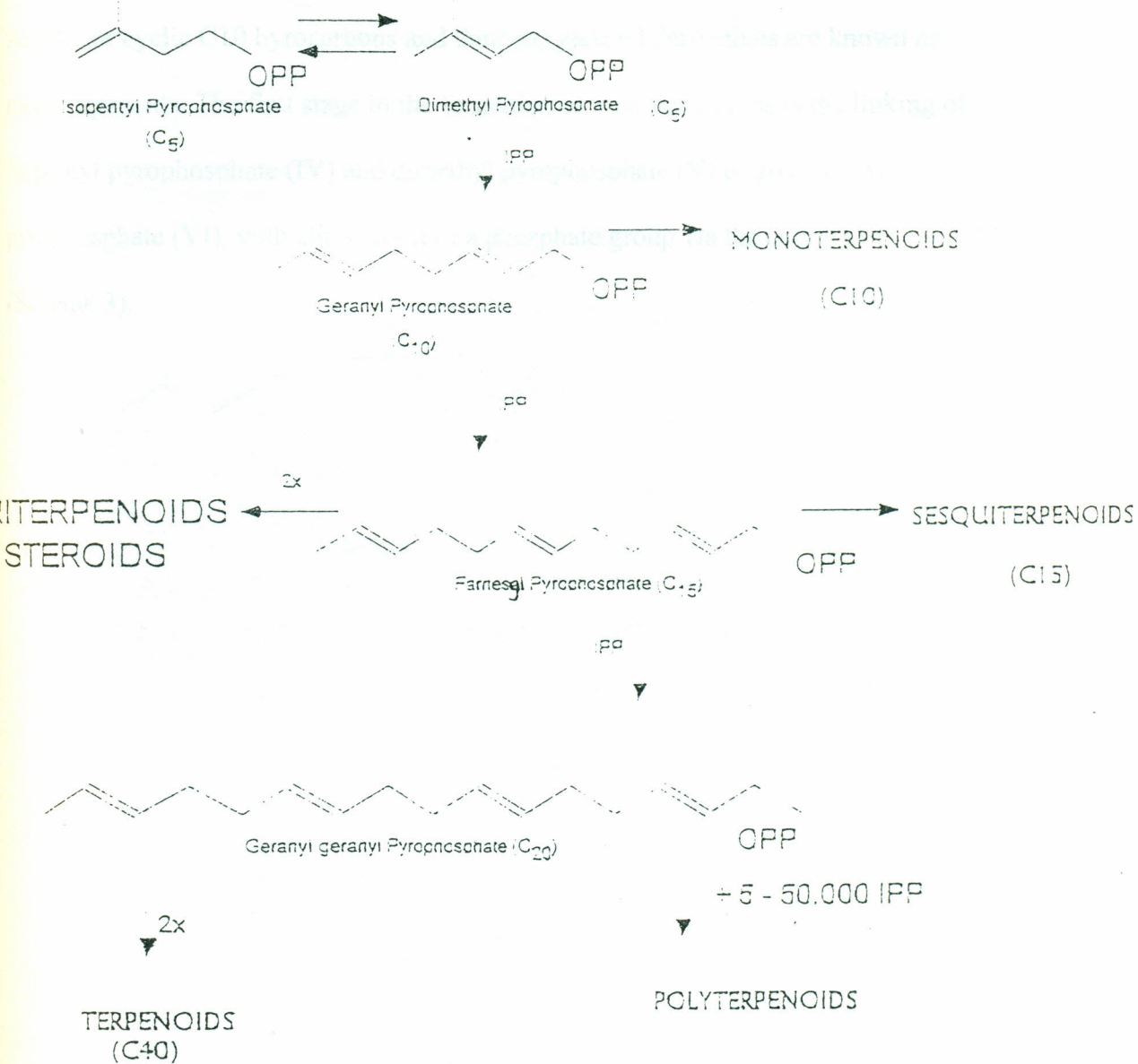


Scheme 1: Biosynthesis of Isopentenyl Pyrophosphate from Acetyl CoA.

The C_3 and C_5 compounds are often referred to as lower terpenoids. C_3 compounds are also referred to as isoprenoids, as they are commonly referred to as essential oils (volatile terpenoids). C_5 compounds are often referred to as higher terpenoids (non-volatile terpenoids).

Mevalonate Pathway

Isoprenoids

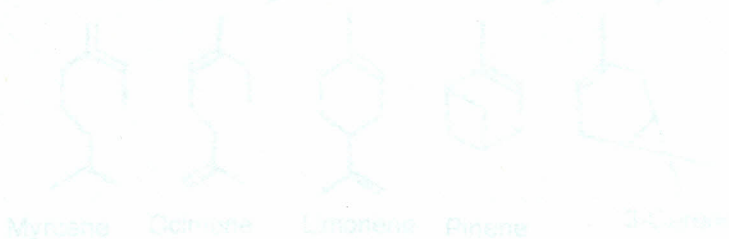


Scheme 2 : Biogenesis of Terpenoids

The C₁₀ and C₁₅ compounds are often referred to as lower terpenoids. When they occur together, they are commonly referred to as essential oils (volatile terpenoids). C₂₀ and above are often referred to as higher terpenoids (non-volatile terpenoids).

2.2.2.1.1 Monoterpenoids

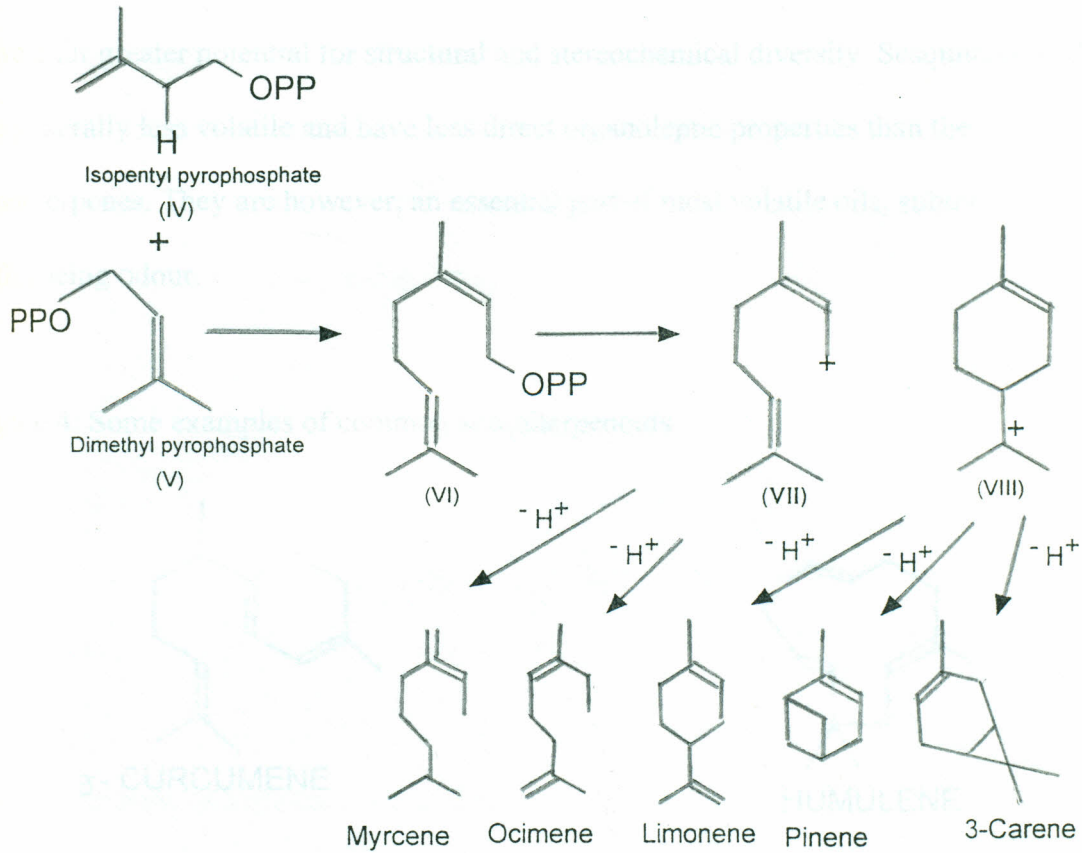
Acyclic or cyclic C₁₀ hydrocarbons and their oxygenated derivatives are known as monoterpenoids. The first stage in the formation of monoterpenoids is the linking of isopentyl pyrophosphate (IV) and dimethyl pyrophosphate (V) to give geranyl pyrophosphate (VI), with elimination of a phosphate group via the cation (VII) or (VIII) (Scheme 3).



Scheme 3: Possible Ways for the formation of Monoterpenoids from Geranyl

Pyrophosphate

Sesquiterpenoids

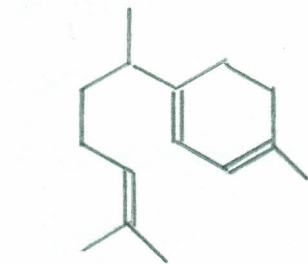


Scheme 3 : Possible Ways for the formation of Monoterpenoids from Geranyl Pyrophosphate.

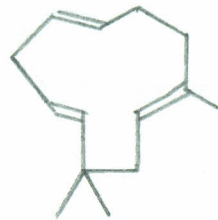
2.2.2.1.2 Sesquiterpenoids

Sesquiterpenoids, because they possess five more carbons than the monoterpenoids, have a far greater potential for structural and stereochemical diversity. Sesquiterpenoids are generally less volatile and have less direct organoleptic properties than the monoterpenes. They are however, an essential part of most volatile oils, subtly influencing odour.

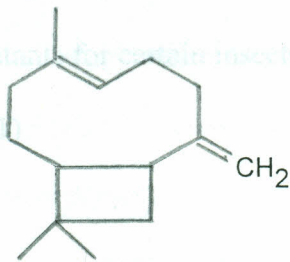
Figure 4: Some examples of common sesquiterpenoids



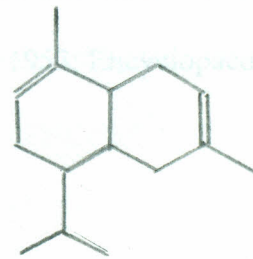
γ -CURCUMENE



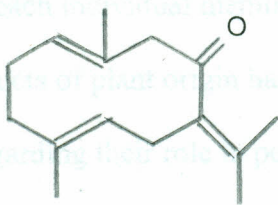
HUMULENE



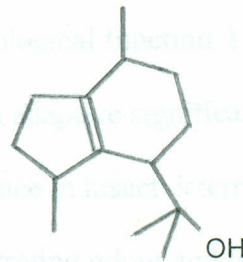
CARYOPHYLLENE



α -CADINENE



GERMACRONE

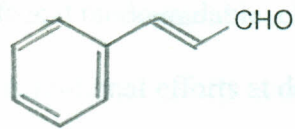


GUIOL

2.2.2.3 Phenylpropenes

The skeleton of phenylpropenes invariably consists of a 6-carbon aromatic ring with a 3-carbon side chain attached. The side chain always contains a double bond but only occasionally an oxygen functional group for example cinnamaldehyde in cinnamon oil.

Figure 5: Structure of Cinnamaldehyde



CINNAMALDEHYDE

2.2.3 The Role of Essential Oils in Plants

The odoriferous principles of leaves and flowers probably aid in natural selection by acting as attractants for certain insects to pollen (Simonsen, 1953; Encyclopaedia Britannica, 1991).

Terpenoids are by far the most dominant constituents of essential oils. Their number in plants exceeds that in any other group of natural products. It would therefore be unrealistic for each individual member to have a specific biological function. However, terpenoid products of plant origin have been associated with adaptive significance particularly regarding their role in pest and pathogen resistance in insect deterrence or attraction (Bridges, 1987; Harborne, 1989). With their penetrating odour and taste, the monoterpenoids present in other parts of the plant, other than in the leaves or flowers,

almost function biologically by repelling predators, while sesquiterpenoids may provide a defence mechanism against fungal attack (Pridham, 1966).

2.3 Essential Oils used in Storage Pest Control

Stored product losses resulting from storage pests have been prevented predominantly through the use of pesticides. The development of resistance to widely used pesticides (Haliscak and Beeman, 1983; Horton, 1984) and concerns over chemical residues in foods dictates that alternative safe and biodegradable chemicals be investigated. This necessity has led to concerted international efforts at developing new sources from the vast store of chemical substances in plants. In the storage ecosystem, the use of plant products have many advantages over synthetic pesticides. Above all, their relative safety.

Repellent plant substances are made of volatile, often aromatic terpenoid constituents (essential oils) which prevent attack and infestation by insects and on long exposure causes deleterious physiological effects. Such substances are extracted in some countries for the use as insecticides. The powder of heart wood of *Juniperus recurva* in Nepal is an example (Marinibetto, 1983). The use of certain plant substances as insect repellents led directly to the extraction and identification of several essential oils that are fairly efficient repellents. Two examples are oil of citronella and oil of camphor (Rice, 1983).

A chemical class conspicuous among secondary plant metabolites and containing chemicals inimical to insects are the terpenoids (Ryan and Byrne, 1988). Plant families particularly rich in terpenoid oils are the Compositae and Labiatae.

Essential oils of some plants and some of their components have been found effective against some stored product pests. Shaaya and Vladimir (1991) investigated the potential use of the essential oil extracted from various species and herb plants and tested some of their major constituents as fumigants. The fumigant toxicity of 26 essential oils and some of their constituents were assessed against the adults of *Rhizopertha dominica*, *Oryzaephilus surinamensis*, *Tribolium castaneum* and *Sitophilus oryzae* (all storage insects). Three groups of active materials were distinguished according to their toxicity against the insects tested. In addition, based on the structure of the effective constituents it was possible to synthesize and select a compound ZP 51 with a potency of 5-10 fold greater relative to all other compounds assayed. A concentration of 1.5 μ l/l of air of ZP 51 was enough to achieve 100% mortality against the *S. oryzae* and 3 μ l/l of air against *O. surinamensis*, *T. castaneum* and *R. dominica*. It should be noted that a concentration of 20-30mg/l of air of methyl bromide is recommended for the control of storage product insect pests. The essential oils of *Origanum vulgare* L. (Oregano), *Ocimum basilium* (Basil), *Marjorana hortensis* (Marjoram) and *Thymus vulgaris* L.(Thyme) have also been reported to be active against *Oryzaephilus surinamensis*. *Salvia officinalis* L. (Sage) oil was active against *S. oryzae* (rice weevil) (Putievsky et al., 1986).

Lippa species, which grow as herbs or shrubs, are among the many odoriferous plants found in Kenya (Agnew, 1974). The following is an account of the reported bioactivity of the essential oil of these species. The essential oil of *L. grandifolia* has been found to exhibit allelopathy to lettuce seedling growth (Elakovish and Oguntimein, 1987). *L. ukambensis* oil from Tanzania has also shown to display weak antimicrobial activity (Chongo and Crank, 1982) while the antimicrobial activity of *L. grandifolia* and *L.*

javanica oils from Kenya have also been reported (Mwangi et al. 1991). The major insecticidal compound from *L. stoechadifolia* oil has been isolated (Grudy and Still, 1985).

