ANALYSIS OF CHEMICAL COMPOSITION OF COWPEA FLORAL VOLATILES AND NECTAR

BY

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DECLARATION

I hereby declare that this is my own original work and it has not been presented for award of a degree in any other university.

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To my husband Godfrey Isaac Ochieng' and my children Mercy, Humphrey and Jeffrey

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LIST OF ABBREVIATIONS

A^0	Armstrong
Amu	Atomic mass units
AFLP	Amplified fragment length polymorphism
ATP	Adenosine triphosphate
APRT	Anthranilate phosphribosyl transferase
BAMT	Benzoic acid carboxyl methyl trasferase
BIR	Biogenic isoprene rule
COMT	Caffeic acid-*O*-methyl transferase
CL	Confidence limit
CM	Chorismate Mutase
CH_2Cl_2	Dichloromethane
CPC	Cyclopropyl carbonyl
CPP	Chrysanthemyl pyrophosphate
CS	Chorismate Synthase
DAHP	3-deoxy-D-arabino-hept-2-ulosonate-7-phosphate
DCM	Dichloromethane
DHSA	Dehydroshikimic acid
DHQA	Dehydroquinic acid
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxy ribonucleic acid
EAG	Electroantenography
EFN	Extra floral nectar
EPSP	5-Enol pyruvate-3-shikimate-3-phosphate
FAS	Fatty acid synthase

Fruc	Fructose
F_1	Filial one generation
FPP	Farnesyl pyrophosphate
Gluc	Glucose
GC	Gas chromatography
GHS	Glycosilated hydroxypulegone schezonepetoside
GPP	Geranyl pyrophosphate
GC-MS	Gas chromatography-mass spectrometry
GLV	Green leaf volatiles
HP	Hewlett Packard
HPLC	High performance liquid chromatography
IGPS	Indole-3-glycerol phosphate synthase
IPM CRSP Project	Integrated Pest Management Collaborative Research Support
IPP	Isopentyl pyrophosphate
IEMT	Isoeugenol-*O*-methyl transferase
LS	Linalool synthase
LC-MS	Liquid chromatography-mass spectrometry
LPP	Linalyl pyrophosphate
MS	Mass spectroscopy
MVA	Mevalonic acid
NADPH	Nicotinamide adenine dinucleotide pyrophosphate
NIR	Near Infrared
NPP	Neryl pyrophosphate
PBAI	Phosphribosyl anthranilate isomerase

PEP	Phosphoenolpyruvate
pH	Hydrogen ion concentration
RAPD	Random amplified polymorphism DNA
R_t	Retention time
SMM	*S*-methylmethionine
Suc	Sucrose
SAM	*S*-adenosyl-*L*-methionine
SAMT	Salicylic acid carboxylmethyl trasferase
SSR	Simple Sequence Repeats
SPME	Solid phase micro extraction
TTC	Teachers Training College
UV	Ultra violet
μ	Micro

ABSTRACT

Cowpea is one of the 14 species of grain legumes. It rewards insects by producing nectar and it advertises to the pollinators by producing floral volatiles. The volatiles act as cues that guide insect pollinators in terms of pollen and nectar. Floral volatile quality also influences the efficiency of pollination. Genetic manipulations involving selection of varieties with high quality floral volatiles and nectar can therefore increase pollination efficiency and hence cowpea yields. It is believed that efficiency of insect pollination in several food crops is dependent on the quality of floral volatiles. Several molecules, including allozyme, co-dominant and isozyme DNA molecular markers (AFLP, RAPD and SSR) among others are useful in the selection of disease resistant and high yielding food crop cultivars in breeding programmes. Floral volatiles and nectar profiles can therefore act as molecular markers in cowpea breeding programmes. This project involved the collection, analysis and characterization of cowpea floral nectar and volatile composition. The flowers bloomed for one day and nectar was secreted between 6.00 and 10.00 am East African time. The flower sizes in the six selected cultivars were measured, nectar withdrawn using microlitre syringe and its characteristics (volume and sugar composition) examined. The sugar composition of the nectar was analyzed using HPLC, LC-MS and co-injection with authentic standards. Hydro-distillation and static headspace trapping with adsorbents (activated charcoal, reverse-phase, C_{18} bonded silica and porapak Q) was done in the six selected cultivars and volatiles concentrated using gentle stream of N_2 while cooling under ice. GC was used to analyze the composition of floral volatiles and GC-MS for identification of the components. Co-injection with authentic standards was used to confirm identity of the components. Nectar volume varied as a function of time. A correlation between nectar production and time was observed. There were significant quantitative differences in the volumes of nectar produced in the different cowpea cultivars. The highest volume collected (18 µl) was from cultivar 219, with a mean of 7.99 ± 0.78 µl and the lowest recorded value (0.2 µl) was from SP46 with a mean of 3.65 ± 0.59 µl. The cultivars showed similar trends in the rate of reduction in the volumes of nectar produced with time. Sucrose (0.104 ± 0.099 mg), glucose (0.0224 ± 0.006 mg), and fructose (0.0225 ± 0.012 mg) occured frequently in the nectars. Lactose (0.003 ± 0.001 mg), raffinose (0.004 ± 0.002 mg), and mannose (0.006 ± 0.004 mg) were present in trace amounts. The biggest flower size recorded (47 mm) was from cultivar 219 with a mean of 42.62 ± 0.65 mm while the smallest size (10 mm) was from SP46, with a mean of 17.13 ± 0.65 mm. The nectar volume is directly proportional to the flower size. The total number of trapped volatile compounds ranged from 43-109 for headspace trapping and hydrodistillation. Porapak Q trapped the largest number of compounds. Quantitative and qualitative differences in volatile composition of various cultivars were noted. Aliphatic compounds were the most abundant followed by benzenoids, monoterpenes, sesquiterpenes, norisoprenoids and other compounds. The most common cowpea floral volatiles were toluene, 1-hexanol, benzaldehyde, acetophenone, limonene, 1-octen-3-ol, artemisia alcohol and nerolidol. Palmitic acid was the most abundant component of steam distillates. Due to the high protein content, grain legumes should help in reducing protein deficiency cases in developing countries. Improved legume yields should be encouraged to address protein deficiency in children. There is need to intensify production of cowpea by developing more efficient and well adapted varieties with good pest and disease resistance through biotechnology.

CHAPTER ONE
INTRODUCTION

1.1 Background

A legume is any plant belonging to the pulse family (Leguminosae) that produces a dry dehiscent fruit in the form of a pod including many important vegetable crops such as pea, bean, lentils, peanut and soybeans and cowpea. The family Leguminosae has approximately 650 genera and 18,000 species (Rachie *et al.*, 1979). It is the third largest family after Compositae and Orchidaceae. They are found in temperate zones, humid tropics, highlands, savannah lowlands and aquatic environments. The sub-families are Caesalpinioideae, Mimosoidea and Papilionoideae (Fabaceae). Most widely used leguminous plants are peanuts, soybeans, peas, lentils, pigeon peas, chickpeas, mung beans, kidney beans, cowpeas, alfalfa (lucerne), sweet cloves (*Melilotus* spp) and other cloves (*Trifolium* spp). Legumes are the only flowering plants that posses root nodules containing bacteria that can take up atmospheric nitrogen and convert it to other nitrogenous compounds that can be used by the plant thereby improving soil fertility. This unique character makes legumes important in soil fertility management by crop rotation (Herrero & Flores, 2008).

Cowpea, *Vigna unguiculata*, is a herbaceous annual plant. Other common names frequently encountered in the literature are southern pea and black eye pea (US), beans in Anglophone Africa, *niebe* in Francophone Africa, lubia, lobid, coupe, frijole, asparagus beans, yard long beans and sitao. The last three names generally, refer to *V. u. sesquipedalis.* It grows by epigeal germination. Cowpea belongs to the sub-family

Papilionoideae within the family *Leguminosae*, the tribe *Phaseolea*, sub-tribe *Phaseolinae* and section *catiang*. The species include cultivated and wild annual forms such as V. *unguiculata var. spontanea* plus ten wild perennial subspecies (Pasquet, 1993a; 1993b; 1997; 1999). *V. u. unguiculata* and *V. u. spontanea* are the likely progenitors of the cultivated cowpea (Pasquet, 1999). The wild ancestors of cultivated cowpea are *V. u. mensensis, V. u. dekindtiana* and *V. u. pubescens*. Numerous cultivated traditional varieties are short day plants (longer dark periods for flowering -12 hours). The flower colors range from white to violet. The immature pods are green or varying pigmentation like pink, red, crimson or black. They have twinning and bushy stems with trifoliate leaves and long petioles (2.5-12.5 cm). The flowers are in auxillary racemes (Baudoin & Vanderborght, 2001). Cowpea is widely spread in West Africa and India (Summerfield, 1978). It was known in India during the first millennium BC (Steele & Merha, 1980) and was widespread in Asia by 2300 BC. Wild cowpea is almost only encountered in Africa. Although Asia is considered the probable center of the cultivated forms, there is enough evidence suggesting that cowpea originated in Africa (Baudoin & Vanderborght, 2001). It was domesticated in Africa in the neolithic age (Murechal *et al.*, 1978). There is still uncertainty as to where the crop was first domesticated: Ethiopia (Vavilov, 1926; Steele, 1976; Pasquet, 2000), West Africa (Murdock, 1959; Rawal, 1975; Vaillancourt & Weeden, 1992; Ng, 1995), eastern and southern Africa (Baudoin & Marechal, 1985) have all been proposed as probable centers of the original domestication and primary center of diversity for the wild forms. Primitive and semi-wild forms (natural hybrids between wild and cultivated forms as well as the escapes) came from West Africa as (Figure 1). Archeological data suggests that cowpea was grown with millet in West Africa (Rachie *et al*., 1979). A diffuse domestication in the savannah after the dispersal of cereals has also been hypothesized (Chevalier, 1974; Steele, 1976;

Garba & Pasquet, 1998). Cowpea was introduced in Europe in 300 BC where it remained a minor crop in the southern part of the continent. Other cultivars were transported directly from Africa to Latin America with slave trade. It reached the United States in the 19th century. Spanish and Portuguese explorers exported it to the new world in 17th century.