Essential oils and their constituents are known to be repellents to various insects or their larvae but repellency against maize weevil (*Sitophilus zeamais*) has rarely been reported (Chongo and Crank, 1982). The constituents of the essential oils of *Ocimum suave* and *Clome monophylla* have been examined for maize weevil repellency with promising results (Hassanali et al., 1990 Ndung'u et al., 1994).

Reproduction retarding, fumigant toxicity and grain protection capability of 31 naturally occurring essential oils of plant origin at the rate of 1000 ppm were studied in the laboratory against the *S. oryzae* (Singh and Agarwal., 1988).

Jambere et al (1995), investigated the effect of dried ground leaves and the essential oil of *Ocimum kilimandscharium* (Labiatae) as post-grain harvest protectants against three stored pests. They found that both the leaves and the essential oil provided protection to maize and sorghum grains against attack by *Sitophilus zeamais*, *Rhyzopertha dominica* and *Sitotroga cerealella*. However, the essential oil in particular was highly repellent to all the three pests tested.

2.4 Plant derived Products used Protectants

Plants have evolved over some 400 million years and to combat insect attack they have developed a number of protective mechanisms such as repellency and insecticidal

action. Thus a large number of different plant species contain natural anti-insect materials. Some of these have been used by man as insecticide since early time although many of them cannot be profitably extracted. However, several of these extracts have provided valuable contact insecticides which possess the advantage that their use does not appear to result in the emergence of resistant strains to the same degree as in the application of synthetic insecticides (Cremllyn, 1978).

The most important botanical insecticide which has been used for a very long time is nicotine and pyrethrum. According to Cremllyn (1978), as early as 1690 water extracts of tobacco leaves were being used to kill sucking insects on garden plants. Nicotine functions as a non-persistent contact insecticide against aphides on a variety of crops. However, it has been replaced by synthetic chemicals, because of its high mammalian toxicity and its lack of effectiveness in cold weather (Cremllyn, 1978).

Pyrethrum is contact insecticide which is obtained from the flower heads of *Chrysanthemum cinerariaefolium* Vis. and owes its importance to its outstanding knock-down action on flying insects combined with its low mammalian toxicity due to its ready metabolism to non-toxic products (Matsui and Yamamoto, 1971).

Some plants, for example, *Ocimum suave* and *Azadirachta indica* have been used as post-grain harvest protectants for centuries. *Ocimum suave* is an indigenous plant of Cameroon commonly used by the farmers for the control of insect pests on stored maize, beans and cowpeas. Leaf branches of the plant are mixed with the grains for the control of storage insects and this practise has been passed on for long (Parh et al., 1990).

Azadirachta indica (neem) is another widely used botanical to control stored product pests. Neem leaves and neem oil have been traditionally used in India to protect grains against infestation (Golob and Webley, 1980). In a recent study done by Baba (1994), the effects of plant-derived products on stored food pests has been compared. The tests carried out in the laboratory showed that a number of plant-derived products have a pronounced insecticidal effects. These included *Ricinus communis*, *Solanum nigrum*, *Cissampelos owariensis* and *Erythrophelelum suaveolens*. However, these plants cause major reductions in grain quality or are toxic to warm-blooded species. The results obtained show that neem and pyrethrum were better alternatives to protect food designed for consumption.

2.5 Importance of Pyrethrum

The growing concern for environmental protection and mounting restrictions on the synthetic chemical insecticides have expanded the sales possibilities for pyrethrum. As a natural insecticide with almost no toxic effects on human beings, pyrethrum has become increasingly attractive to insecticide manufactures in recent years. Pyrethrum flowers (Plate 1) are in the genus *Chrysanthemum* (family-Compositae), the commercially important species being *cinerariaefolium*. Pyrethrum is a small perennial crop grown for

Plate 1: *Chrysanthemum* flowers



Plate 1: Pyrethrum Flowers

the production of natural insecticide which is found mainly in the flower head. In Kenya, the pyrethrum plants flower continuously for nine months in a year during which time flower heads are in various stages of development (Head, 1966).

Although synthetic substitutes have been developed that duplicate many of pyrethrum's desirable properties, pyrethrum has been able to improve its position in the market. The substitutes are not capable of replacing pyrethrum in all its end uses, as they do not possess its entire range of characteristics.

The pyrethrum flower head consists of a female outer ring of white ray florets and the male yellow disc florets. The florets open from the outer row and progressively open towards the centre of the flower. Eight distinct stages of flower development (from bud to seed stage) have been identified and described (Head, 1966; Parlevliet and Brewer, 1968). These stages have been reported to be of great economic importance in the harvesting of pyrethrum flowers. The insecticidal action of the plant is due to the presence of pyrethrins, which are concentrated in the flower head, with the concentration increasing with maturity.

The flowers of the pyrethrum plant have many properties necessary for insect control such as rapid 'knock-down' and killing action, a good 'flushing-out' effect (that is forcing the insect into the open), strong repellency action (detering insects from entering the area), effectiveness against a wide spectrum of insects and a low-level of insect immunity. In addition, and perhaps most important insight of the growing concern over chemical accumulation in the environment, pyrethrum is virtually non-toxic to human and mammalian life (Lindholm and Fock, 1978).

2.5.1 Suppliers of Pyrethrum

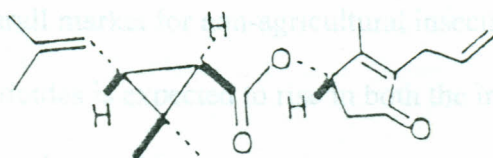
Pyrethrum is produced mainly in developing countries and most of its output is exported to the industrialized markets. World production of pyrethrum in terms of dried flowers totalled 23,000 tons in 1974-75. During the past 20 years annual output has been rising at an average 6.7% in value. The five main producing countries (in order of production) are Kenya, Tanzania, Ecuador, Rwanda, and Japan (Japan's position on the market, however, is rapidly declining).

A number of other countries producing pyrethrum but on a smaller scale are Bolivia, Brazil, Hungary, Indonesia, India, Papua New Guinea, Peru, Zimbabwe, Taiwan and Zaire (Lindholm and Fock, 1978).

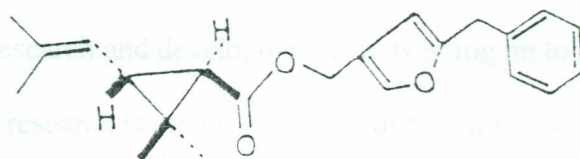
2.5.2 Substitutes for Pyrethrum

As with other natural products, synthetic substitutes have been developed for pyrethrum. The closest synthetic substitutes are the pyrethroids, which have some of the properties of pyrethrum and therefore compete with it in several end uses. There are three major synthetic pyrethroids which are commercially available and are blended with natural pyrethrins. They are allethrin, resmethrin, and tetramethrin (figure 6). These are all esters of chrysanthemic acid that may be in the *cis*, *trans* form (Casida, 1973).

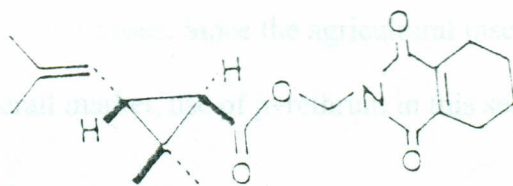
Figure 6: Structures of three synthetic pyrethroid insecticides.



Allethrin



Resmethrin



Tetramethrin

Pyrethroids, like pyrethrum, do not pose the environmental and health hazards that many other synthetic chemical insecticides do. Pyrethroids, however, cannot be fully substituted for pyrethrum in all its uses. Although they closely resemble pyrethrum in chemical structure, none of the pyrethroids available commercially nor any of the known laboratory formulations are exactly the same as pyrethrum in insecticidal activity (Lindholm and Fock, 1978).

Composition of Pyrethrum

2.5.3 Pyrethrum - Longer term outlook

In the non-agricultural sector of insecticides, and particularly for the households, pyrethrum appears to have good prospects over the longer term because of its

nontoxicity. As more restrictions are introduced on the use of certain man-made insecticides and as the overall market for non-agricultural insecticides expands, demand for pyrethrum based insecticides is expected to rise in both the industrialised and the developing countries during the coming years.

A considerable amount of research and development is now going on to find new outlets for pyrethrum. Much of the research is directed towards overcoming the problem of pyrethrum's rapid break down when exposed to direct sunlight. This has limited the use of pyrethrum for agricultural purposes. Since the agricultural insecticides account for about four fifths of the overall market, use of pyrethrum in this sector would greatly increase sales.

A dramatic change in the relative prices of pyrethroids and pyrethrum could adversely affect the future of pyrethrum. But price is only one of a number of factors that determine the choice of active insecticidal ingredients in insecticidal products. It seems probable that the nontoxic, nonpersistent characteristics of pyrethrum, combined with its large spectrum of insecticidal efficiency, will prove to be the main positive factors affecting pyrethrum's future on the market (Lindholm and Fock, 1978).

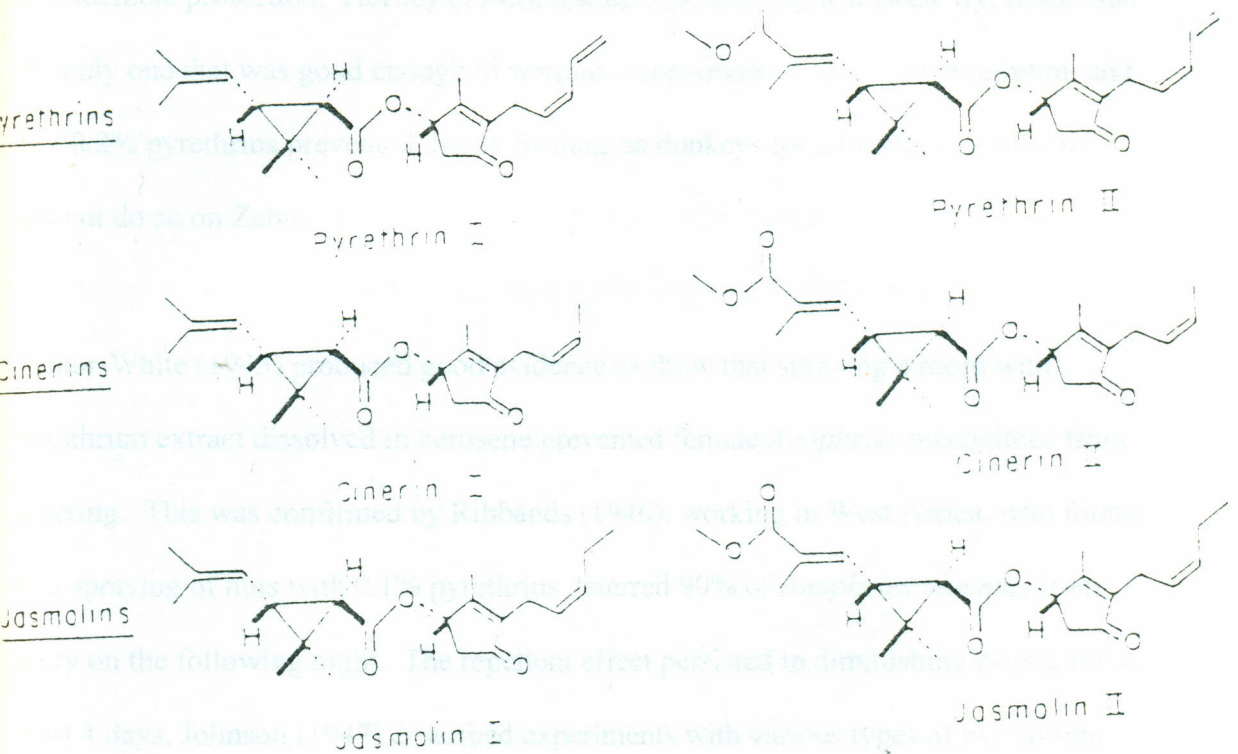
2.5.4 Composition of Pyrethrum

The insecticidally active constituents of pyrethrum extract, the pyrethrins, are esters formed by a combination of two acids, chrysanthemic acid and pyrethric acid, and three alcohols namely pyrethrolone, cinerolone, and jasmololone. The esters of chrysanthemic acid are called pyrethrin I, cinerin I, and jasmolin I, respectively and are together known as the pyrethrins I fraction, whereas the esters of pyrethric acid are called

Pyrethrin II, cinerin II, and jasmolin II, these representing the pyrethrins II fraction.

These structures are shown in figure 7. These six components together account for the kill and knockdown properties of pyrethrum extract (Casida, 1973).

Figure 7: Structures of the six insecticidal constituents of pyrethrum extract.



2.5.5 Repellent Properties of Pyrethrum and Pyrethrin

Dethier (1947) stated that "as pyrethrum is an effective insecticide, not much emphasis has been placed upon its repellent qualities although these are of high order". One of the earliest references on the subject was by Rudolfs (1926) who observed that pyrethrum mixed with vaseline gave protection from mosquitoes for 100-120 minutes. Macnay (1939) observed that pyrethrum was a most promising material for use against mosquitoes, biting flies (simuliids) and other blood-sucking insects. It is difficult to

estimate accurately the amount of pyrethrum present in Macnay's formulations but it was probably about 0.8% w/v, applied in castor oil. Kearns (1942), working with biting flies and mosquitoes in the United Kingdom, found that 1% pyrethrins in petroleum or olive oil gave protection for 5 hours. Rey (1942), working with mosquitoes, particularly *Aedes aegypti*, recorded that any preparation containing pyrethrum extract will afford considerable protection. Hornby (1943), testing repellents against tsetse fly, found that the only one that was good enough to warrant comprehensive tests was pyrethrum, and that 0.2% pyrethrins prevented the fly feeding on donkeys for 24 hours or more but it did not do so on Zebu cattle.

Senior-White (1945) produced good evidence to show that spraying a room with pyrethrum extract dissolved in kerosene prevented female *Anopheles* mosquitoes from entering. This was confirmed by Ribbands (1946), working in West Africa, who found that spraying of huts with 0.1% pyrethrins deterred 90% of *Anopheles minimus* from entry on the following night. The repellent effect persisted in diminishing degree for at least 4 days. Johnson (1947) described experiments with various types of pyrethrum-based anti-mosquito repellent creams and demonstrated that a cream based on gum tragacanth containing 1% pyrethrins was superior to an ointment containing the same pyrethrins concentration based on hard and soft paraffin wax. Tests with even the most promising formulation failed to give complete protection after 1 hour, but 70% protection was still available after 7 to 8 hours. McCulloch (1947), working in India, also observed the repellency effect on *Anopheles* mosquitoes produced by pyrethrum extract in sprays and aerosols. Smith and Chadwick (1964), using a modern water-based emulsion of synergised pyrethrum containing a light stabiliser, found that the residual repellent action of pyrethrum in an experimental hut extended for at least one

month. They felt that there might be promise in the future use of pyrethrum as a residual repellent and suggested that further studies of this property would be justified.

The literature, therefore, establishes without doubt the marked repellent properties of pyrethrum towards a variety of species of mosquitoes and as might be expected, the type of formulation affects the repellent action. The work of Zucker (1966), using modern, refined extracts, pointed to the elimination of the former disadvantage of skin irritancy occasioned by early crude materials. Zucker's conclusions have had considerable confirmation from commercial practice in Australia where insect repellent aerosols containing up to 0.5% pyrethrins (for example giving 2% on the skin) have reached an estimated sale in excess of a million cans. No reports have reached the formulators of irritancy which can be attributed to the pyrethrum extract, of which over 98% was refined in Kenya.

Lately, there has been a world revival in mosquito coils and a rational explanation of their action, necessitates a theory by which exceedingly minute amounts of pyrethrum in the vapour phase effect the movement and food-seeking reaction of mosquitoes long before they show symptoms of poisoning (Maciver, 1964).

2.5.5 Protection of Durable Commodities using Various Pyrethrin

Formulations

Pyrethrum repellency studies are complicated by the fact that not only are the pyrethrins powerful repellents, but unlike many of the synthetic repellents, are at higher concentrations, also strongly insecticidal. This renders it difficult to distinguish between true repulsion of the insect and onset of knockdown, a major feature of

pyrethrum poisoning. It is not surprising, therefore that the word 'repellent' tends to be avoided in much of the published experimental work on repellency; in the following review an attempt has been made to select reported cases where pyrethrins have been correctly interpreted as true repellents.

The protection afforded to clean stored grain by very low concentrations of synergised pyrethrins has been well documented. (Watts and Berlin, 1950; Goodwin-Bailey and Holborn, 1952; Le Pelley and Kockum, 1954; Dove and Schroeder, 1955). Goodwin-Bailey and Holborn (1952) concluded that an initial application, in an organic filter, of 1.3 ppm pyrethrins and 27 ppm piperonyl butoxide w/w gave complete protection to wheat from *Sitophilus granarius* and *Oryzaephilus surinamensis* for 11 months. These workers reported a repellent effect for the first few months of the experiment, before conditions were made difficult due to the presence of dead insects, which accumulated on the treated bags.

Laudani and Swank (1954) developed an apparatus for testing the repellency of treated grain to a range of insect species using low concentrations of pyrethrins synergised at a 1:10 ratio with piperonyl butoxide. A marked repellency was observed against all species tested. *Tribolium* spp. were almost completely repelled by a pyrethrins level of only 0.37 ppm. A sub-lethal effect was observed at a range between 0.5 and 1.0 ppm. The residual behaviour of pyrethrins/piperonyl butoxide films on wheat has been investigated by Blinn et al. (1959). They showed that the degradation rates of the two components were independent and that under the conditions of their tests, grain containing 13% moisture and stored at 90°F, the pyrethrins degraded more rapidly than the synergist. The film, initially analysed at 0.8/7.7 ppm pyrethrins/synergist, had

degraded to 0.2/3.5 ppm after a period of 90 days. Further unpublished data from this laboratory suggest that initially these low-concentration films act as an insecticide, but that a concentration level is maintained as the pyrethrins slowly degrade the proportion of synergist increases and thus with it, up to a certain point, the factor of synergism, so maintaining biological effectiveness over a lengthy period of time. On the other hand, it has not been established definitely that piperonyl synergises the repellent effect of pyrethrins.

Chadwick (1962), studying the repellent and sub-lethal effects of pyrethrum on the grain weevil, found that unsynergised pyrethrins acted as a repellent at 3 to 4 ppm, and at lower concentrations the effect disappeared but at very low concentrations of pyrethrins 0.5-0.6 ppm synergised at a ratio of 1:10 with piperonyl butoxide were used, no repellent action under similar experimental conditions was observed.

Davis (1957) showed that bacon could be effectively protected from oviposition and spoiling by blowflies during processing and storage by the application of pyrethrum dusts. Since no observations of mortality were reported, protection was presumably conferred by the repellent action of pyrethrins. More notable has been the application of synergised pyrethrins to fish which in many parts of the world are sun-dried and such conditions are ideal for spoiling of the commodity by blowflies. Dipping the fish after gutting, in low concentration aqueous emulsions gives adequate protection for several days during the drying period (McLellan, 1963). The fish can similarly be protected against beetles after drying and during storage by further dipping in very low concentrations of synergised pyrethrum, which affords protection for at least six weeks (McLellan, 1963).

Synergised pyrethrins films on cardboard packets and waxed liners in boxes have been highly successful in protecting flour-based and meat-based food products and confectionery between the time they leave the manufacturer and the time they reach the consumer (Goodwin-Bailey and Brooke, 1957). Laudani and Davis (1955) considered that the greater part of the protection conferred by pyrethrins-treated paper was due to true repellency, as opposed to toxicity. A deposit of 10 mg. pyrethrins and 100 mg. piperonyl butoxide per square foot gives protection for many months, particularly under the flaps of the carton where ingress of the insects normally occurs. Here, of course, the pyrethrins are protected from light. High effectiveness of pyrethrins as a repellent can be judged by the fact that, of 534 experimental compounds screened in the U.S.A. in 1959 as alternatives to pyrethrum in this connection, only one was found to have a degree of repellency greater than the pyrethrins/piperonyl butoxide standard; a further 11 were approximately equivalent (Lindholm and Fock, 1978).

2.6 The Maize Weevil, *Sitophilus Zeamais*

(Coleoptera: Curculionidae)

The maize weevil, *Sitophilus zeamais* is a primary and most destructive pest of maize, rice and other grains, and their processed products. It is more cosmopolitan in distribution from tropical areas to temperate zones (Maeshima et al. 1985; De Pury, 1973.; Tipping et al. 1987) and therefore causes serious economic losses of grain throughout the world (Phillips et al., 1985).

The adult maize weevil are brownish-black with the head prolonged into a typical snout or rostrum. The adults are long-lived (several months to one year) and eggs are laid throughout the adult life, although about 50% maybe laid in the first four to five weeks.

The eggs are laid individually in small cavities chewed into the cereal grain by the female. Each egg is protected by a waxy secretion produced by the female (Birch, 1944). Infestation of produce typically starts in the fields and is later carried to the stores. This is especially so if the moisture content of the stored grain is high, at least 12.5%. They mate and lay eggs very rapidly and increase their numbers very quickly (Hill and Waller, 1990; De Pury, 1973).

The infected grain becomes very light with holes on the seeds. The grain also attains a bad smell and the flour goes bad in only a few days. All these together with the ugly sight of floating larva and adult weevils on cooked food makes such maize grains unfit for human consumption.

Storing the grain in a dry and clean conditions in insect--proof containers has been used as a control method. If the grain moisture content is below 9% the insect is unable to breed. This method is not very practicable and fumigation has been the only possible protection (ICRISAT, 1983). Since use of methyl bromide as a fumigant has been banned, hence safer alternatives are being sought for.

2.7. Brief description of the Analytical Techniques for the Chemical Analysis of Essential Oils

The constituents of essential oils can be confirmed by using various analytical techniques. The most important being gas chromatography (GC), mass spectrometry (MS), and combined gas chromatography-mass spectrometry (GC-MS), gas chromatography-infrared spectroscopy (GC-IR), and or nuclear magnetic resonance (NMR).

2.7.1 Gas Chromatography (GC)

Gas chromatography (GC) is one of the most important tools in the analysis of essential oils. In the GC there are two phases, a stationary liquid phase and a mobile gas phase (usually nitrogen or helium). The oil is volatilized in the injection port of the instrument and then entrained with the mobile phase, before being passed through a column packed with a stationary phase. The components of the oil are partitioned between the stationary and mobile phases, the speed of the process through the column being dictated by the affinity of each component for the former. Thus the components of the oil are split up during the passage through the column and do not all emerge at the other end together (Hay and Waterman, 1993).

Because of the characteristic instability of terpenoids, that is, easy polymerization due to the presence of double bonds and dehydration of tertiary alcohol, it is therefore necessary to determine carefully the most suitable gas chromatographic conditions to achieve good separation. Although GC is very suitable in the separation of monoterpenoids and generally sesquiterpenoids, the analysis of the latter is complicated by the lower volatility of these compounds and their occurrence in nature as complex mixtures (Heftmann, 1967).

One of the principle factors influencing separation is a suitable stationary phase. For the separation of monoterpenoids and sesquiterpenoids, a great number of different non-polar and polar phases have been developed (Heftmann, 1967). The most frequently recommended non-polar phases are squalene and silicone elastomers. More polar phases, polyethylene and polypropylene glycol as carbowax are common. It is however

difficult to make the best choice because no phase is universal and even the working conditions will affect the separation.

Due to the instability of terpenoids, their decomposition products formed in the preheater zone of the column may cause some difficulty. So there is need to use lower column temperatures and consequently increase the detector sensitivity and thus reduce the amount of the sample required in the analysis. The incorporation of the flame ionization detector, which is sensitive to organic compounds, thus constitutes an important step forward in the qualitative analysis of terpenoids.

For the analysis of complex mixtures of terpenoids, chromatography in a capillary column is very advantageous. However, some of the components present in a natural essential oil in very low quantities may escape detection while the individual components may not be obtained preoperatively. This method is not applicable to the identification of the unknown compounds, and even the identification of known compounds may be difficult because authentic samples of terpenic compounds are very difficult to obtain. For less complex essential oils whose chromatographic separation can be easily achieved, the combined use of preparative gas chromatography, combined gas chromatography-mass spectroscopy (GC-MS), infrared spectroscopy (IR) and nuclear magnetic resonance spectroscopy (NMR) can lead to the identification of new compound (Heftmann, 1967).

2.7.1.1 Interpretation of Retention Time (R_t) data in GC

There are three fundamentals concerning retention times on a given instrument with a given column operating under specific conditions:

- (i) If the retention time of component A, $(R_t)_A$ is equal to retention time of unknown component $(R_t)_{UK}$, this does not confirm that the unknown component is A. This prevents GC from being an exceptional qualitative method of analysis.
- (ii) If $(R_t)_A$ does not equal $(R_t)_{UK}$, then indeed with absolute certainty it can be said that the unknown component is not component A.
- (iii) If there is no discernable peak at $(R_t)_A$, this component A is not present in the sample within the limits of detection.

The systematic method for expressing retention data uses the Kovats retention indices (RI). These indices indicate where components will appear on a chromatogram with respect to straight chain alkanes injected with the sample.

The index of an unknown compound X is described as:

$$I_X = 100 \left[n \frac{\log R_X - \log R_Z}{\log R_{Z+N} - \log R_Z} + Z \right]$$

where,

$R_X = R_t$ for unknown X

$R_Z = R_t$ for normal alkane having z carbons

$R_{z+N} = R_t$ for normal alkanes having $z+n$ carbons

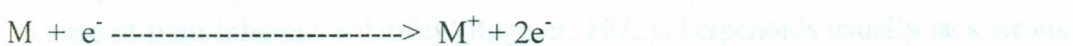
n = the difference in the number of carbon atoms for the normal alkanes.

Retention Indices (RI) for a normal paraffin is 100 times the number of carbon atoms in the compound regardless of the column used for chromatographic conditions.

2.7.2 Mass Spectrometry

In mass spectrometry a sample is introduced into a highly evacuated chamber, of pressure between 10^{-6} - 10^{-7} mm Hg, maintained by the use of diffusion pumps.

Molecules are then bombarded with a beam of electrons with sufficient energy to effect its ionisation (70 eV). This may lead to the ionisation of the sample molecule resulting in the formation of the molecular ion.



The electron removed from this sample may originate from :

- (1) a lone pair of electrons on a heteroatom such as oxygen, sulphur or nitrogen.
- (2) a pair of electrons in the π orbital of a multiple bond, or
- (3) a pair of electrons in the σ orbital of a single bond.

Energy in excess of that required to ionize a molecule may be dissipated by fragmentation of the molecular ion, into smaller ions (fragment ions, or daughter ions).



The molecular ions, the fragment ions and the fragment radical ions are separated by deflection in a variable magnetic field according to their mass and charge, and generate a current (the ion current) at the collector in proportion to their relative abundance. A mass spectrum is a plot of relative abundance against the ratio mass/charge (m/e value).

The preference of fragment ions in a spectrum provides valuable information concerning the structure of the molecule.

Basically compounds are identified using mass spectrum by comparing the spectrum of the unknown with those in reference collection (NIST and NBS) to find one with ions of similar m/e value and relative abundance (Rose and Johnstone, 1982).

2.7.3 Combined Gas Chromatography-Mass Spectrometry (GC-MS)

The sensitivity of the mass spectrometer and the resolving power of the combined gas chromatography-mass spectrometer (GC-MS) has made mass spectrometry a useful tool in the elucidation of the structure of diminishing small concentrations of compounds isolated in essential oils. Such compounds are particularly sensitive to GC-MS analysis because of their inherent volatility (Regnier, 1972). Terpenoids usually lack strong fragmentation directing groups and in favourable cases complete structure assignment may be made by mass spectra alone but coupled with co-chromatography, identification can be achieved. When run under the same conditions the mass spectra of terpenoid isomers, although generally very similar, frequently exhibit small but significant differences in the relative intensities of some peaks. Such differences can be used in the identification of compounds.

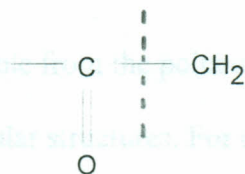
2.8 A Guide to the Interpretation of Mass Spectra Data of Terpenes

The analysis of the mass spectra requires knowledge of the chemical behaviour as well as the structural features of the molecule (Rose and Johnstone, 1982). Generally,

(i) for acyclic alkanes, the loss of a methyl group is never observed unless it is present at a point of chain branching, even so, the longer chain is preferentially removed.

of the filament. In these circumstances the opposite order is observed in the facility with which the water is removed. Both occurrences may well lead to the same olefin ion, and hence deductions based upon the facility of this elimination may well lead to erroneous conclusions.

(viii) The presence of the keto group again gives rise to fragmentation processes characteristic of this group. The loss of 28 mass units which is thought to be neutral carbon monoxide and formation of a formyl ion $m/e = 29$ are observed. The predominant fissions in a chain are those which occur α - to the carbonyl, that is



Hydrogen transfer as a concomitant process will yield the neutral molecule, acetylene and an olefinic or cyclic $C_3H_6^+$ ion. The ion, $C_3H_3^+$ has been considered to be of particular stability since it is found to be a prominent ion in the fragmentation patterns of many diolefins and this may readily be derived either by the loss of two hydrogen atoms, or a hydrogen molecule, from the ion $m/e = 41$, or ethyl radical. Thus, the parent molecular ion may split into



or



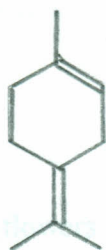
2.8.1 Acyclic Monoterpenes

These are considered to be derived from the isoprene units by dimerization which takes place between the C-1 of one unit and C-4 of another unit to yield a ring system. This

ring structure may be further modified by group migrations. In certain cases, the second stage of the addition does not occur in this way and the resultant product may be an acyclic compound such as myrcene. The cracking pattern of this compound may be correlated with its known structure. The low abundance of the parent molecular ion is related to the presence of two quaternary centres as well as a doubly allylic C-C bond in the molecule. The rather prominent ions at $m/e = 39, 41, 69$ and 91 may also be considered to arise from these features (Rose and Johnstone, 1982).