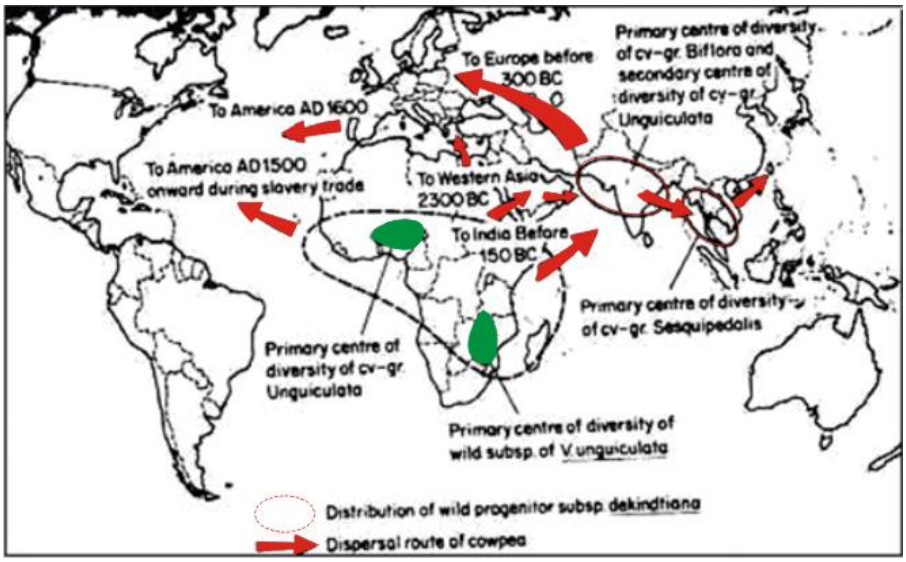


Figure 1: Global dispersal pattern and distribution of cowpea (Chevalier, 1974)

Vigna unguiculata is a diverse seed, vegetable and fodder legume of the tropics. The major economic sub-species are of West African origin and are especially important for its protein contribution. Cowpea is an ancient neolithic African crop now grown throughout the tropics and sub tropic as a pulse, vegetable, fodder and cover crop (Rajapakse & Van Emden, 1997). It is mainly important in the cereal farming systems of Africa and India where people eat the mature seeds, young leaves, pods and feed the halms to livestock (Summerfield, 1978). There are no accurate estimates but the annual world production and acreage probably exceed 3 million tons and 12.5 million hectares, respectively (Singh *et al.*, 1997). Cowpea is cultivated in 80,000 hectares of land in the USA (Fery, 1990). In Africa, the total annual production is estimated at 1.5 million tons,

with East Africa and Kenya producing 130,000 tons and 48,000 tons, respectively (Singh *et al.,* 1997). Cowpea is an important source of protein to the people of northern and eastern Uganda where 90% of the crop is grown. The crop is grown under low input subsistence farming, in mono- or mixed -cropping systems. The yield is lower (500 kg per hectare) in Uganda (Baudoin & Vanderborght, 2001).

Cowpea crop still suffers from several insect pests like the pod borer, flower bud thrips and the pod sucking bug complex since there is no good resistance against these diseases in cultivated cowpea (Ng, 1995). *V. vexillata*, the wild cowpea species, has good resistance to pod borers, pod sucking bugs and flowering thrips (Singh *et al.,* 1997), the cultivated cowpeas, *V. unguiculata,* have high levels of resistance to aphid borne mosaic virus (Ng, 1995). Many accessions of wild species; such as *V. vexillata* (L.), *V. reticulata* (L.), *V. oblongifolia* (L.) and *V. luteolia* (L.) are highly resistant to cowpea storage weevil (Ng, 1995).

Vigna leutolia (L.) has a fast growth rate and its ability to produce seeds in a relatively short time is good towards improving the food in Africa and developing world in particular (Ng, 1992). *Vigna* v*exillata* (L.) also has medicinal properties. *Vigna marina* (L.) grows along the seashores where it exhibits a tremendous resistance to salty environments (Sonnante *et al*., 1997; Padulosi & Ng, 1993; 1991). The wild cowpea and relatives have great potential and requires serious and detailed research in such areas as taxonomy, cytology, genetic variability and genetic affinity between cultivated and wild cowpea species (Ng, 1995). Many attempts are being made to transfer pest resistant traits that are present in wild species into cultivated cowpea with the aim of improving food production, enhancing food security and reducing poverty levels. Histological

studies have shown that after crossing *V. unguiculata* (L.) with *V. vexillata* (L.) the F_1 embryos started to develop but collapsed at globular stage (Barone & Ng, 1990; Ng, 1992). The cultivated form, *V. u. unguiculata*, is interfertile with its wild sub-species such as *V. u. dekindtiana* var. *dekindtiana*, *V. u. dekindtiana* var. *mensensis* and *V. u. dekindtiana* var. *pubescens* (Steele, 1976). Successful crossing of *V. luteolia* (L.) and *V. oblongifolia* (L.) resulted in hybrid plants that can be used as bridges of crosses to cowpea (Padulosi & Ng, 1991; Schnapp *et al*., 1990).

1.2 Wild and cultivated cowpea species

There are various sub-species of wild cowpea: *V. u. dekindtiana, V. u. tenuis, V. u. stenophylla* but varieties *protracta* and *pubescens* have also been raised to two distinct sub-species because of their distinctive characteristics like hairy pods and morphology of other plant parts such as flowers, pollen grains, leaves and root nodules. *Vigna u. protracta* has several varieties such as *protracta, rhomboidea* and *kgalagadiensis*. *Vigna u. protracta* var. *rhomboidea* has been reinstated to a sub-species because of its strong incompatibility with other taxa within *V. unguiculata* (Ng, 1995). Similarly, *V. u. tenuis* has several varieties like *tenuis, oblongifolia* and *parviflora*. Likewise, *V. u. dekindtiana* has several varieties including *huillensis, congoliensis, ciliolate* and *grandiflora* (Sonnante *et al*., 1996). All the weedy forms and the intermediates between true wild *V. u. dekindtiana* and cultivated cowpea have been named *V. u. unguiculata* var. *spontanea*. *Vigna ungiculata burundiensis* is a variant of *V. u. dekindtiana* var. *ciliolate* and is found in the mid-latitudes in Zaire, Burundi, Kenya and Uganda (Pasquet, 1993a). Wild cowpea basically is of African origin (Figure; 2)

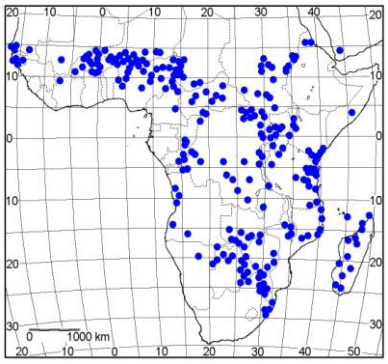


Figure 2; A map of the of distribution of wild cowpea in Africa (Pasquet, 1993a)

1.3 Growth types

Genotypes and environments interact to produce a bewildering diversity of growth, which is difficult to rationalize. The classification is based upon genotypic differences in flowering response to photoperiod and night temperatures. Three genotypes have been broadly identified (Summerfield, 1978). They include reproductively photo-insensitive determinate cultivars, which have apical inflorescence on main stem and branches. In some cultivars flowering is as early as five weeks and day length and night temperatures affect first flower. Reproductively photo-insensitive indeterminate cultivars are common in large collections of germplasm. The flowers appear 5-6 weeks delayed by cold nights and long days. They require short days for flowers to appear. Photo-sensitive indeterminate cultivars include oldest varieties of cowpea; in which flowering begins at the end of rainy season regardless of the sowing date (Baudoin & Vanderborght, 2001). Under the predominant influence of human selection, there are photo-sensitive and photo-insensitive cultivars in which vegetative growth, plant architecture and reproductive ontogeny are mainly determined by the interaction of genotypes with day length and night temperatures. Reproductively short day cultivars in West Africa are

precisely adapted to local environments through a range of location, specific day length requirements for inflorescence initiation and expansion.

1.4 Cultigroups (cultivars) of cultivated cowpea

Classification of cultivated cowpea, *V. unguiculata,* was based on three groups for a long time (Westphal, 1974) but it is now based on five cultivars (Pasquet, 1998; 2000). These include *textilis* with long inflorescence peduncle (0.4-1 cm) found in West Africa. *Sesquipedalis* formerly *V. unguiculata,* but also known as yard-long bean and comprises Asian cultivars with fleshy pods wrinkled when ripe, longer than 0.3 cm, kidney shaped seeds spaced within the pods, more than 7 ovules and are mainly found in eastern Asia. The third one is *melanophthalmus* with thin seed testa partly white and often wrinkled, flower and seed partly white with less than 7 ovules, flowers quickly from the first nodes under inductive conditions and was originally found and majorly grown in West Africa *Biflora* formerly *V. unguiculata cylindrica* but also known as catjang bean has thick seed testa, often coloured shinny flowers, less than 7 ovules, flowers quickly from the first nodes under inductive conditions and cultivated in Africa and South East Asia. The fifth one is *unguiculata* formerly *sinensis* with shinny, thick, coloured seed testa and often coloured flower, with more than 6 ovules, flowering late even under inductive conditions and largely grown in Africa (Baudoin & Vanderborght, 2001).