2.8.2 Cyclic Monoterpenes

Cyclic structures are more intelligible from the point of view of correlating fragmentation patterns with molecular structures. For example, Terpinolene,



The observed mass spectrum contains ions of $m/e = 121, 119, 93, 91, 77, 55, 45$ and 39 . The parent molecular ion $m/e 136$ is quite abundant, which is consistent with the known cyclic nature of the molecule, whereas $m/e = 93$ is the base peak. The peak of $m/e 55$ is present in accordance with the trend in cyclic molecules, particularly those which contain a cyclohexane ring. This compound and others possess two tertiary carbon atoms, therefore, fission may well be favoured at one of these. Since the molecule is cyclic, such a change will not alter the molecular weight of the ion (Rose and Johnstone, 1982).

CHAPTER 3

MATERIALS AND METHODS

3.1 General Experimental Procedures

All recyclable glassware used were washed with hot water and soap and rinsed with distilled acetone. The glassware was then dried at 110° for one hour.

3.1.1 Reagents and Reference compounds

The acetone and the authentic samples of the compounds identified in the essential oil were purchased from Aldrich Chemical Co., Gillingham, U.K. and Sigma Chemical Co., Poole, U.K.

3.2 Plant Materials

Chrysanthemum cinerariaefolium Vis. flowers were collected from pyrethrum plantations in Molo, Nakuru District, Rift Valley Province, Kenya. The flowers were packed in plastic bags and taken to the laboratory for extraction in the minimum time possible to avoid loss of essential oil through evaporation. The oil was extracted at the Pyrethrum Board of Kenya laboratories in Nakuru, Rift Valley Province, Kenya. The fresh flowers were ground to a grist. Three kilogrammes of the grist were weighed and placed in a 10 litre round bottomed flask and steam distilled using a Clevenger-type apparatus for 8 hours. This exercise was repeated until all the 36 kilogrammes of the grist was extracted.

Figure 8. Schematic diagram of the olfactometer used for the bioassay.

The steam distillate was collected every two hours, and extracted with diethyl ether. This prevented possible decomposition of the oil due to heat and exposure to light. The ether was finally removed by distilling over a steam bath. The oil was stored in sealed amber coloured sample vials at about 0°C.

3.3 Test Organism

To start the colony of the *Sitophilus zeamais*, laboratory reared *S. zeamais* were obtained from the Kenya Agricultural Research Laboratories (K.A.R.I), Nairobi, Kenya. These *S. zeamais* were placed in glass jars containing sterilized insecticide-free maize. The maize was sterilized by placing in an oven at 60°C for six hours. The glass jars containing the maize and the *S. zeamais* were covered with a muslin cloth and tied with rubber bands. The weevils were reared at 25°C and at a relative humidity of 65%. The colony took one month to establish.

3.4 Repellency Bioassay

A Y-shaped olfactometer was used for the bioassay (Figure 8). It is constructed of glass and consists of three compartments A, B and C which are connected by means of glass tubing (6mm I.D.). The three compartments are fitted with ground glass female joints D, E and F each fitted with a glass stopper of an appropriate size. Stoppers for E and F have narrow grooves along their length which allows airflow into the olfactometer during the bioassay. Maize weevils were introduced into the compartment A and were induced to migrate to a choice of B or C.

The test samples for the bioassay were prepared by dissolving 1000µl of the test compound in 1000µl of acetone. Subsequent lower concentrations (0.1, 0.01, 0.001,

0.0001 $\mu\text{l}/\mu\text{l}$) were made by diluting the stock solution 10, 100, 1000, 10000 fold, respectively. 100 μl of the diluted test material was applied to the 'treated' filter paper discs (1.8 cm diameter) while acetone alone was applied onto the 'control' filter paper discs. The solvent was allowed to evaporate and the resulting treated and control discs were then be placed in one or the other compartments B or C.

Prior to the introduction of the test material at G, air suction was applied by means of an aspirator pump, Cole-Parmer Air Cadet (model 7059-60), at a flow of 1.5 ml/min. This ensured that the olfactometer did not become saturated with the test material, which was confined to the olfactometer arm that contains the treated filter paper disc (treated arm). All odourous air from the olfactometer to the aspirator pump, was carried away in a Tygon tube (8mm diameter) from the pump outlet to the fume hood.

For the assay, 80 randomly selected adult maize weevil of mixed sex and age were introduced into compartment A. The assay was run for 1 hour in a dark room and then the number of weevils in the control arm (N_c) and in the treated arm (N_t) of the olfactometer were recounted. The olfactometer was washed and dried at 100°C. The assay for each dose of the test material was replicated six times. The assay was repeated using the standard N,N-diethyl-m-toluamide (DEET).

The percentage repellency (PR) was computed using the formula:

$$\text{PR} = \frac{(N_c - N_t)}{(N_c + N_t)} \times 100$$

3.5 Analysis of the essential oil

The essential oil was analysed by gas-chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

3.5.1 GC Analysis

Gas chromatography of the essential oil was performed on a Hewlett Packard model HP5890A series Gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID) both of which were maintained at 250° C. All the GC analysis were performed in a splitless mode. Chromatographic separation were achieved using a fused silica capillary column (Ultra 1 Hewlett packard (50 m x 0.32 mm I.D.)) coated with crosslinked methyl silicone (0.17µm film thickness). Nitrogen (WSN) at a flow rate of 3.45 cm³/min was the carrier gas, while hydrogen and medical air at flow rates of 45cm³/min and 360 cm³/min respectively was used as fuel gas. The GC oven temperature was programmed to run from 45°C where it was maintained for 5 min then rose to 5°C/min to 180°C then rose to 20°C/ min to 280°C at which it was maintained for 15 min. The whole run took a cumulative 52 minutes. The peak areas, the percentage compositions and the retention times were calculated on a Hewlett Packard model HP3393A integrator.

3.5.2 GC-MS Analysis

GS linked electron impact ionization mass spectrometric (GC-MS) analysis were performed on a Hewlett packard model HP5790A series gas chromatograph coupled to a VG-Masslab 12-250 analytical organic mass spectrometer equipped with a data library system. Chromatographic separations was achieved using a fused silica capillary column (50 m x 0.2 mm I.D.) coated with crosslinked methyl silicone (0.33 mm film

thickness). All the GC-MS analysis were performed in the splitless mode with helium as the carrier gas at a linear flow of $20 \text{ cm}^3/\text{sec}$. The oven temperature program was the same as that used during the GC analyses except for the final temperature (280°C) which was maintained for 20 mins, while the ion source and injector temperatures were 180°C and 240°C respectively.

3.6 Identification of the Components of the Essential Oil

The chemical analysis utilized conventional techniques routinely used in the separation and characterization of components of essential oils (Klock et al., 1985 ; Komai and Tang, 1989). Chromatographic separations of the components of the essential oil were done using the conventional gas chromatography under the conditions previously described in section 3.5.

The components were tentatively identified by Gas chromatography-mass spectrometry through computerized matching of the acquired electron impact ionization mass spectra with the stored National Bureau of Standards (NBS) mass spectral library in the data system of the GC-MS and also by comparison with the published mass spectral data in the Wiley/NBS registry of mass spectral data (McLafferty and Stauffer, 1989). The identifications were confirmed by their order of elution on the GC column, comparison of their relative retention time (R_t) values with those of authentic samples and co-injection of the essential oil with the authentic samples.

3.6.1 Co-injection and Peak Enhancement

After the MS library search it was necessary to confirm the identity of the candidate compounds. This was done by injecting a known volume V_1 of the sample into the GC;

the peak area of the candidate compound was noted (A_1). Then its corresponding authentic sample was diluted from a stock solution prepared in the ratio 1 ml of authentic sample :5ml Dichloromethane (DCM), such that the volume V_2 of the authentic sample, injected into the GC had approximately the same area (A_2) as the candidate compound. This was done on condition that the two had the same retention time.

Volume (V_1) of the test sample and volume (V_2) of the authentic sample were drawn from their respective vials in that order using a 10 ml syringe and co-injected into the GC. If the peak area of the candidate compound was enhanced by almost twice its original peak area, then, its identity was confirmed.

The GC oven temperature program and the column were the same as those used in the GC analysis, section 3.5.

3.7 Further analysis of the oil

Due to the unavailability of many of the authentic samples, the essential oil was sent to the University of Barcelona, Barcelona, Spain, for further analysis. The identification of the oil components was done from their retention time indices (RI), determined in relation to a homologous series of fatty acid methyl esters and from their mass spectra (McLafferty and Stauffer, 1989).

GC analysis were carried out using a Hewlett-Packard gas chromatograph model 5890A equipped with a flame ionization detector and fused silica capillary columns of two different stationary phases: Carbowax 20M (CW-20M) and methyl silicone (SE-30),

(25m x 0.2mm, 0.25 μ m film thickness). Analytical conditions used were as follows: injector temperature 250°C, detector temperature 270°C, split ratio 1:60. Oven temperature from 80°C to 250°C (4°C/min), using helium as a carrier gas at a working flow rate 1ml/min. Quantitative data were obtained from FID area values on the two columns.

GC-MS analysis were performed with a HP 5890 gas chromatograph coupled to a HP 5971A mass selective spectrometer. GC-MS operating conditions were: columns, supelcowax 10 (30m x 0.25mm, 0.25 μ m film thickness) and methyl silicone SE-30 (25m x 0.2mm, 0.25 μ m film thickness); oven temperature programmed from 80°C to 220°C at a rate of 6°C/min; carrier gas helium, flow rate 1ml/min. Mass spectra were taken every 5s over m/e 35-400, using an ionizing voltage of 70 ev.

The retention indices were calculated using the formula:

$$RI_A = RI_N + \frac{\text{distance between A and n}}{\text{distance between n and n+1}} \times 100$$

where,

RI_A = RI for unknown

RI_N = RI for fatty acid methyl ester

A = Peak of unknown compound

n = Peak of fatty acid methyl ester

Distance is taken as a function of retention time.

3.8 Data Analysis

The repelling effects of the different compounds at varying doses (1.0, 0.1, 0.01, 0.001, 0.0001 $\mu\text{l}/\text{disc}$) were tested against the *Sitophilus zeamais*. Due to the nature of the sampling technique, a repeated-measure analysis of variance was applied so as to test the effects of different dosages (DOSE), different repellents (REPEL) and their interactions (DOSE*REPEL) on the response variable (PERCENT REPELLENCY) on the *S. zeamais*.

The percent repellency variable was transformed using angular transformation so as to normalize the data and to minimize the co-relation between the mean and the variance of the data. Since levels of dosages represent quantitative factor whose effect can often be explained through polynomial relations, hence orthogonal polynomial contrasts were used to investigate repellency trends with concentrations. Mean comparison was performed by use of adjusted treatment (REPEL) means computed by least square method (Munyinyi, 1995).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction of the Essential Oil

The essential oil was extracted from the fresh flowers by steam distillation using a Clevenger-type apparatus as described in section 3.2.

From 36 kg of pyrethrum flowers from Molo, 11gms of the essential oil was obtained. Thus the percentage yield of the oil was 0.0306%. The marshy residue grist left after the extraction of the essential oil was sun-dried and then extracted with hexane. The hexane was removed by distillation and the oleoresin obtained was U.V. analyzed for pyrethrin. The U.V. analysis were done by determining the absorbance of the oleoresin at 225nm and then derermining the concentration. This was found to contain 25% pyrethrins.

4.2 Chemical Composition of the Essential Oil

Figure 9 shows the gas chromatogram of the essential oil of *Chrysanthemum cinerariaefolium* Vis. when the oil was run on a methyl silicone column. Table 3 indicates the chemical composition of the essential oil and the method of identification.

Figure 9: Gas chromatogram of the essential oil from *Chrysanthemum cinerariaefolium* Vis.

Figure 9: Gas Chromatogram of the essential oil from *Chrysanthemum cinerariaefolium* Vis.

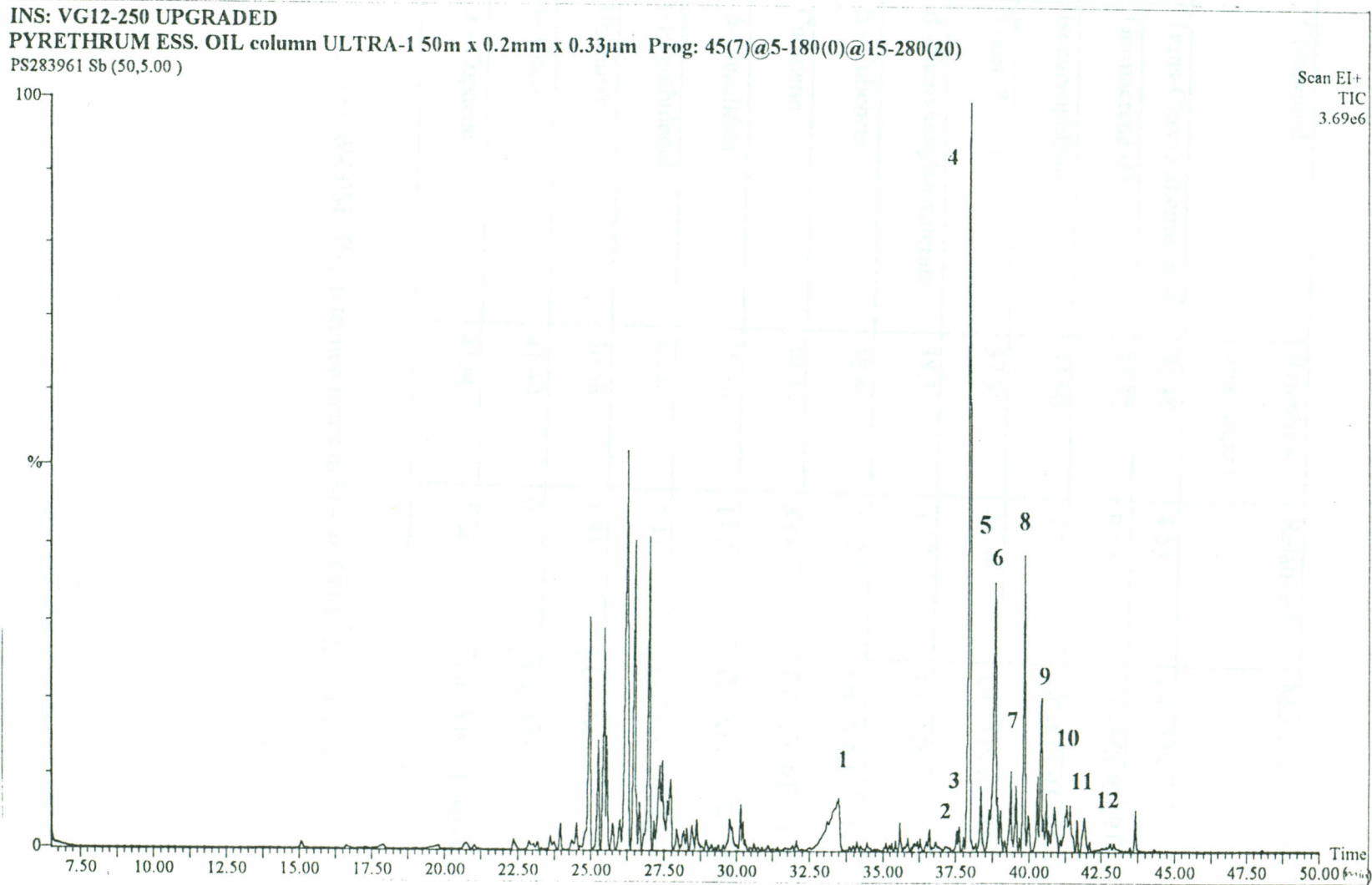


Table 3: Chemical composition of the essential oil from *Chrysanthemum cinerariaefolium* Vis.

Peak No.	Compound	Retention time (mins)	Relative %	Method of identification
1	Trans-Chrysanthemic acid	33.48	4.53	GC-MS
2	Germacrene -D	37.52	trace	GC-MS, RI ₁ , RI ₂
3	Isocaryophyllene	37.60	0.71	GC-MS, RI ₁ , RI ₂ , CO-INJ
4	Trans β - Farnesene	37.95	41.36	GC-MS, RI ₁ , RI ₂
5	β -Phenylethylisovalerate	38.33	3.37	GC-MS
6	β - Cubebene	38.80	17.27	GC-MS, CO-INJ
7	Cadinene	39.35	4.06	GC-MS, RI ₁ , RI ₂
8	δ - Nerolidiol	39.80	14.23	GC-MS, RI ₁ , RI ₂ , CO-INJ.
9	(-)-Spathulenol	40.40	7.41	GC-MS, RI ₁ , RI ₂
10	Unknown	40.58	1.81	GC-MS
11	δ - Cadinol	41.40	0.75	GC-MS
12	α - Copaene	41.90	3.04	GC-MS, RI ₁ , RI ₂ , CO-INJ

RI₁: retention index in CW-20M; RI₂: retention index in SE-30; CO-INJ; co-injection

The mass spectrum of *trans*-Chrysanthemic acid (figure 10 a) displayed a molecular ion at m/e 168 and the table 4 shows the characteristic peak intensities and peak ions.

Figure 10a: Mass spectrum of *trans* -Chrysanthemic acid.

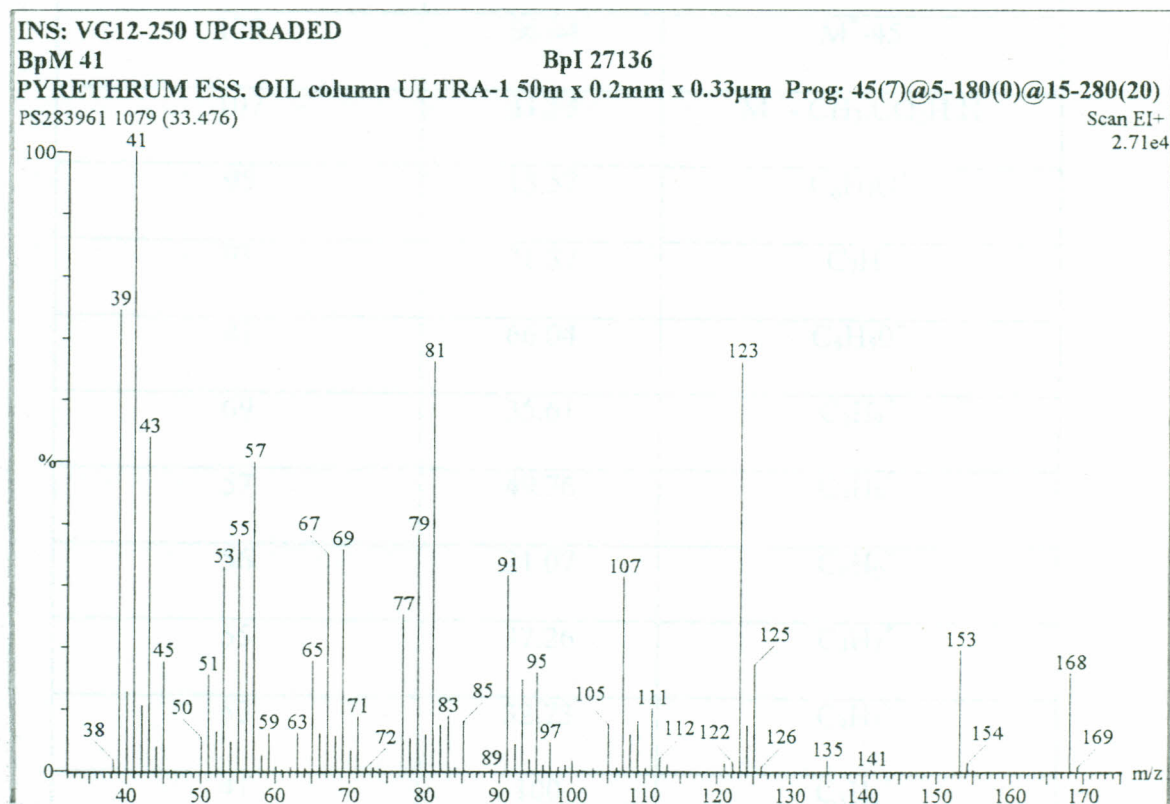
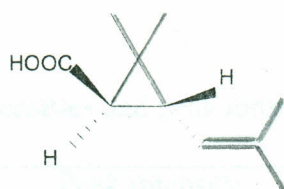


Table 4: Characteristic peak intensities and peak ions of *trans* -Chrysanthemic acid.

Retention Time (mins)	Peak Intensity	Peak Ions
168	15.57	M ⁺
153	19.34	M ⁺ -15
125	16.98	M ⁺ -43
123	66.04	M ⁺ -45
107	31.13	M ⁺ - CH ₃ .CO ₂ H.H
95	15.57	C ₆ H ₇ O ⁺
91	31.37	C ₇ H ₇
81	66.04	C ₅ H ₅ O ⁺
69	35.61	C ₅ H ₉ ⁺
57	49.76	C ₄ H ₉ ⁺
56	21.07	C ₄ H ₈ ⁺
55	37.26	C ₄ H ₇ ⁺
53	32.73	C ₄ H ₅ ⁺
41	100	C ₃ H ₅ ⁺
39	74.53	C ₃ H ₃ ⁺

The structure of *trans* -Chrysanthemic acid (10b) shows cleavage of the bond adjacent to the carbonyl group (α - cleavage) results in the formation of M-CO₂H which is a characteristic peak from McLafferty rearrangement.

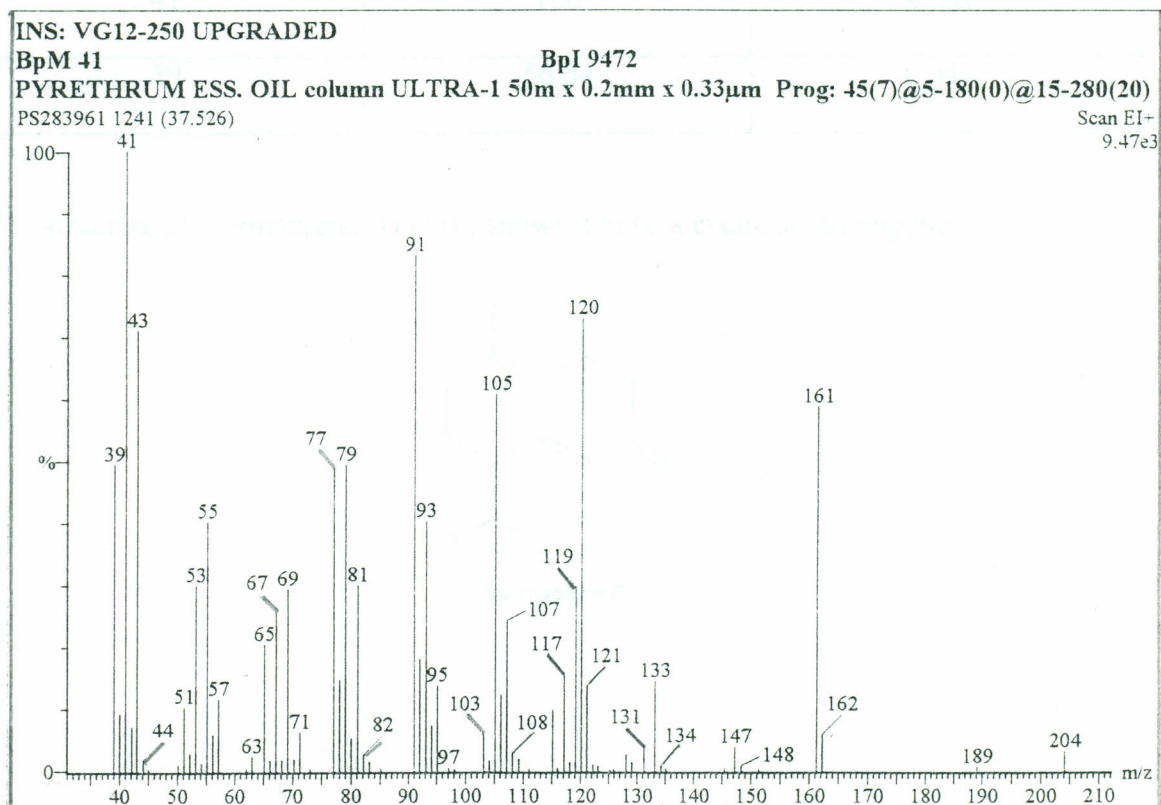
Trans⁻ Chrysanthemic acid

(10b)

Generally, esters of both the *cis* and *trans* form are widely used in commercial insecticides. *trans*-Chrysanthemic acid does not conform to the Biogenetic Isoprene Rule. The irregular monoterpene chrysanthemic acid is found in esterified form in the pyrethrins found in the *Chrysanthemum cinerariaefolium* flowers (Dewick, 1997).

The mass spectrum (figure 11a) displayed a molecular peak at 204 and the table 5 shows the characteristic peak intensities and peak ions of Germacrene-D.

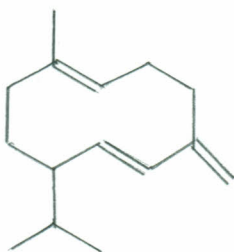
Figure 11a: Mass spectrum of Germacrene-D.



. Table 5: Characteristic peak intensities and peak ions of Germacrene-D.

Retention Time (mins)	Peak Intensity	Peak Ions
204	3.03	M^+
162	12.78	$M^+ - C_3H_6$
161	59.29	$M^+ - C_3H_7$
133	15.49	$M^+ - C_5H_{11}$
120	26.99	$M^+ - C_6H_{12}$
79	45.35	$C_6H_7^+$
77	50.88	$C_6H_5^+$
69	25.88	$C_5H_9^+$
67	30.09	$C_5H_7^+$
55	44.47	$C_4H_7^+$
43	53.32	$C_3H_7^+$
41	100	$C_3H_5^+$
39	56.64	$C_3H_3^+$

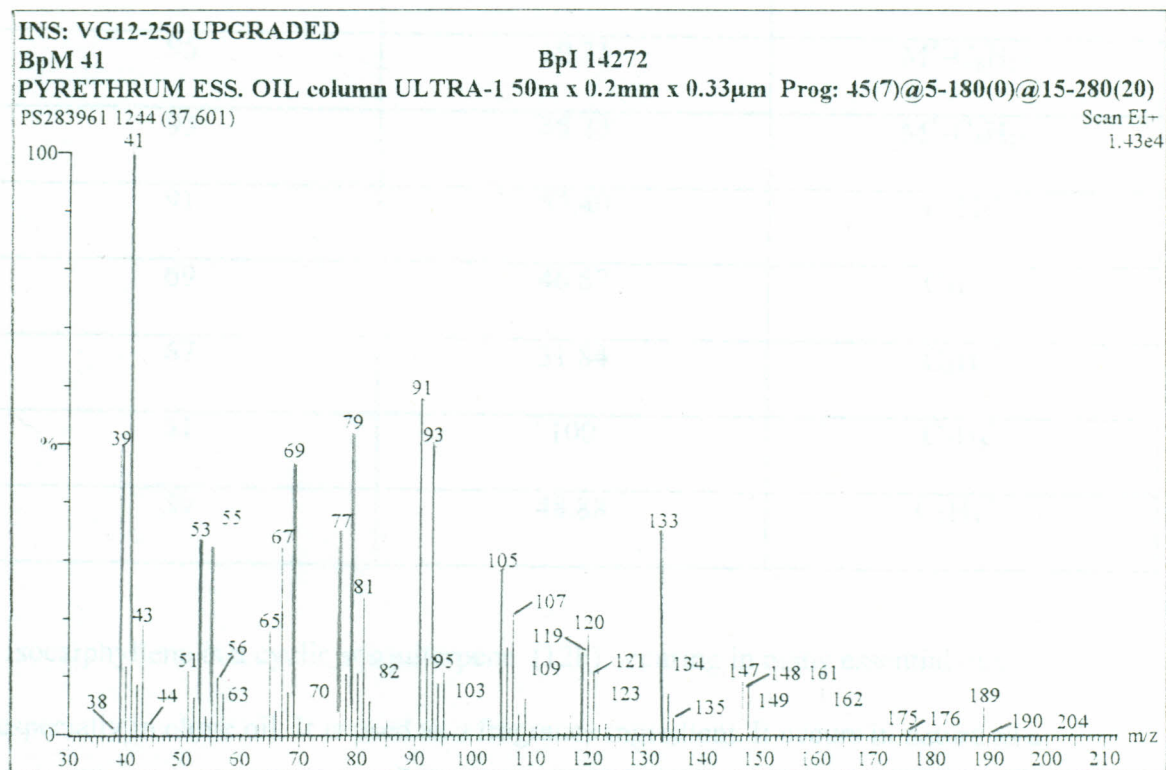
The structure of Germacrene -D (11b) shows it to be a cyclic sesquiterpene.



Germacrene D

The mass spectrum (figure 12a) displayed a molecular ion peak at m/e 204.

Figure 12a: Mass spectrum of Isocaryophyllene.

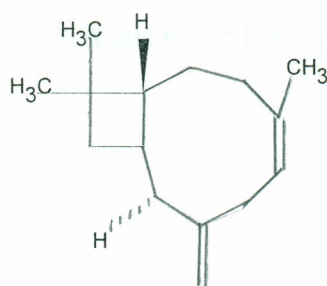


The table 6 shows that the base peak appeared at m/e 41 which corresponds to the loss of two hydrogen atoms from the isopropyl group. This is a characteristic peak of the cyclic alkanes.