1.5 Medicinal and economic significance of legumes

Legumes and grains grow together in the field and often go well together in the plate. A special type of bacteria, rhizobium, develops in the roots of plants in the family, leguminoseae. These bacteria convert atmospheric nitrogen, an inert gas into nitrogenous compounds such as ammonia and nitrates (Herrero & Flores 2008). For this

reason, cultivation of legumes needs no nitrogenous fertilizers. They also supplement the soil for other crops without this ability. Legumes are important sources of nectar for foraging honeybees (Furgala *et. al*., 1958). They are ecologically profitable since one hectare dedicated to legume production provides up to seven times more calories and protein than if it were used to raise livestock for milk or meat. If the same amount of money used to buy meat were to buy legumes the amount purchased would be several times greater in terms of calories, vitamins and minerals. Amino acids; valine, leucine and isoleucine are contained in pollen and are of great nuitritional quality for honey bees (Cook *et al*., 2003)

To prevent or help alleviate certain health problems and adequately feed people, there is need for added contributions from legumes. Legumes produce primary and secondary metabolite and other phytochemicals such as nutraceuticals, pharmaceuticals, pesticides and industrial products (Brad, 2003). Bio-functional legumes have ben used in the past for forage, pasture, minor food, green manuring and erosion control. Hyacinth bean is used as ornamental and wildlife feed plant (Brad, 2003).

1.6 The statement of the problem

The role of pollinators and floral volatiles in pollination efficiency and cowpea yield has never been investigated. Cowpea yields may be positively associated with pollination efficiency while the amount of and quality of floral volatiles and nectar may enhance pollinator visitation. The use of volatiles and nectar as molecular markers for the useful traits in cowpea could be essential in selection of appropriate cultivars.

1.7 Hypothesis

Floral volatiles and nectar in cultivated, wild and inbred cowpea are qualitatively and quantitatively different and may have effects on pollination and crop yields.

1.8 Objectives

1.8.1 General objective

Analysis of floral volatiles and nectar from six different cultivars of cowpea (wild out crossed, wild inbred and cultivated forms) and their effect on pollination.

1.8.2 Specific objectives

- To determine the floral sizes of the six cowpea cultivars.
- To trap the floral volatiles from six cowpea cultivars using different adsorbents and also by steam distillation and to analyze the collected volatiles by GC and GC-MS to establish any quantitative or qualitative differences between the six cowpea cultivars.
- To identify the components of the floral volatiles of the six cowpea cultivars.
- To collect nectar from the six cowpea cultivars and to establish qualitative and quantitative differences in the nectar from the six cowpea cultivars using HPLC.

1.9 Justification

Legumes are primarily important as sources of protein in diets in many parts of the world. Cowpea is economically useful as vegetable (leaves), a protein source (seeds and young pods), fodder and pulse crop. Increase in vegetable protein, vitamin and soluble carbohydrates supply from cowpea in malnourished areas presents a less difficult, less expensive and more energy efficient solution. Consequently, cowpea production should be enhanced. If a link between floral volatiles and pollination efficiency can be demonstrated and the direct impact on cowpea yield assessed, then, bee keeping and

cowpea cultivation could be encouraged as complimentary farming practices. Breeding resistant cultivars is an important means of pest and disease control in tropical farming systems where monetary inputs are limited. The wild cowpea has got useful traits (high yields, drought resistance, medicinal value, pest and disease resistance) which if trasfered to the cultivated cowpea would be of great importance.

CHAPTER TWO
LITERATURE REVIEW

2.1 Plant-insect interactions

There is a wide variety of flowers and insects. Flower pollinator insect combinations are often very precise and often quite general. Insects recognize flowers that provide for their nutritional needs. The flowers in return advertise to the insects that fit their needs (Pichersky & Gershenzon, 2002). Flowers attract insects from varying distance through interplay of visual and chemical stimuli which by virtue of their species-specific patterns allow insects to discriminate between flowers of different species (Menzel, 1985). Plant volatiles, together with some other compounds, are determinants in insect-plant interactions (Dobson, 1994).

Insect-flower associations have long been a pivotal subject of interest to entomologists, due to their economic importance in agriculture and the co-evolutionary history between flowers and pollinating insects. Animal-pollinated plants offer rewards in several ways, such as pollen, nectar, stigmatic exudates and essential oils, sexual attractants, resins, gums, food tissues and brood places (Simpson & Neff, 1983). Among these, nectar (secreted by a localized glandular nectariferous tissue) is the most common (Baker *et al*., 1978). Floral nectarines occur on various flower parts and have been used as key characteristics in plant taxonomy and phylogeny (Fahn, 1979). Most butterfly species are effective pollinators and tend to visit broad spectrum of plant species for nectar. The quantity of available sugars (caloric content) is an important factor influencing insect visitation patterns since they prefer plants or flowers that offer high rewards (Devlin & Stephenson, 1985).

Flower visit varies from one species of butterfly to the other and appears to be an outcome of learning through recognition of rewarding flowers. In foraging for food sources, insects make use of a variety of sensory cues (visual, olfactory and gustatory). Although generalists are visited from an array of insects, different visitors vary considerably in pollination efficiency (Corbet, 1978a; 1978b). For effective pollination, the efficiency must be enhanced and hence the specialized pollinators for particular plant species. Characteristic scents produced by particular flowers therefore will attract efficient pollinators.

2.2 Floral scent

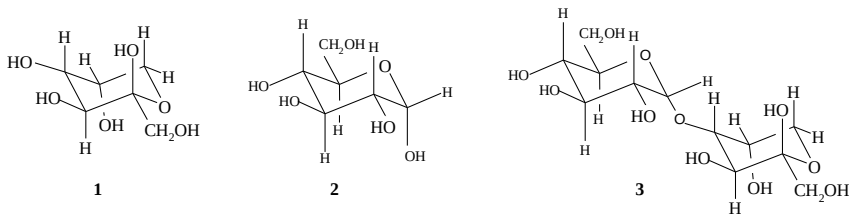
Floral scent is an important component of reproductive biology of many flowering plants, advertising the presence of rewards (nectar or pollen) to foraging pollinators (Heinrich & Raven, 1972; Kevan & Baker, 1983; Robacker *et al.,* 1988). They can serve as attractants in plants pollinated by bees, beetles, butterflies, moths and probably bats (Dodson *et al.,* 1969; Galen & Kevan, 1983; Nilsson, 1983; Pellmyr, 1986; Williams, 1983; Williams & Whitten, 1983; Dudareva & Pichersky, 2000). In most associations between plants and pollinators, floral scent acts as a long-range attractant (Faegri & Van der Pijl, 1979; Williams, 1983) or a close range orientation guide within the flower (Dobson *et al.*, 1990; Knudsen & Tollsten, 1991).

In nocturnal moth species, attraction to flowers is guided mainly by floral scent (Gabel *et al.,* 1992; Dobson, 1994). Olfactory modulation in butterflies influences their attraction to floral odour. In some flowering plants the scent has a highly specific role and determines the relationship between the flower and pollinators. Examples are the co-adaptation of many Neotropical orchids, perfume collecting male euglossine bees

(Williams & Whitten, 1983) and the deception of male Hymenoptera by Orphyress orchids (Borg-Karlson, 1990). The purpose of floral scent in the former is to attract and reward the pollinating bee. In the latter, it is a sexual stimulant giving way to pseudo-copulation, which results in pollination. In a number of pollination syndrome, the adaptation of flowers to specific groups of pollinators is believed to depend heavily on scents. However, little is known about the chemical compositions and the exact role of the scent in the different syndromes (Faegri & Van der Pijl, 1979). Many plant species share characters from different pollination syndrome and are visited by more than one group of pollinators (generalists or promiscuous). Many flower volatiles are pleasant to human sensory system and have potential application in perfumery. Most common floral scents are composed of essential oils.

2.3 Nectar

Nectar is a sugar rich solution, which is also thought to be a reward and pollinator attractant, produced by plants (Nepi *et al*., 2003). The sugar content, type of sugars present and their relative amounts have been identified as the main factors that dictate nectar quality in plant-insect interactions. Three main sugars that are usually in nectar are fructose (Fruc) (**1**), glucose (Gluc) (**2)** and sucrose (Suc) (**3**) (Baker & Baker, 1983a; Baker *et al*., 1998).



1 **2** **3**

Nectar may also act as an attractant to the pollinators. Pollinator visits have been assumed to be independent of the rate of nectar production by the plant. This assumption is based on the belief that pollinators are unable to remember the location of individual plants in a dense population (Summerfield, 1978). Bees prefer bright flowers (Reinhard *et al*., 2004) though colours can be associatively learnt with rewards (Menzel, 1990). Bumble bees descriminte against white flowers when floral rewards are comparable (Odell *et al*., 1999). Familiar scents trigger navigational and visual memories in experience to bees. Acquired visual and chemical associations facilitate honeybee navigation in the field enhancing foraging efficiency (Reinhard *et al*., 2004). Pollinators have been found to favour larger plants over smaller ones. It is possible that if nectar production is associated with some other phenotypic trait, then pollinators might selectively visit plants with high rates of nectar production.

2.3.1 Factors that influence nectar production

Production and composition of nectar vary widely according to species (Fahn, 1979; Baker & Baker, 1983a; 1983b). Variations in environmental conditions can influence the volume of nectar that is produced by a flower and the overall concentration of solutes in the nectar (Shuel, 1955; 1957). Photoperiods and environmental conditions influence the amount of nectar produced (Baker & Baker, 1982). At a photoperiod of 8 hours and 10 ^{o}C, no nectar is produced. Low temperatures and low light intensity decreases nectar production (Pleasants, 1983). Corbet *et al*. (1979) and Plowright (1981) studied the importance of humidity in controlling nectar concentrations and found that at a given humidity, nectar-containing sucrose (**3**) has a higher concentration than nectar consisting of hexoses in order to maintain equilibrium with air (Corbet *et al*., 1979). This explains why hexose rich nectar is found in shallow exposed nectarines. High relative humidity

and high temperatures can stimulate nectar production (Butler *et al*., 1972). The presence of other compounds may also influence vapour pressure and biochemistry of nectar. Carbon dioxide increases nectar production (Lakes & Hughes, 1999; Pan *et. al*., 1998). The quantity and composition of nectar vary widely from species to species (Baker & Baker, 1983a). Patterns of nectar sugars are influenced by several factors, including removal by insects (Silva & Dean, 2000). Flowers exposed to insect visitors often have much lower sugar amounts than individual whose nectar sources are experimentally protected and unavailable to insects (Davis, 1997). There is wide intra-species variability resulting from environmental factors like temperature, soil moisture and humidity. Physiological factors such as flower age, health of plants and damage to floral parts also affect the quality and composition of nectar. High moisture content or soil water increases nectar volume (Wyatt *et al.*, 1992). Nectar composition and nectary cytology are generally studied during anthesis, when nectar is available to pollinators. A complication in nectar composition during aging is due to reabsorption of nectar not collected by pollinators (Zimmerman, 1988; Burquez & Corbet, 1991; Davis, 1997). Although some species do not reabsorb nectar (Pleasants, 1983; Burquez & Corbet, 1991), nectar sitting in flowers may be reabsorbed into nectarines and sometimes reincorporated into concentrated nectar (Corbet, 1978b; Burquez & Corbet, 1991). The latter suggest that nectar may not be reabsorbed if it is stored in an area remote from the nectary or if the nectarines are abscised along with the corolla shortly after fertilization. In monoecious and dioecious zoophilous plants, nectary position and nectar composition may differ in the flowers of the two sexes (Delph & Lively, 1992).

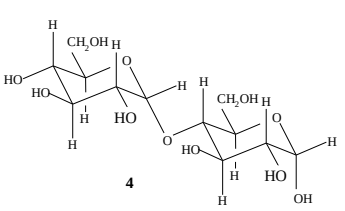
Nectar production increases with plant age and also nectary depth. Nectar volume increases with flower depth for example in Asclepiadaceae (*Asclepias syriaca*) (Pyke,

1978), though not in all species as in Polemoniaceae (*Ipomopsis aggregata*) (Pleasants, 1983). The flower length is correlated with nectary size and total volume of nectar secreted. Structural constraints play a major role in determination of nectar traits (Galetto & Bernardello, 2004; Herrera & Alonso 2006). In some flowers, surfactants on the surface of the nectar pool retard nectar evaporation. Periodic nectar harvesting from some tropical hummingbird flowers appears to increase total nectar yield (Gill, 1988). Removal of nectar stimulates further production (Corbet, 1978b). The production of nectar is an active energy requiring process, which is curbed by respiratory inhibitors (Southwick, 1984; Heinrich, 1983). There is a strong genetic component in nectar production characteristics (Hawkins, 1971). Day-to-day environmental variations influence metabolic process of nectar production (Gardener & Gillman, 2001b). Large amounts of nectar sugars at night may be the result of increased nectar secretion (Wyatt & Shannon, 1986), selective reabsorption during the day (Nepi *et al*., 1996b) and reduced insect visitation during the night due to adverse temperatures (Deppe *et al*., 1999).

2.3.2 Nectar sugars

Most nectar contains some proportion of all three major sugars: fructose (**1)**, glucose (**2**) and sucrose (**3**). Others contain sucrose and very little glucose. Some also contain sucrose, fructose and no glucose (Percival, 1961; Hainsworth & Wolf, 1976). Sucrose and glucose with an apparent absence of fructose; and glucose and fructose with sucrose at undetectable levels occur fairly frequently Glucose more frequently outweighs fructose (Baker *et al*., 1998; Baker & Baker, 1973). Fructose and glucose are secreted from the nectariferous tissues and are not products of hydrolysis (Baker & Baker, 1982). No nectar has been found to contain fructose only (Baker & Baker, 1982). Some sugars

like sucrose (**3**), maltose (**4**), glucose (**2**), fructose (**1**), trehalose (**5**) and melezitose (**6**), all of which are present in some nectars taste sweet to honeybees.



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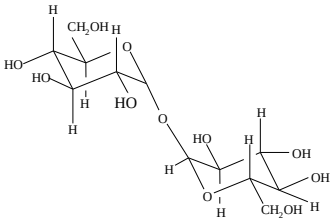

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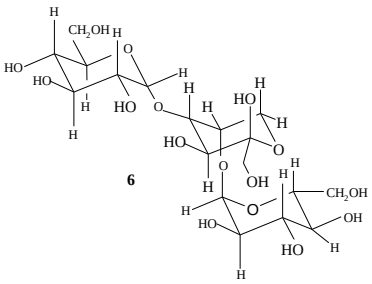

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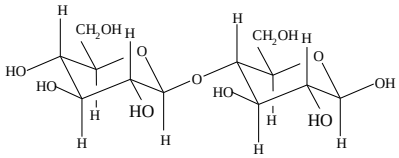
Sugars that are apparently tasteless to bees include: lactose (**7**), melibiose (**8**), raffinose (**9**), xylose (**10**), and arabinose (**11**) (Von Frisch, 1950).



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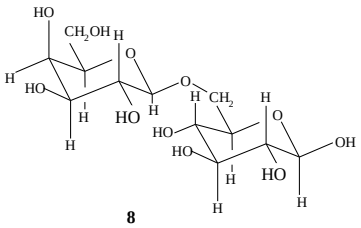

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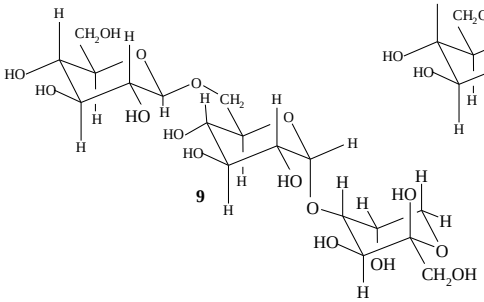

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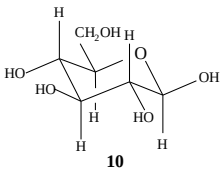

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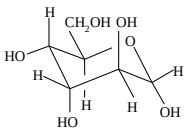

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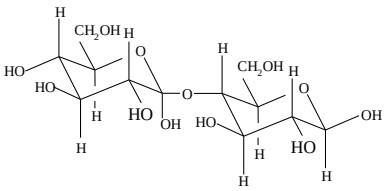
The sugars that are repellant to bees include cellobiose (**12**) and gentiobiose (**13**) (Von Frisch, 1950).



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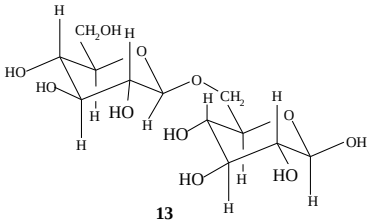

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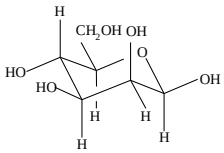
Galactose (**14**) and mannose (**15**) are toxic to bees (Barker & Lehner, 1974b; Cane, 1977).



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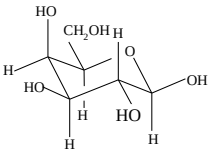

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Other unusual oligosaccharides are present in some fresh nectar. They may increase in amount if nectar is allowed to stand as a liquid (Mauritzio, 1959).

2.3.3 Nectar sugar ratios

Baker & Baker (1983a) distinguished four classes of nectar on the basis of sucrose: hexose ratios. They include hexose dominant, hexose-rich, sucrose-rich and sucrose-dominant. Perfectly balanced nectar contains equal quantities of sucrose, fructose and glucose by weight. The ratio would include sucrose/hexose ratio of 0.333/0.667 = 0.5. If much sucrose is present, the ratio may be 1.0. Sucrose-dominant nectars have sucrose/hexose a ratio of 0.999. The nectars with sucrose/hexose ratios between 0.5 and 0.999 are sucrose-rich and while those between 0.1 and 0.499 are hexose-rich. Ratios of sucrose/hexose which are less than 0.1 are characteristic of hexose-dominant nectars, (Herrera & Alonso, 2006; Silva & Dean, 2000; Baker & Baker, 1982; Southwick *et al*., 1981).

2.3.3.1 Nectar sugar ratios and pollination biology

Flowers have played a role in eliciting behavioural responses in several adult phytophagous insects (Waller, 1972). Among soybean cultivars, floral characteristics that influence attractiveness to honeybees include flower size, colour, abundance, clustogamy, aroma and nectar (Erickson, 1975). Of all the floral rewards offered by flowers to animal visitors, nectar is considered the most important (Simpson & Neff, 1983). Bees exhibit definite preferences for nectar with respect to carbohydrate composition (Bachmann & Waller, 1977). Concentration and abundance of nectar in flowers affect honeybee foraging activity (Kauffield & Sorensen, 1971). Nectar composition is influenced by time of sampling, and the total carbohydrate content increases with time of sample collection (Erickson *et al.*, 1973). Nectar production and blossom sequence vary along a continuum between extreme limits for most of these characteristics. There are co-evolutionary relationships between the nectar sugar ratios and the types of pollinators that are attracted by the plant (Baker, 1975; Baker & Baker, 1979; 1983a; 1983b; Lammers & Freeman, 1986). To explain those cases where nectar sugar composition and pollinators are not correlated, it has been suggested that, some plant taxa have a phylogenetic constraint and therefore do not develop distinct pollination syndromes (Baker & Baker, 1983a). Sucrose-rich nectars are associated with pollination by humming birds, hawk moths, butterflies and long-tongue bees like honeybee. Hexose-rich nectars are likely to be used by passaring birds, bats, flies and short-tongue bees (Baker & Baker, 1983a). Honeybees prefer sucrose-rich nectar (Waller, 1972; Wykes, 1952a; 1952b; Barker & Hurd, 1969). Balanced nectar is rare (Percival, 1961). Starved honeybees would take glucose, fructose and sucrose when offered singly. More loading would occur if sucrose were combined with fructose or glucose (Barker & Lehner, 1974a; 1974b). Long-tongue bees remove sucrose rich

nectar. Little is known about inter-relationships among the constituents or characters of cross-pollinated legume flowers that attract pollinators.