Table 6: Characteristic peak intensities and peak ions of Isocaryophyllene.

Retention Time (mins)	Peak Intensity	Peak Ions
161	8.41	$M^+ - C_3H_7$
133	34.53	$M^+ - C_5H_{11}$
95	10.31	$M^+ - C_8H_{13}$
93	49.33	$M^+ - C_8H_{15}$
91	57.40	$C_7H_7^+$
69	46.67	$C_5H_9^+$
67	31.84	$C_5H_7^+$
41	100	$C_3H_5^+$
39	48.88	$C_3H_3^+$

Isocaryophyllene is a cyclic sesquiterpene (12b) occurring in many essential oils especially in clove oil. It is used as a fragrance ingredient. It occurs in nature as a mixture with caryophyllene (Merck Index).



Isocaryophyllene

(12b)

The mass spectrum of *trans*- β -Farnesene (figure 13a) displayed a molecular ion peak at m/e 204.

Figure 13a: Mass spectrum of *trans*- β -Farnesene.

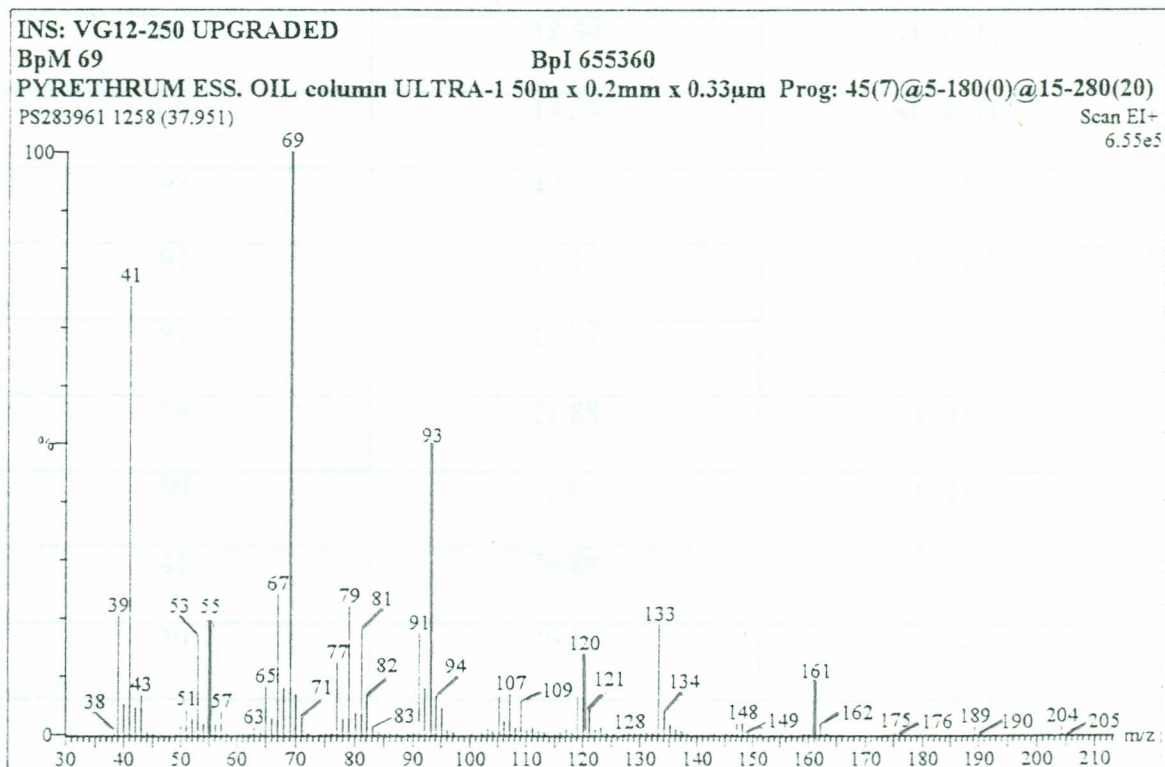
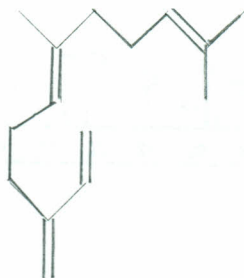


Table 7 shows the base peak appeared at m/e 69, arising from the loss of the isopentyl end group by allylic cleavage which is due to the loss of isopentyl end group by allylic cleavage.

Table 7: Characteristic peak intensities and peak ions of *trans*- β -Farnesene.

Retention Time (mins)	Peak Intensity	Peak Ions
204	1.08	M^+
161	8.79	$M^+ - C_3H_7$
133	18.59	$M^+ - C_6H_{11}$
120	13.59	$M^+ - C_7H_{12}$
93	49.38	$C_7H_9^+$
91	17.19	$C_7H_7^+$
81	17.97	C_6H_6
79	21.88	$C_6H_7^+$
69	100	$C_5H_9^+$
41	76.88	$C_3H_5^+$
39	20.16	$C_3H_3^+$

Its structure (13b) shows it to be a sesquiterpene hydrocarbon.



trans- β -Farnesene

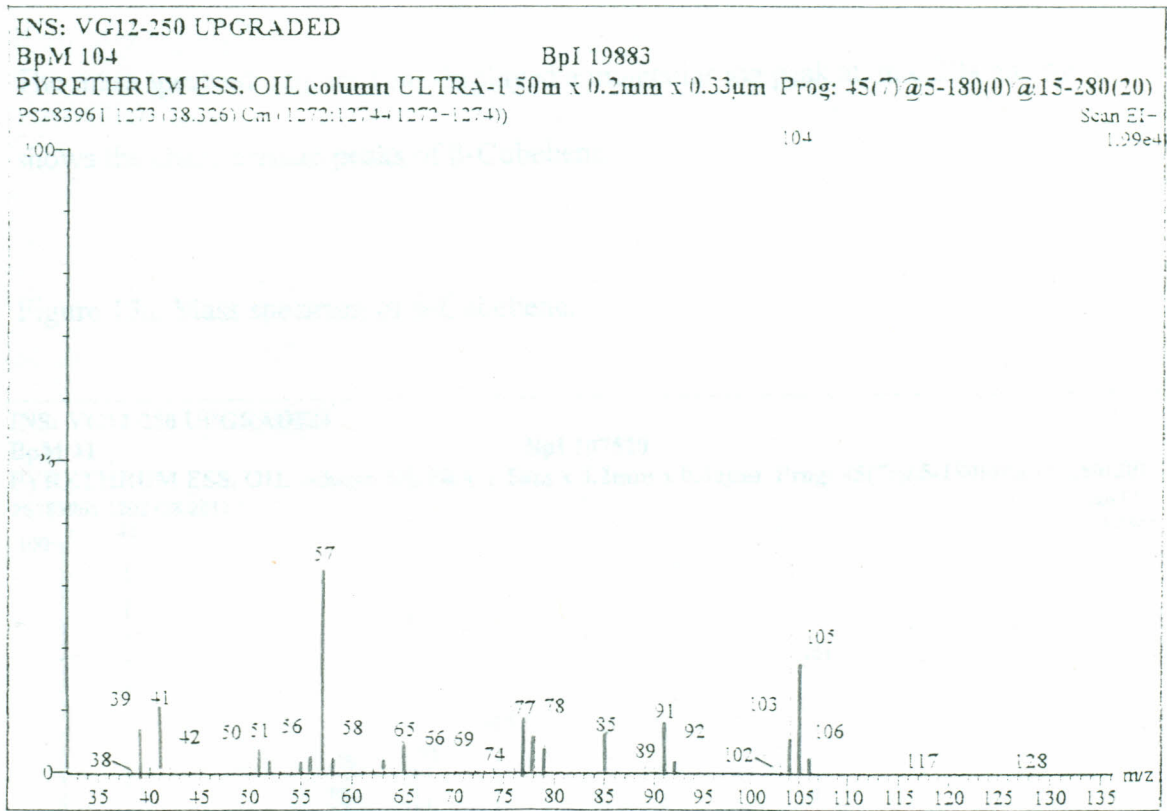
(13b)

trans- β -Farnesene is one of the hydrocarbons obtained by the action of dehydrating agents on Nerolidiol. It is a constituent of the essential oils of hop and camomile. It is

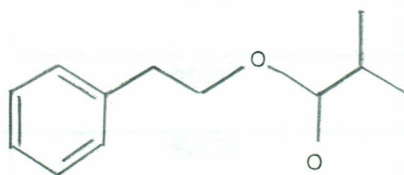
used as an alarm pheromone of aphids. *trans*- β -Farnesene has been reported to occur in male mouse urine. Major constituent of the oil of *Chrysanthemum cinerariaefolium* Vis. (Saggar et al.,1997).

The mass spectrum (figure 14a) displayed a molecular ion at m/e 106 the base peak appeared at m/e 104 which corresponds to β -phenylethylisovalerate.

Figure 14a: Mass spectrum of β -phenylethylisovalerate.



The structure (14b) shows it to be a typical phenyl ester.



Phenyl ethyl isovalerate

(14b)

The mass spectrum (figure 15a) displayed a molecular ion peak at m/e 204 and table 8 shows the characteristic peaks of β -Cubebene.

Figure 15a: Mass spectrum of β -Cubebene.

INS: VG12-250 UPGRADED

BpM 41

BpI 107520

PYRETHRUM ESS. OIL column ULTRA-1 50m x 0.2mm x 0.33 μ m Prog: 45(7)@5-180(0)@15-280(20)

PS283961 1292 (38.801)

Scan EI-
1.08e5

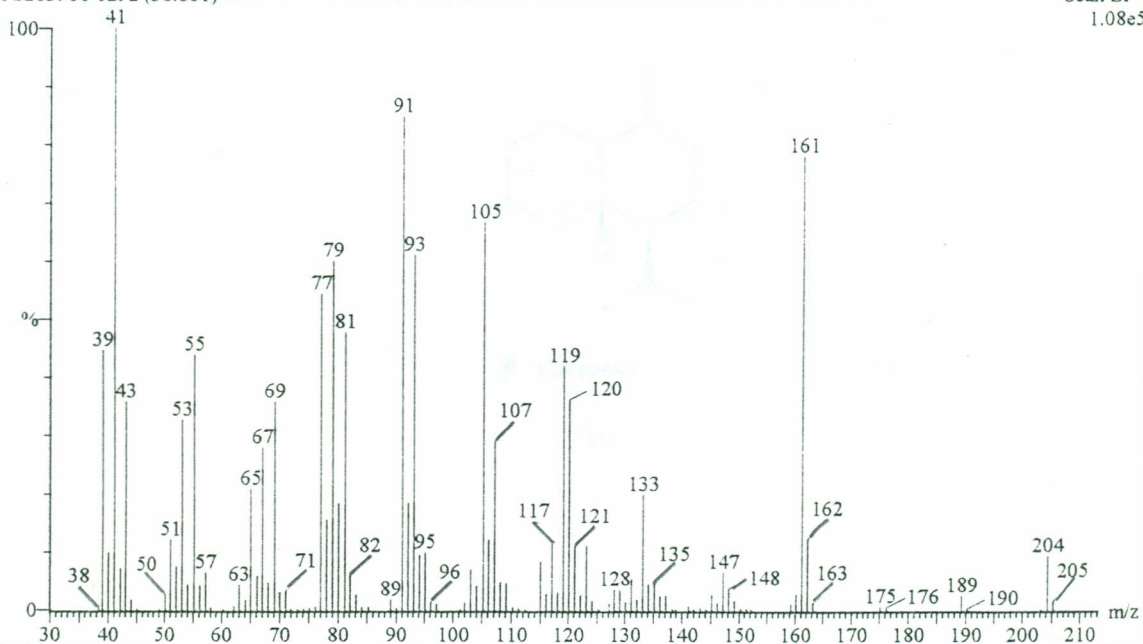
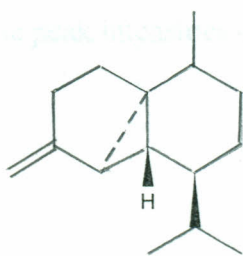


Table 8: Characteristic peak intensities and peak ions of β -Cubebene.

Retention Time (mins)	Peak Intensity	Peak Ions
204	9.92	M^+
105	66.67	$C_8H_9^+$
91	84.76	$C_7H_7^+$
79	60	$C_6H_7^+$
69	35.71	$C_5H_9^+$
55	43.81	$C_4H_7^+$
41	100	$C_3H_5^+$
39	44.52	$C_3H_3^+$

It is a constituent of the oil of cubeb. Major constituent of the oil of *Chrysanthemum cinerariifolium* Vis (Saggar et al. 1997).

The figure (15b) shows it to be a typical sesquiterpene hydrocarbon.

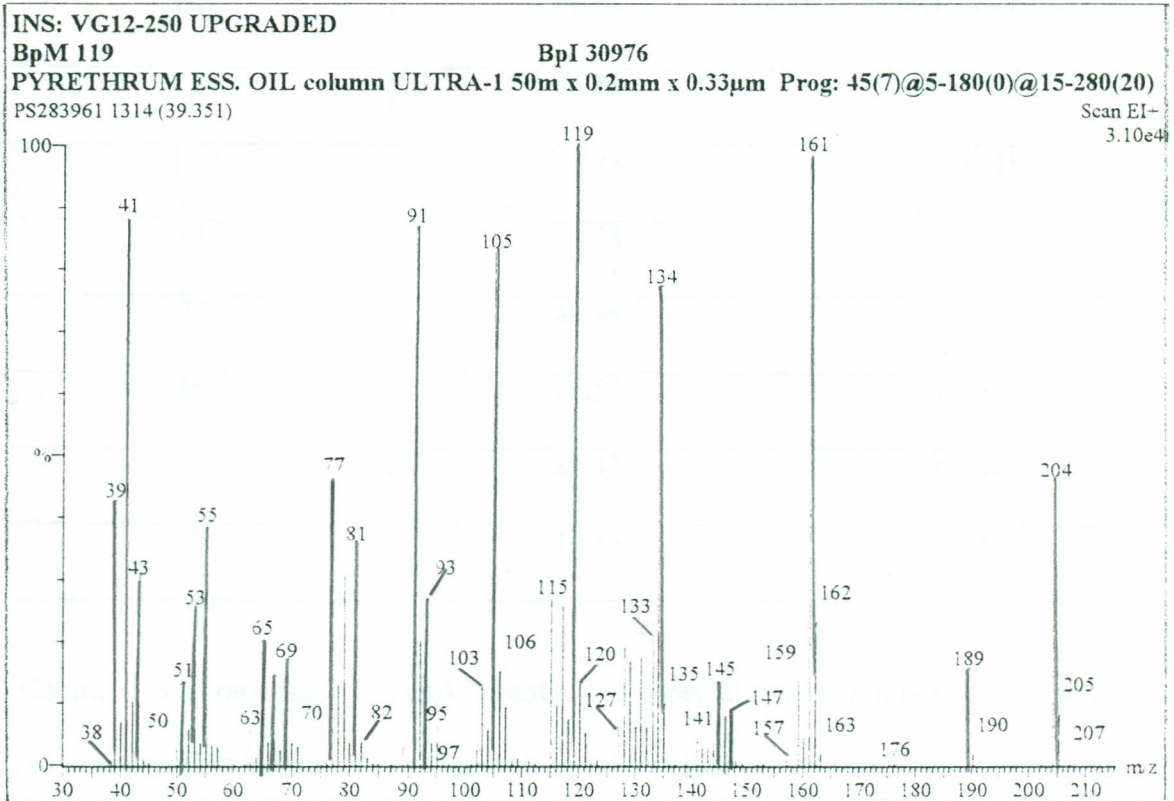


β -Cubebene

(15b)

The mass spectrum of Cadinene (figure 16a) displayed a molecular ion peak at m/e 204.