2.3.4 Roles of the sugars

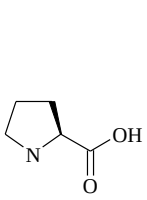
Carbohydrates or sugars occupy a central position in plant metabolism, such that the methods for their detection and estimation are very important to plant scientists. Sugars act as source of respiratory energy (maltose), stored energy (starch), energy (sucrose) and building blocks of cell wall (cellulose). Nucleic acids, like DNA and RNA, contain sugars as essential features of their structures. Sugars also play a number of ecological roles in plant-animal interactions (flower nectar is mainly composed of sugars). They also protect plants from wounding and infection. Sugars are also involved in detoxification of foreign substances (Harbone, 1998). Sugars can be classified as monosaccharides, disaccharides, oligosaccharides and polysaccharides. Nectar is mainly composed of monosaccharides, which are characterized by low molecular weight and optical activity. They are cyclic, water soluble, polyhydroxy compounds that are difficult to crystallize even when pure (Chatwaal, 1988).

2.3.5 Other nectar constituents

Nectar is not merely sugar solution in water providing energy for pollinators. Other constituents in nectar include amino acids, alkaloids, vitamins, phenolics, terpenes, proteins, inorganic ions, micro-organisms, iridoid acids and floral oils (Baker, 1977; 1973).

Amino acids are the second most abundant class of compounds in nectar after sugars (Gardener & Gillmann, 2002). Amino acid composition can be modified by soil nutrient

conditions (Gardener & Gillmann, 2001a). Concentrations of amino acids can vary between species (Baker & Baker 1973) and within single species (Gardener & Gillmann, 2001b). All twenty common amino acids like proline (**16**) and histidine (**17**) found in proteins have been identified in various nectars, with the non-aromatic ones like alanine, arginine, serine, proline, glycine, isoleucine, threonine, valine being the most prevalent (Baker & Baker 1973; Cook *et al*., 2003).



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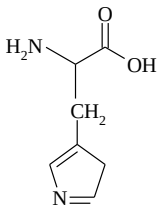

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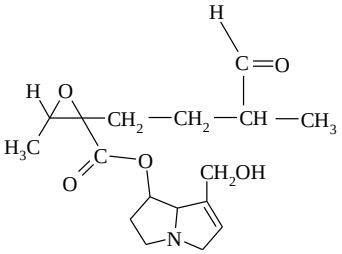
They contribute to the overall taste of nectar (Gardener & Gillmann, 2001a) and elicit various responses in insect taste receptors. Plants adapted to pollination by butterflies show high concentrations of amino acids while those pollinated by birds show low concentration of amino acids in their nectar (Baker & Baker, 1975; 1982). Plants pollinated by bees, which are able to eat and digest pollen, as their source of amino acids, form an intermediate group (Baker & Baker, 1973; Cook *et al*., 2003).

Alkaloids are also present in nectars frequented by bees but do not discourage them from foraging (Baker & Baker, 1975). Adult Lepidoptera are intolerant to alkaloids. Ithiomine butterflies derive protection from predators from pyrrolizidine alkaloids like jocabine (**18**) and heliotrine (**19**) found in nectars of some flowers they visit (Masters, 1990). Vitamin C (**20**) has been identified in the nectar of several plant species. However, the reducing agents identified in nectar may not always be vitamin C (Baker & Baker, 1975).



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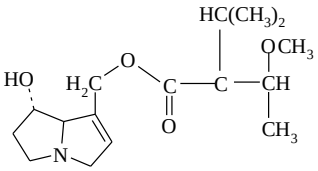

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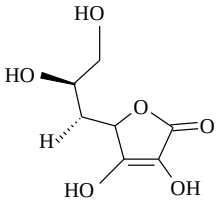

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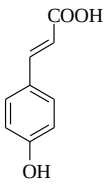
Enzymes (transglucosidases, transfructosidases, esterases and malate dehydrogenases) have also been detected in nectar. Other proteinous contaminants in nectar may be from pollen, micro-organisms or glandular secretions from bees (Baker & Baker, 1973).

Phenolics protect plants from wounding (Buchmann & Buchmann, 1981). Many phenols including ferullic acid (**21**), vanillic acid (**22**) and 2, 5-dihydroxyphenylacetic acid (**23**) are broad-spectrum allelopathics (Johnson *et al*., 2006; Cabras *et al*., 1999; Mann, 1978).



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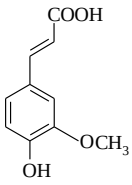

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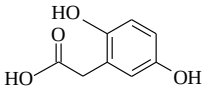

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Yeasts and bacteria can grow in nectars of low sugar concentrations exposed for longer period of time (Buchmann & Buchmann, 1981). They are at times introduced through contamination by floral visitors (Gilliam *et al*., 1983). *Anthomyces reukaufii* is common floral yeast (Meeuse, 1982). In some cases, yeast hydrolyzes sucrose (**3**) in nectar to yield glucose (**2**) and fructose (**1**) (Meeuse, 1982; Heil *et al*., 2005). The presence of micro-organisms (yeast) can increase the levels of amino acids, alcohols and other fermentation products (Kevan *et al*., 1988). Honey often harbors microorganisms. Bees

visiting milkweed flowers cannot differentiate nectar with and without yeast (Spencer *et al*., 1970).

Catalpa (Bignoniaceae) nectar contains iridoids which act as phagostimulants for leaf feeding larvae (Wilkins & Bohin, 1976). The larvae take *Catalpa* nectar but not sucrose solution of the same concentration (Stephenson, 1982b). Examples of iridoids are iridodial, iridodiol, nepetalactone, dolicholactone among many others (Otsuka *et al*., 1989).

Carboxylic acids (Buchmann & Buchmann, 1981), lipids (Baker & Baker, 1975) and other organic compounds are also present in floral nectar. Water, present in nectar is also important to nectarivores (Willmer, 1986).

Some floral nectar has significant amounts of ions like K^+ (Waller *et al*., 1972). Onion, *Allium cepa* (Liliaceae), nectar contains K^+, Na^+, Mg^{2+} and Ca^{2+}. High levels of K^+ deter honeybees from collecting onion nectar (Silva & Dean, 2000; Waller *et al*., 1972). The major cation of most nectar is K^+, making up 35 to 74% of the total cation content (Heinrich & Raven, 1972). Other notable cations include; Na^+ (17.9%), Ca^{2+} (12.8%), Mg^{2+} (5.9%), Al^{3+} (4.6%), Fe^{3+} (1.2%) and Mn^{2+} (0.8%) (Heinrich & Raven, 1972).

No work on the quality and quantity of cowpea nectar has been reported.

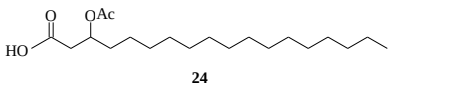
2.4 Extra-floral nectar (EFN)

Extra-floral nectar (EFN), produced by nectarines on leaves, petioles, stipules and stems also attract insects. Like floral nectar, they contain sugars, amino acids, water and other chemical constituents which provide insects with necessary nutrients and water (Baker *et*

al., 1978; Dress *et al*., 1997). EFN reduce herbivory and seed predation (Pembertone & Lee, 1996). Most studies have focused on the mutualistic system between plants and ants whereby ants reduce herbivory by aggressively preying on adult herbivores, their eggs or larvae (Bentley, 1977; Stephenson, 1982b). Relatively few studies have investigated EFN production patterns, primarily due to difficulty involved in accurately collecting and measuring them (Wunnachit *et al*., 1992).

2.5 Floral oils

Nectar and pollen are not the only floral rewards. There are flowers offering fatty oil instead of nectar (Simpson & Neff, 1983; Vogel, 1969). Five major families have been confirmed to have special oil secreting elaiophores (glandular hairs or specialized regions of the floral epidermis). Their flowers offer fatty oils instead of nectar or pollen (Buchmann, 1987). The families include Iridaceae, Orchidaceae, Malpighiaceae, Scrophulariaceae and Gesneriaceae (Buchmann, 1987). Floral oils contain free C_{14} - C_{20} fatty acids that are, substituted with acetoxy at C-3. Examples include 3-acetoxy-octadecanoic acid (**24**), from *Calceolaria pavonii* and 1, 2-diglyceride of 3-acetoxy-11*E*- octadecenoic acid (**25**), the major component of *Calceolaria* and *Lysimachia* species. Floral oil may also contain smaller amounts of long chain hydrocarbons, aldehydes and esters (Zinkl & Preuss, 2000; Buchmann, 1987).



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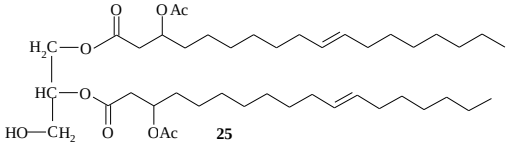

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Lipids are important mediators of pollen hydration (Zinkl & Preuss, 2000; Hülskamp *et al*., 1995b; Preuss *et al*., 1993). The long chain lipids are important components of pollen coats (Ross & Murphy, 1996; Roberts *et al*., 1994).

2.6 Essential oils

Essential oils are odoriferous volatile secondary metabolites that plants produce for their own needs such as defense and attraction (Corbet, 1978a). They can be obtained by gently heating or by steam distilling certain plant materials (flowers, leaves, roots, fruits) (Solomons, 1997; Pinder, 1960). They constitute the characteristic floral scents of the various plants and therefore enable their recognition by pollinators. The composition of flower compounds that give characteristic odour and flavor to each plant may change with physiological state of the plant. Chemical examination of the essential oil has shown that they consist of complex mixtures of acyclic, alicyclic, aromatic and heterocyclic compounds and they may be classified broadly as: aromatics terpenes, nitrogen and sulphur containing compounds (Pinder, 1960). They are usually referred to as floral volatiles.

2.7 Floral volatiles

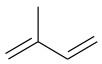
Volatile compounds mediate many interactions between organisms, including plant response to pathogen infection (Shulaev *et al*., 1997), plant-parasitoid signaling in response to herbivory (Turlings *et al*., 1990) and plant-pollinator communication during flowering. As pollinator attractants, volatiles are important cues that help insects locate flowers and signal the presence of food or mates (Knudsen *et al*., 1993).

Plants synthesize and emit a large variety of volatile organic compounds with terpenoids, fatty acids and derivatives as the dominant classes. Floral scent composition of a plant is thought to have evolved partly from adaptations towards the olfactory requirements of efficient pollinators. Some volatiles are probably common to almost all plants while others are specific to only one or a few related taxa (Pichersky & Gershenzon, 2002; Visser, 1986). Plant odour specificity is achieved by a characteristic ratio of the constituent chemical compounds, which are generally distributed among the plant species (Visser, 1986). Anthers and pollen release distinctive odours (Barkmann, 2003; Blight *et al*., 1995). Floral volatiles are formed via plant biosynthetic pathways. The rapid progress in elucidating the biosynthetic pathways, enzymes and genes involved in the formation of plant volatiles allows their physiological activity and function to be rigorously investigated at the molecular and biochemical levels. Floral volatiles act as attractants for species-specific pollinators. However, the volatiles emitted from the vegetative parts, especially those released after herbivory, protect plants by deterring herbivores and/ or attracting the enemies of herbivores (Pichersky & Gershenzon, 2002). Most of the floral fragrance compounds are terpenoids, simple aromatics, amines and hydrocarbons. The most common floral fragrance compounds are monoterpenes (William & Whitten, 1983; Vickery & Vickery, 1981).

2.7.1 Terpenes

Terpenes and terpenoids (oxygen containing compounds) are the most important constituents of essential oils (Solomons, 1997). They are products of secondary metabolism synthesized in various cellular organelles but stored in specialized secretory structures. Apart from the relatively rare hemiterpenoids (a group of natural products

containing a single C_5 skeleton) there exist monoterpenes. Isoprene (**26**) is a classic example of hemiterpenoids (Agarwaal, 1998).



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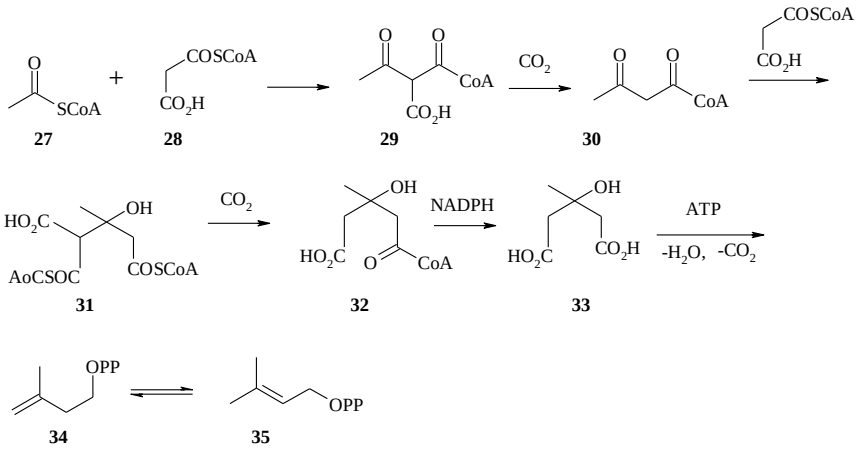
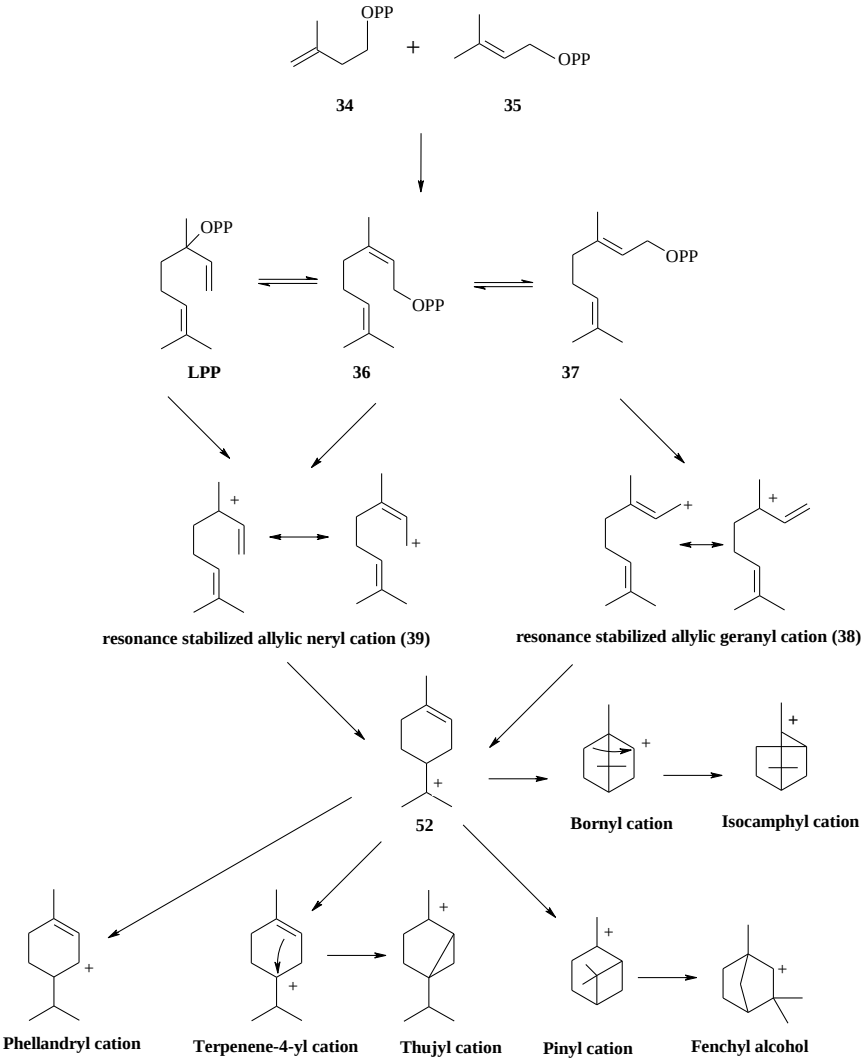
Monoterpenes may be envisaged as consisting of two isoprene (C_5 carbon) units, formed by head-to-tail condensation to produce a C_{10} branched chain or ring. The metabolic pathway of terpenes is believed to start with the condensation acetyl coenzyme A (**27**) from acetic acid and malonyl coenzyme A (**28**) from malonic acid, (Scheme 1). They condense to form (**29**) which decarboxylates to form aceto acetyl coenzyme A (**30**). Another molecule of malonyl coenzyme combines with aceto acetyl coenzyme to form (**31**). The latter then decarboxylates to form hydroxyl methyl glutarate (**32**), on addition of NADPH, mevalonic acid (**33**) arises. The isoprene unit, normally in the form of isopentyl pyrophosphate (IPP) (**34**) readily converted to dimethylallylpyrophosphate (DMAPP) (**35**), Scheme 1.



Scheme 1: Biosynthesis of IPP from acetyl coenzyme A (Mann, 1978)

IPP (**34**) is readily converted to monoterpenes by condensation with DMAPP (**35**) to give nerylpyrophosphate (NPP) (**36**) and geranylpyrohosphate (GPP) (**37**) (Scheme 2). The

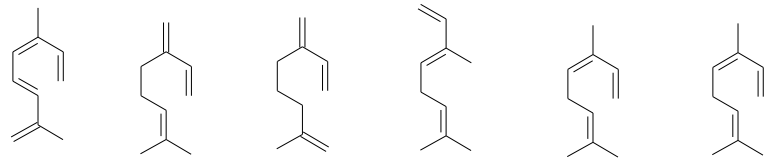
cyclic, bicyclic and acyclic species are derived from NPP (**36**) and GPP (**37**) (Scheme 2). When the phosphate group is eliminated, linalyl (**38**) and neryl (**39**) cations are formed. NPP, LPP and GPP are in isomerism. They form cations which are in isomerism and they form menthyl (α-terpenyl) cation which also forms various cations (scheme 2).



Scheme 2: Biosynthesis of monoterpenes (Pinder, 1960; Charlwood & Charlwood, 1998; Dewick, 2002).

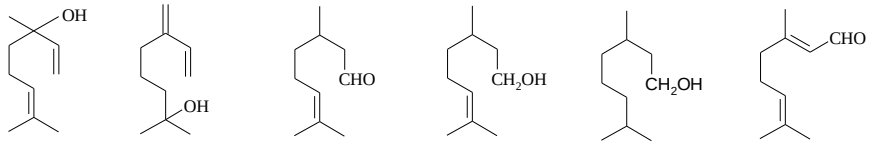
Linalyl cation (**38**) gives rise to the acyclic species (Charlwood & Charlwood, 1998). The aliphatic monoterpene hydrocarbons are represented by: cosmene (**40**), *β*-myrcene (**41**) *α*-myrcene (**42**) (isolated from bay oil, verbena, hops and terpentine oils), (*E*)-

ocimene (**43**), (*Z*)-ocimene (**44**) and allo-ocimene (**45**). Ocimene was first isolated from the oils of the leaves of *Ocimum basilicum* (Agarwaal, 1998; Charlwood & Charlwood, 1998) and has been found to attract nocturnal Lepidoptera species (Dotterl *et al*., 2005).



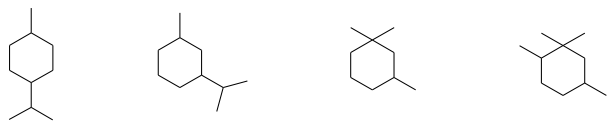
40 **41** **42** **43** **44** **45**

Aliphatic oxygenated monoterpenoids are represented by: linalool (**46**), myrcenol (**47**), citronellal (from citronella oil) (**48**), citronellol (**49**), (from rose oil, geranium oil and citronella oil), dihydrocitronellol (**50**) and geranial (**51**) also referred to as *E*-citral amongst many others (Agarwaal, 1998; Charlwood & Charlwood, 1998).