Figure 16a: Mass spectrum of Cadinene.



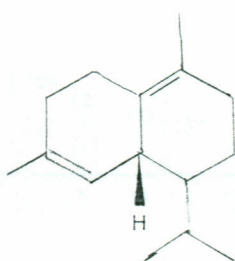
The table 9 shows the characteristic peak intensities of Cadinene.

Table 9: Characteristic peak intensities and peak ions of Cadinene.

Retention Time (mins)	Peak Intensity	Peak Ions
204	45.66	M^+
189	98.35	$M^+ - CH_3$
161	98.35	$M^+ - C_3H_7$
134	76.86	$M^+ - C_5H_{10}$
119	100.00	$M^+ - C_6H_{14}$
91	86.78	$C_7H_7^-$
77	46.49	$C_6H_5^-$
69	16.53	$C_5H_9^-$
41	88.43	$C_3H_6^-$
39	42.36	$C_3H_3^-$

Cadinene is a constituent of many essential oils especially from *Juniper* species and cedar. It was first isolated from ylang-ylang oil. Orchids of the genus *Ophrys* produce scents tht are rich in the sesquiterpene hydrocarbons of the cadinene series. Its *cis* sesquiterpene is called muurolene (Merck Index).

Its structure 16b shows it to be a bicyclic sesquiterpene.

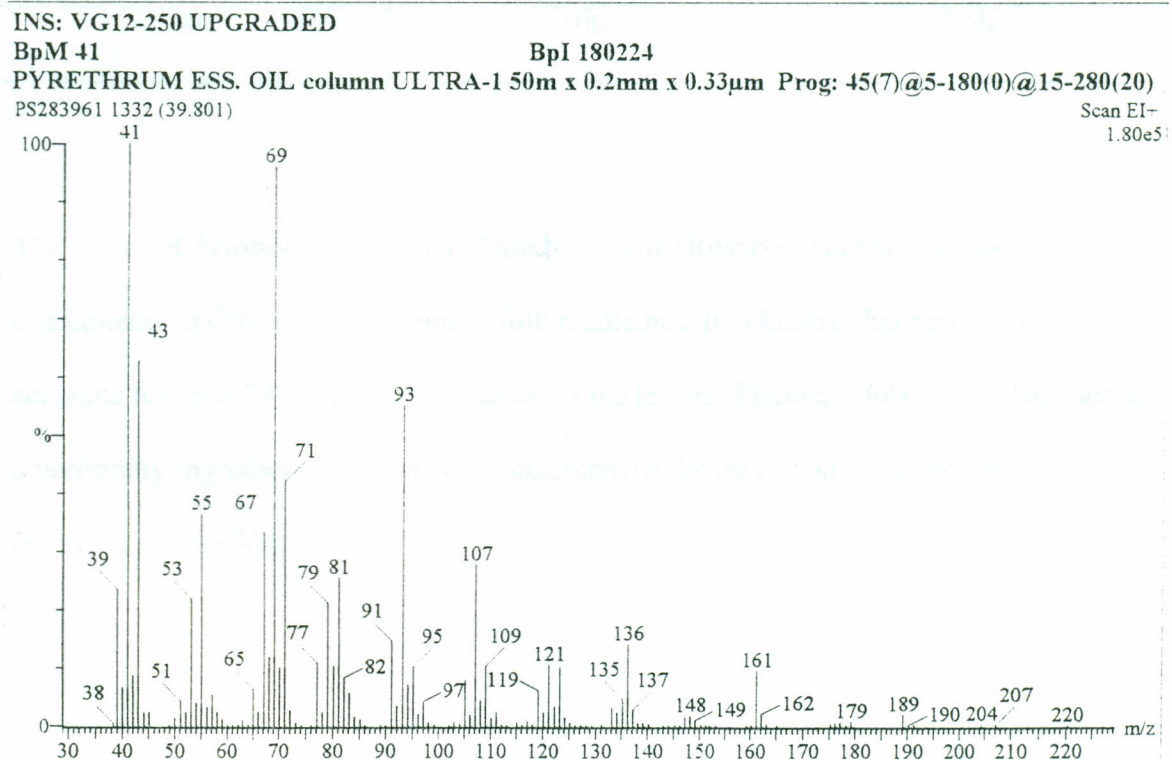


Cadinene

(16b)

The mass spectrum of Nerolidiol (figure 17a) displayed a molecular ion peak at m/e 220. Spectra of Nerolidiol shows that the main peaks belong to one or the other of the two groups, one containing the hydroxyl function and the other containing the hydrocarbon skeleton only. Peaks m/z 207 and 179 correspond to the fragment containing the hydroxyl function whilst peaks m/z 189 and 161 belong to the hydrocarbon skeleton. The 1,1-dimethyl-propenyl moiety is lost readily by allylic cleavage resulting in the m/e 69. Co-injection with an authentic of δ -Nerolidiol showed peak enhancement.

Figure 17a: Mass spectrum of Nerolidiol.



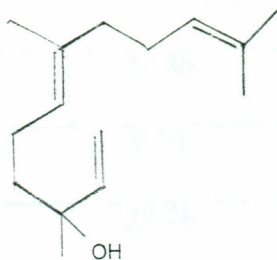
The table below shows the characteristic peak intensities.

Table 10: Characteristic peak intensities and peak ions of δ -Nerolidiol.

Retention Time (mins)	Peak Intensity	Peak Ions
205	0.19	$M^+ - 15$
197	2.54	$M^+ - 18 + 15$
177	1.31	$M^+ - 43$
159	12.09	$M^+ - C_3H_9O$
69	81.35	$C_5H_9^+$
55	31.97	$C_4H_7^+$
43	54.10	$C_3H_7^+$
41	100	$C_3H_5^+$

The leaves of *Eriobotrya japonica* (Thunb.) Lindl (Rosaceae) are well known components in Chinese and Japanese folk medicines. It is known that nerolidiol accounts for upto 74% of the essential oil of the leaves (Pfander, 1988). It is also used as a perfumery ingredient. It is a major constituent of the oil of *Chrysanthemum cinerariaefolium* Vis.

The structure (17b) shows Nerolidiol to be a sesquiterpene alcohol.



Nerolidol

(17b)

The mass spectrum of (-)- Spathulenol (figure 18a) displayed a molecular ion peak at m/e 220.

Figure 18a: Mass spectrum of (-)-Spathulenol.

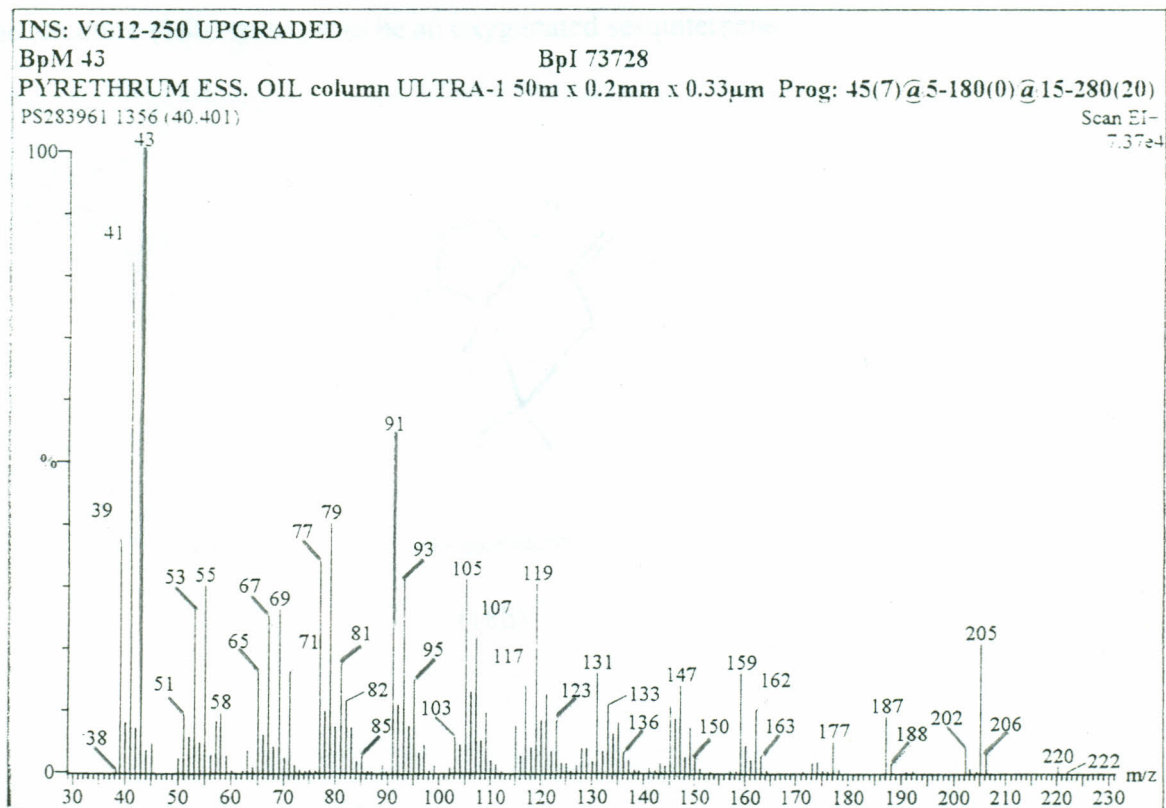
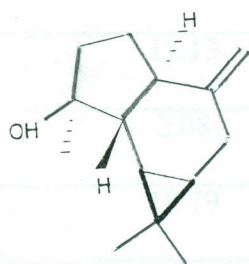


Table 11: Characteristic peak intensities and peak ions of (-)-Spathulenol.

Retention Time (mins)	Peak Intensity	Peak Ions
220	20.86	M^+
202	9.79	$M^+ - H_2O$
189	29.98	$C_7H_9^+$
91	57.33	$C_7H_7^+$
77	32.22	$C_6H_5^+$
69	26.99	$C_5H_9^+$
67	23.13	$C_5H_7^+$
43	100	$C_3H_7^+$
41	77.11	$C_3H_5^+$
39	36.44	$C_3H_3^+$

The structure (18b) shows it to be an oxygenated sesquiterpene.

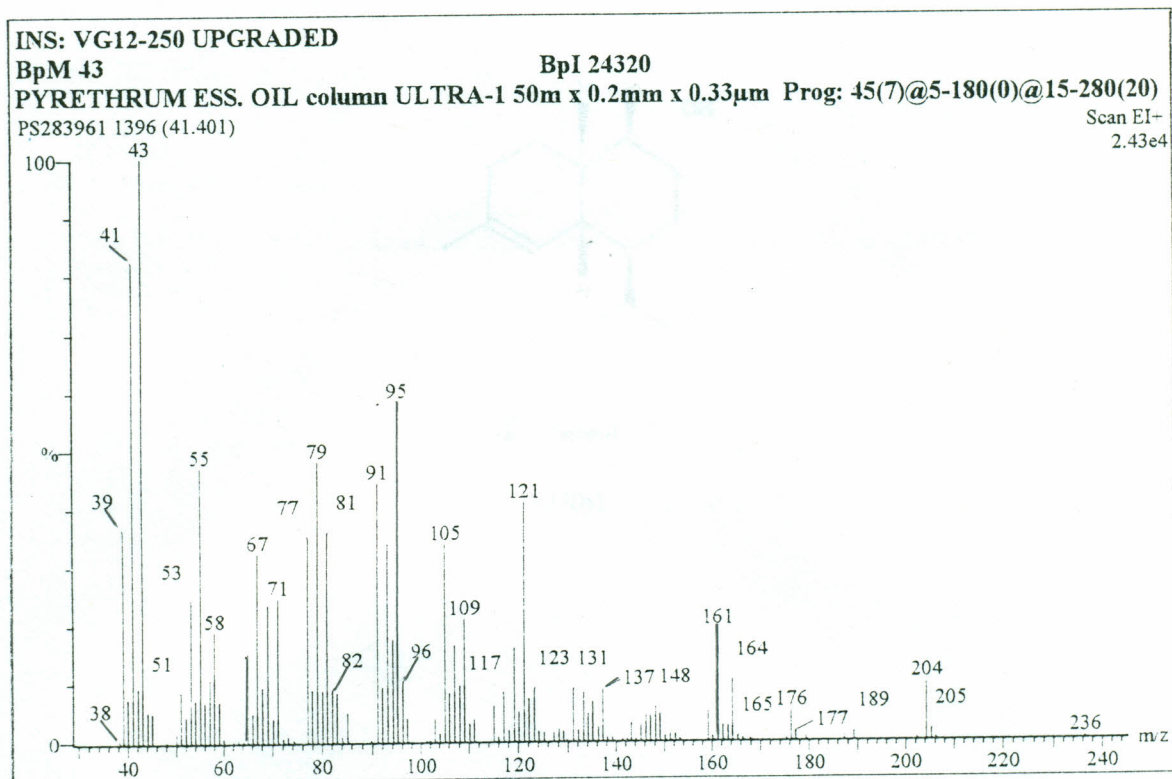


(-)- Spathulenol

(18b)

The mass spectrum of δ -Cadinol (figure 19a) shows a molecular ion peak at m/e 204.

Figure 19a: Mass spectrum of δ -Cadinol

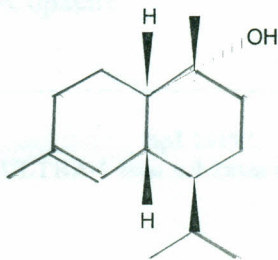


The table below shows the characteristic peaks of δ -Cadinol.

Table 12: Characteristic peak intensities and peak ions of δ -Cadinol.

Retention Time (mins)	Peak Intensity	Peak ions
204	13.13	M^+
189	2.08	$M^+ - 15$
161	19.79	$M^+ - 43$
91	27.36	$C_7H_7^+$
77	21.83	$C_6H_5^+$
43	100.00	$C_3H_7^+$
41	45.28	$C_3H_5^+$
39	17.25	$C_3H_3^+$

The structure 19b shows δ -Cadinol to be an oxygenated sesquiterpene.