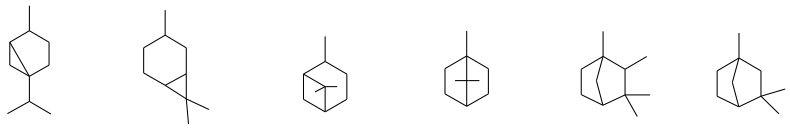
46 **47** **48** **49** **50** **51**

Neryl cation (**39**) gives the cyclic classes. It has cisoid stereochemistry required for cyclisation by intermolecular electrophyllic attack at the isoprenyl double bond yielding α-terpenyl cation (**52**). Rationalization of many monoterpenoid skeletons is made possible through hydride shifts, internal additions and rearrangements. The monocyclic monoterpenoids are classified on the basis of carbon skeleton consisting; *p*-menthane (**53**), *m*-menthane (**54**), 1,1,3-trimethylcyclohexane (**55**) and 1,1,3,6-tetramethylcyclohexane (**56**) (Dewick, 2002; Agarwaal, 1998).



53 **54** **55** **56**

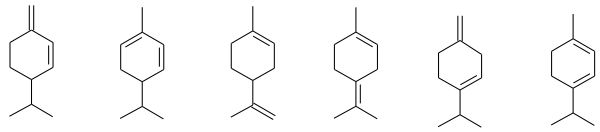
Bicyclic monoterpenes are classified into six groups on the basis of saturated parent hydrocarbons namely thujane (**57**) from thujyl cation, carane (**58**), pinane (**59**) from pinyl cation, bornane (camphane) (**60**) from bornyl cation, isocamphane (**61**) from isocamphyl cation and fenchane (**62**) from fenchyl cation. The last two are derivatives of norbonane (Dewick, 2002; Agarwaal, 1998). They are formed through hydride shifts, internal additions and rearrangements.



57 **58** **59** **60** **61** **62**

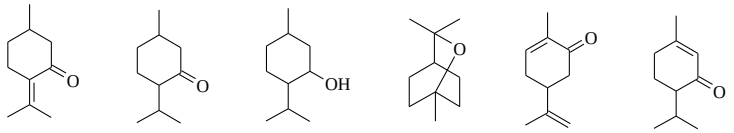
Monoterpenoids ($C_{10}H_{16}$) and its oxygenated derivatives are the simplest among the naturally occurring isoprenoids and form the important constituent of the essential oils obtained from leaves, roots, and flower sand barks of various plants. They have different pleasant odours and hence are frequently used in perfumery (Agarwaal, 1998; Charlwood & Charlwood, 1998). Other exploitable properties of monoterpenoids are anti-bacterial, anti-fungal and anti-cancer activities and in chemotherapy (Charlwood & Charlwood, 1998).

The hydrocarbon monoterpenoids with monocyclic menthane skeletal include *β*-phellandrene (**63**), (from water fennel, eucalyptus, Japanese pepper mint and Canadian balsam oils) *α*-phellandrene (**64**), (from fennel, aniseed, eucalyptus and biter ginger grass oil), limonene (**65**) (from lemon, orange, caraway, peppermint and pine needle oils), terpenolene (**66**) (coriander and *Manila elemi* oils), *β*-terpenene (**67**) and *α*-terpenene (**68**) (from cardamon, coriander and marjoram oils) (Charlwood & Charlwood, 1998).



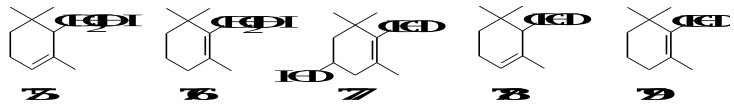
63 **64** **65** **66** **67** **68**

The oxygenated monocyclic menthane skeletal monoterpenoids are represented by pulegone (**69**) (from penny royal oil), menthone (**70**) (from geranium and penny royal oils), menthol (**71**) from pepper mint *Mentha piperita* oils), 1, 8-cineol (**72**) (from wormseed, cajaput and eucalyptus oils), carvone (**73**) (from dill, spearmint and caraway oils) and piperitone (**74**) (from broad leaf peppermint and the Himalayan grass, *Andropogon snerancus* among many others) (Gershenzon & Croteau, 1991; Agarwaal, 1998).



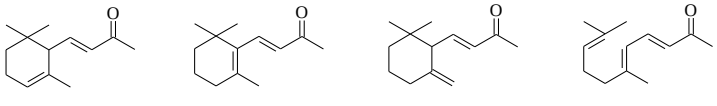
69 **70** **71** **72** **73** **74**

The monocyclic monoterpenoids derived from 1,1,3 trimethylcyclohexane skeleton are represented by cyclogeraniols and cyclocitrals. The examples of cyclogeraniols are *α*-cyclogeraniol (**75**) and *β*-cyclogeraniol (**76**). Cyclocitrals do not occur in nature but are derivatives of naturally ocuring safranal (**77**). Examples of cyclocitrals are: *α*-cyclocitral (**78**) and *β*-cyclocitral (**79**) (Agarwaal, 1998).



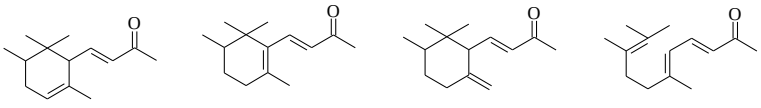
The 1,1,3,6-tetramethylcyclohexane (**56**) derivatives include the ionones and irones. The examples of ionones include; *α*-ionone (**80**) and *β*-ionone (**81**). The *α*-ionones and *β*-

ionones have been isolated from essential oil of *Baronia megastigma*. Other examples include *γ*-ionone (**82**) (ambergris oils) and *Ψ*-ionone (**83**) (Agarwaal, 1998).



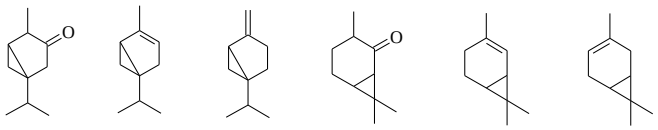
80 **81** **82** **83**

Examples of irones are: *α*-irone (**84**), *β*-irone (**85**), *γ*-irone (**86**) (in the roots of *Iris florentina*) and *Ψ*-irone (**87**) (Agarwaal, 1998).



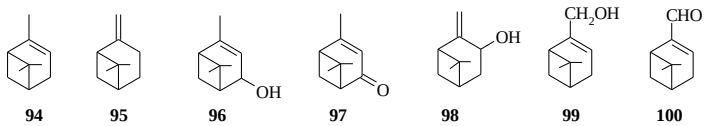
84 **85** **86** **87**

Thujane skeletal structures include thujone (**88**), *α*-thujene (**89**) and sabinene (**90**) while the carane skeletal structures include carone (**91**), 2-carene (**92**) and 3-carene (**93**). The carenes have been isolated from pine needle oils (Agarwaal, 1998; Charlwood & Charlwood, 1998).



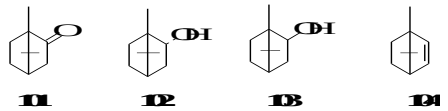
88 **89** **90** **91** **92** **93**

Bicyclic monoterpenoids of pinane skeletal structure include *α*-pinene (**94**), *β*- pinene (**95**), verbenol (**96**), verbenone (**97**), pinocarveol (**98**), myrtenol (**99**) and myrtenal (**100**). (Agarwaal, 1998; Charlwood & Charlwood, 1998)

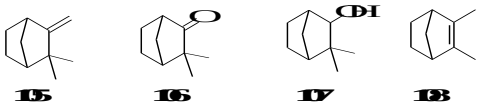


94 **95** **96** **97** **98** **99** **100**

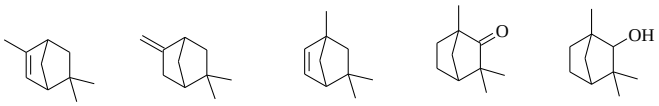
Bornane (camphene) derivatives include: camphor (**101**) (from oil of camphor), isoborneol (**102**) (from valerian, rosemary and spike oils), borneol (**103**) (from oil of *Dryobalanops camphore* tree), and born-2-ene (**104**) among many others (Agarwaal, 1998; Charlwood & Charlwood, 1998).



The isocamphene skeletal structures include camphene (**105**), camphenilone (**106**), camphenelol (**107**) and santene (**108**) among many others (Charlwood & Charlwood, 1998).



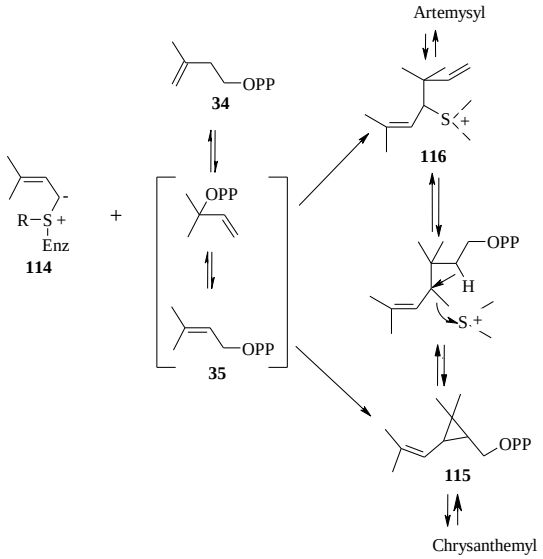
Fenchane skeletal structures are thought to be derived from the pinane skeletal but are rearranged (Charlwood & Charlwood, 1998). Examples include *α*-fenchene (**109**), *β*-fenchene (**110**), δ-fenchene (**111**), fenchone (**112**) and *α*-fenchol (**113**) among many others (Charlwood & Charlwood, 1998).