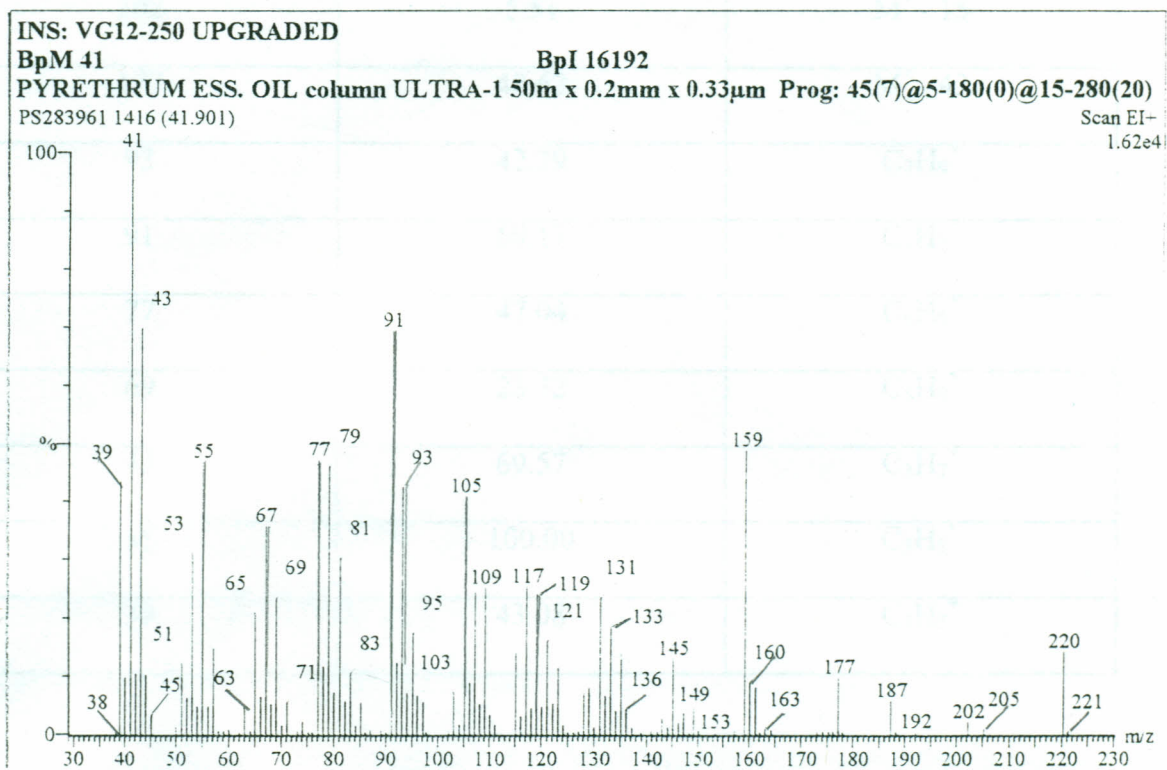


δ -Cadinol

(19b)

The mass spectrum of α -Copaene (figure 20a) displayed a molecular ion peak at m/e 220 and the table 13 shows the characteristic peaks.

Figure 20a: Mass spectrum of α -Copaene.



The structure of α -Copaene is shown to be a bicyclic sesquiterpene



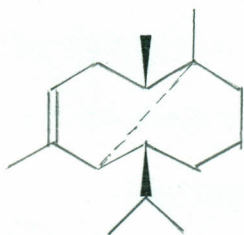
α -Copaene

(2-1)

Table 13: Characteristic peak intensities and peak ions of α -Copaene

Retention Time (mins)	Peak Intensity	Peak
220	1.90	M^+
202	5.51	$M^+ - 15$
177	48.62	$M^+ - 43$
93	42.29	$C_7H_9^+$
91	69.17	$C_7H_7^+$
77	47.04	$C_6H_5^+$
69	23.32	$C_5H_9^+$
43	69.57	$C_3H_7^+$
41	100.00	$C_3H_5^+$
39	43.08	$C_3H_3^+$

The structure (20b) shows α -Copaene to be a tricyclic sesquiterpene

 α -Copaene

(20b)

Isocaryophyllene, δ -Nerolidiol, β -Cubebene, α -Copaene were identified using GC-MS, retention time indices and co-injection. Isocaryophyllene, δ -Nerolidiol and α -Copaene were bioassayed, using the Y-tube olfactometer, against the *S. zeamais*. Germacrene-D, trans- β -Farnesene, Cadinene, (-)-Spathulenol were identified using the GC-MS and retention time indices.

trans-Chrysanthemic acid, β -phenylethylisovalerate and δ -Cadinol were identified by GC-MS.

Mixture of the essential oil and Pyrethrins

4.3 *Sitophilus zeamais* Activity against the Compounds Tested

Table 14 below gives the repellent activity of *Chrysanthemum cinerarefolium*

Vis.essential oil, pyrethrin, DEET and a mixture of the essential oil and pyrethrins

against the *S. zeamais* as determined by the Y-tube olfactometer as mentioned in section

3.4..

1.1 times more active than pyrethrins. The essential oil was 1.61 times more active

than pyrethrins. The mixture of the pyrethrins and the essential oil at this

concentration was 1.15 times more active than DEET due to the essential oil. At this

concentration, the mixture of the essential oil and pyrethrins was 1.15 times more active

than DEET.

At this concentration, the essential oil was 1.15 times more active than DEET.

At this concentration, the essential oil again exhibited the highest percentage

repellent activity. The essential oil was 1.2 times more repellent than DEET, and 1.61

times more repellent than pyrethrins. At this concentration, the mixture of the essential

Table 14: Mean Percentage Repellency Activities \pm Standard error of the Essential oil, Pyrethrin, DEET and a mixture of the Essential Oil and Pyrethrin against the *Sitophilus zeamais*.

Extract	DOSE μ l/disc				
	1.0	0.1	0.01	0.001	0.0001
Essential oil of pyrethrum	77.72 \pm 4.0a	59.60 \pm 1.6a	54.85 \pm 1.3a	50.31 \pm 1.5 a	43.12 \pm 2.0a
DEET	54.13 \pm 1.5b	49.86 \pm 1.6 bc	34.25 \pm 1.3b	24.89 \pm 2.1 b	22.12 \pm 1.1b
Pyrethrins	48.15 \pm 1.5 b	35.93 \pm 2.2c	23.59 \pm 1.9b	10.16 \pm 2.2c	-----
Mixture of the essential oil and Pyrethrins	56.65 \pm 3.5 b	32.80 \pm 3.0 ab	22.52 \pm 1.8b	17.18 \pm 2.2 b	-----

Mean values with the same letters within the same dose level are not significantly different at the 5% level. (Duncan's test on transformed mean values).

At a concentration of 1 μ l/ disc the essential oil of pyrethrum exhibited a percentage of 77.72 which was the highest of all the extracts tested. At this concentration the essential oil was 1.44 times more active than the DEET. At the same concentration DEET was 1.1 times more active than the pyrethrins. The essential oil was 1.61 times more active than pyrethrins. However, the mixture of the pyrethrins and the essential oil at this concentration exhibited enhanced repellency due to the essential oil. At this concentration, the mixture of the essential oil and pyrethrins was 1.05 times more active than DEET.

At a concentration of 0.1 μ l/ disc the essential oil again exhibited the highest percentage repellency of 59.60. The essential oil was 1.2 times more repellent than DEET, and 1.66 times more active than pyrethrins. At this concentration, the mixture of the essential oil

and pyrethrins exhibited a lower repellency than DEET

At a concentration of 0.01 $\mu\text{l}/\text{disc}$ the essential oil of pyrethrum exhibited a percentage repellency of 54.85. DEET at this concentration exhibited a much lower concentration than the oil. The essential oil of pyrethrum was 1.6 times more active than DEET, whilst DEET was 1.45 and 1.52 more active than pyrethrin and the mixture of the essential oil and pyrethrins respectively.

At a concentration of 0.001 $\mu\text{l}/\text{disc}$ the essential oil exhibited a percentage repellency of 50.31 which was the highest for all the compounds tested at this concentration. The essential oil was 2.02 times more active than DEET. At this concentration the pyrethrins and the mixture of the essential oil and pyrethrins exhibited very weak repellency.

At a concentration of 0.0001 $\mu\text{l}/\text{disc}$ the essential oil exhibited a percentage repellency of 43.12. The oil was 1.95 times more active than DEET. At this concentration the pyrethrins and the mixture exhibited no repellency.

The oil exhibited the highest repellency at all doses against all the compounds tested.

The repellency exhibited by the oil was fairly consistent at doses 0.1, 0.01, 0.001, 0.0001 $\mu\text{l}/\text{disc}$. This could be attributed to the fact that the oil consisted mainly of sesquiterpenoids. Sesquiterpenoids are known to subtly influence odour and this could have deterred the *S. zeamais* (Hay and Waterman, 1993).

Pyrethrins exhibited a lower repellency than DEET and the essential oil of pyrethrum. Pyrethrins consist mainly of esters formed by the combination of two acids, chrysanthemic acid and pyrethric acid and three alcohols, pyrethrolone, cinerolone and jasmololone. These esters account for the kill and knockdown properties of the pyrethrium extract (Casida, 1973). However, the repellency exhibited by the pyrethrins was lower than that of the essential oil of pyrethrum. This was due to the fact that repellency is an olfactometric response and the oil having a higher concentration of sesquiterpenoids than pyrethrins had a stronger odour than the pyrethrins thus exhibiting a higher repellency.

4.4 Repellency Bioactivity of Some of the Constituents of the Essential Oil

Some of the compounds that were identified were subjected to repellent bioassay tests against the *S. zeamais*. This was done using the Y-tube olfactometer as mentioned in section 3.4. The repellent activity of the individual compounds and the standard DEET against *S.zeamais* is given in table 15.

Due to the unavailability of authentic samples, only three constituents could be bioassayed. β - Cubebene was confirmed by co-injection but could not be bioassayed because the authentic sample was contaminated .

Table 15 : Mean percentage repellencies of some of the constituents in the essential oil *Chrysanthemum cinerariaefolium* Vis.

Sample	Dose $\mu\text{l}/\text{disc}$				
	1.0	0.1	0.01	0.001	0.0001
Essential oil of pyrethrum	77.72 \pm 4.0a	59.60 \pm 1.6a	54.85 \pm 1.3a	50.31 \pm 1.5 a	43.12 \pm 2.0a
Nerolidiol	67.50 \pm 4.6b	37.41 \pm 2.1d	-----	-----	-----
α -Copaene	44.21 \pm 4.0b	32.35 \pm 1.5d	30.09 \pm 1.3b	24.08 \pm 2.8c	19.76 \pm 2.5b
Isocaryophyllene	-----	42.77 \pm 5.7c	36.96 \pm 1.8b	26.62 \pm 2.2c	12.02 \pm 1.5bc
DEET	54.13 \pm 1.5b	49.86 \pm 1.6 bc	34.25 \pm 1.3b	24.89 \pm 2.1 b	22.12 \pm 1.1b

Mean values with the same letters within the same dose level are not significantly different at the 5% level. (Duncan's test on transformed mean values).

At a concentration of 1 $\mu\text{l}/\text{disc}$ the essential oil of pyrethrum was 1.15 times more active than Nerolidiol and 1.76 times more active than α -Copaene. Isocaryophyllene could not be bioassayed at this concentration because of the unavailability of authentic sample at this concentration. At this concentration DEET was 1.22 times more active than α -Copaene. However, at this concentration Nerolidiol was 1.25 times more active than DEET.

At a concentration of 0.1 $\mu\text{l}/\text{disc}$ the essential oil of pyrethrum was 1.59 times more active than Nerolidiol and 1.84 and 1.39 times more active than α -Copaene and Isocaryophyllene respectively. At this concentration, DEET was 1.33 times more active than Nerolidiol and 1.54 and 1.17 more active than Copaene and Isocaryophyllene respectively.

At a concentration of 0.01 $\mu\text{l}/\text{disc}$, the essential oil of pyrethrum was 1.82 and 1.48 times more active than α -Copaene and Isocaryophyllene respectively. Nerolidiol could not be bioassayed at concentrations of 0.01, 0.001 and 0.0001 $\mu\text{l}/\text{disc}$ because of the unavailability of enough authentic sample. Isocaryophyllene was 1.08 times more active than DEET.

At a concentration of 0.001 $\mu\text{l}/\text{disc}$, the essential oil of pyrethrum was 2.09 and 1.89 times more active than α -Copaene and Isocaryophyllene respectively. Isocaryophyllene was 1.07 times more active than DEET while DEET was 1.03 times more active than α -Copaene.

At a concentration of 0.0001 $\mu\text{l}/\text{disc}$, the essential oil of pyrethrum was 2.18 and 3.59 times more active than α -Copaene and Isocaryophyllene respectively while DEET was 1.12 and 1.84 times more active than α -Copaene and Isocaryophyllene, respectively.

4.5 Conclusion

One of the basic requirements of the Montreal Protocol is that ozone depleting substances shall be phased out once alternatives to replace the substance are available. Anxious to phase out methyl bromide because of its ozone depleting potential, several presentations have been made indicating that a large number of essential oils have the potential of replacing methyl bromide as a grain fumigant.

Chrysanthemum cinerariaefolium Vis. (Pyrethrum) is a well known natural product insecticide whose active principles are commercially available for the control of insect

pest. Pyrethrum is a strong knock down agent, kill agent, has a good flushing out effect and is an acknowledged repellent against a number of insect pests. Unlike many pesticides, pyrethrum is bio-degradable in the environment and animal tissues, thereby making it non-persistent and relatively safe to warm blooded animals. It is also free from potentialities to cause the development of resistance to many insects. All these facts taken together make pyrethrum a potential alternative to methyl bromide in stored grain fumigation.

Repellency factor in pyrethrum flowers has received little attention. It has been amply demonstrated in the case of stored products insects for example stored grain can be kept free of insects pests for up to 11 months (Glynne Jones, 1966). This research was undertaken to establish the nature and identity of this repellent component.

The repellency of the essential oil of *Chrysanthemum cinerariifolium* Vis. was established first. This was done using the Y-tube olfactometer (Saggar et al. 1996). Since the results obtained showed that the oil exhibited a higher repellency than the commercial repellent, DEET, GC-MS analysis were carried out to determine the chemical constituents of the oil.

The chemical analysis of the oil showed that it consisted mainly of sesquiterpenes namely, *trans*-chrysanthemic acid, germacrene-D, isocaryophyllene, *trans*- β -farnesene, β -phenylethylisovalerate, β -cubebene, cadinene, δ -nerolidiol, (-)-spathulenol, δ -cadinol and α -copaene. The analysis of the oil showed that *trans*- β -farnesene, β -cubebene and δ -nerolidiol were the major constituents of the oil. *trans*- β -Farnesene and β -cubebene could not be bioassayed because of the unavailability of the authentic samples thus

further studies would be justified to establish the constituent most responsible for the repellency in the oil. Supercritical fluid extraction is one technique that would be advantageous because this technique uses carbon dioxide which would prevent any reaction with components.

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