109 **110** **111** **112** **113**

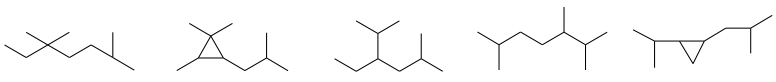
Irregular monoterpenes do not obey the biogenic isoprene rule (BIR) (Mann, 1978; Nakanishi *et al.*, 1980). In irregular monoterpenes the biosynthetic route suggests the involvement of enzyme sulphydril group (**114**) since IPP (**34**) and DMAPP (**35**) are incorporated but not GPP (**37**) and NPP (**36**) (Scheme 3). They are derived from hypothetical process from chrysanthemyl pyrophosphate (CPP) (**115**) by

cyclopropylcarbinyl (CPC) ion rearrangement (**116**) or they are derived from MVA (**33**) via intermediacy of the two units of IPP (**34**) and DMAPP (**35**). The skeletal types are to some extent interconvertible and could probably arise from a common cationic species (Mann, 1978).



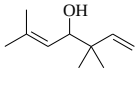
Scheme 3: Biosynthesis of irregular monoterpenoids (Mann, 1978)

Subsequent rearrangements give rise to structures, which are not obviously isoprenoid in character. The most common monoterpenoid skeletones include; artemisyl (**117**), chrysanthemyl (**118**), santolinyl (**119**), lavandulyl (**120**) and rothrockyl (**121**).



117 **118** **119** **120** **121**

Examples in this class include artemesia alcohol (**122**), artemisia ketone (**123**), chrysanthemyl alcohol (**124**), santolina alcohol (**125**) (from santolina oils), and lavandulol (**126**) (from lavandula oils) and rothrockene (**127**) (from *Artemisia tridentate* var. *rothrockii* oils) (Mann, 1978). Compounds of this nature are commonly found in the family Compositae (Mann, 1978).



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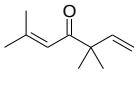

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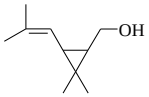

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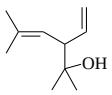

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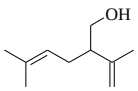

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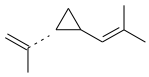
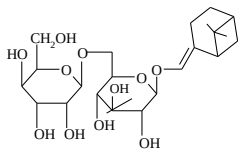


127

Glycosidic monoterpenes have been isolated from *Ocimum*, *Pelargonium*, *Rosa*, *Syneilesis*, *Mentha*, *Thymus* and *Vitis* species, (Jerkovic & Mastelic, 2001; Vorun *et al*., 1990; Van Dries & Svendesen, 2006, Radonic & Mastelic, 2008; Min *et al*., 2009; Watanabe *et al*., 2002; Wende *et al*., 2001). Of particular note are the pinane type enol glycosides (**128**-**130**), which have been isolated from the roots of *Paeonia lactiflora* (Winter & Skouroumounis, 2007; Inoshiri *et al*., 1988; 1987).



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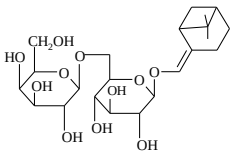

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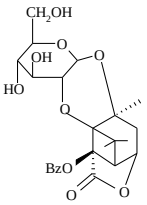
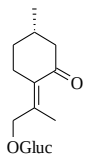


130

Glycosilated hydroxypulegone schizonepetoside (GHS) (**131**) was isolated from *Schizonepeta tenuifolia* (Kubo *et al*., 1986). Angelicoidenal-2-O-β-D-glucopyranoside (**132**) was found in the stems of *Berchemia racemosa* (Inoshiri *et al*., 1988). Agarwaal (1998) has also reported picrocronin (**133**).



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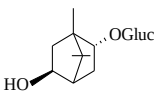

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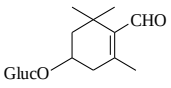
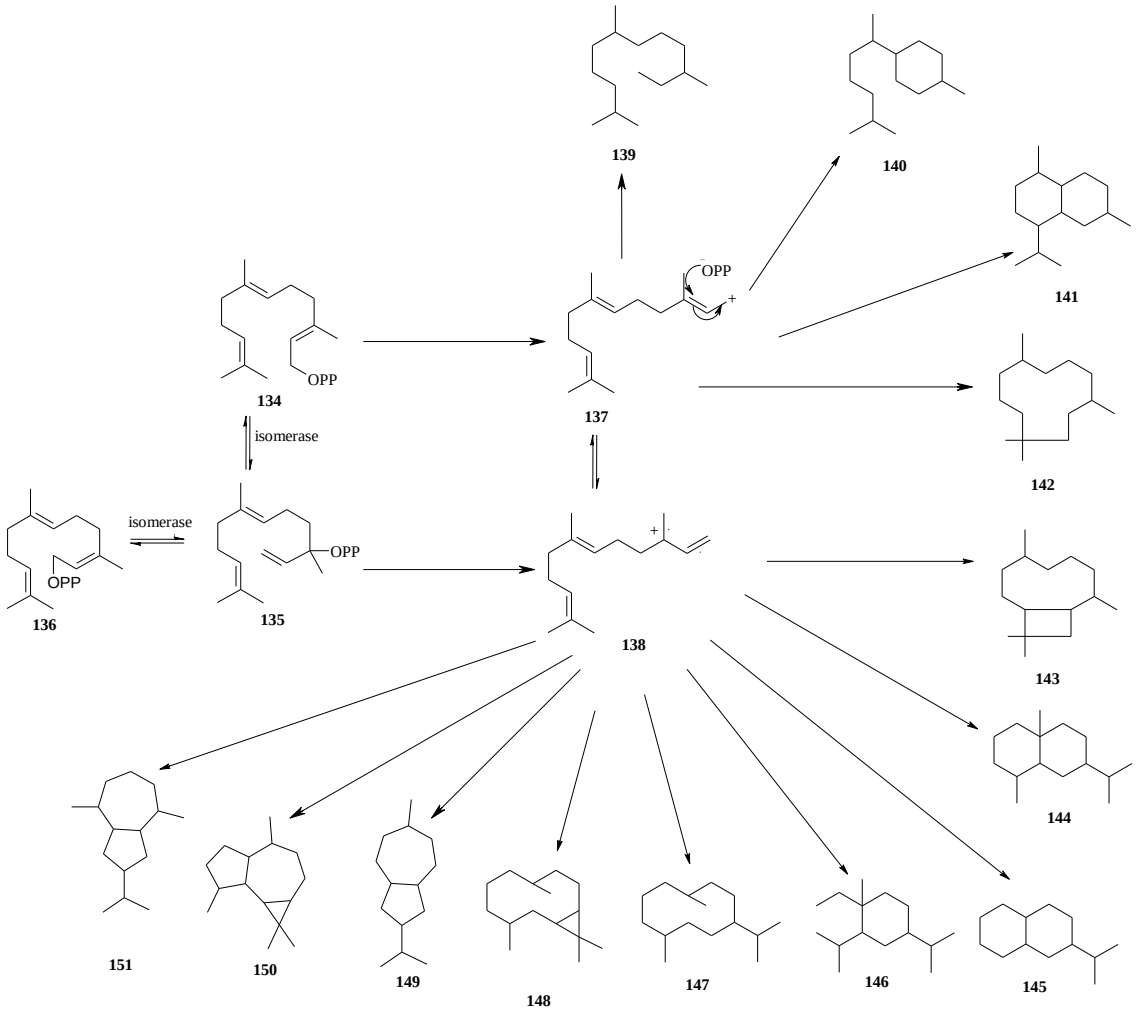


133

2.7.2 Sesquiterpenes

Sesquiterpenes are C_{15} hydrocarbons or their oxygenated analogues. They arise from the cyclisation of 2*E*, 6*E*-farnesyl pyrophosphate (FPP) (**134)** with nerolidyl

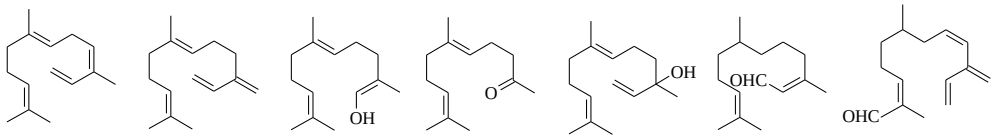
pyrophosphate (NPP) (**135**) and 2*Z*, 6*E*-FPP (**136**) which are in isomerism. Subsequent rearrangements results in farnesyl (**137**) and nerolydyl cation (**138**) (Bouwmeester *et al*., 1999; Mckaskill & Croteau, 1997). Cyclization followed by hydride shifts results in numerous sesquiterpene skeletons: farnesane (**139**), bisabolane (**140**), cadinane (**141**), humulane (**142**), caryophyllane (**143**), eudesmane (**144**), eremophillane (**145**), elemane (**146**), germacrane (**147**), bicyclogermacrane (**148**), carotaene (**149**), aromandrane (**150**) and guainane (**151**) (Scheme 4) (McCaskill & Croteau, 1997; Fraga, 1998).



Scheme 4: Biosynthesis of sesquiterpenes, (Bouwmeester *et al*., 1999; Mackaskill & Croteau, 1997; Fraga, 1998).

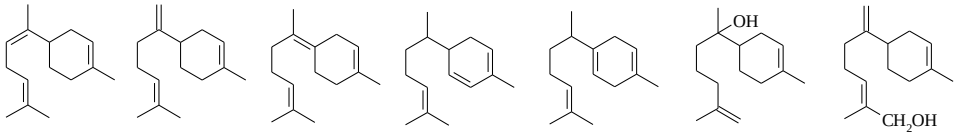
Ionization of FPP to the farnesyl cation (**137**) is the first step in the biosynthesis of a large number of sesquiterpenes (Bouwmeester *et. al*., 1999). For a large number of other sesquiterpenoids, the enzymatic reaction is initiated by isomerization of FPP (**134**) to the isomer NPP (**135**), which is ionized to generate the nerolidyl cation (**138**). The intramolecular attack of the cationic centre formed in the solvolysis of phosphate on one of the double bonds that are not allylic to this group, followed by cyclisitions, rearrangements and deprotonation leading to cyclic sesquiterpenoids such as germacranes, bisabolanes, guiananes, caryophyllanes: and pantalananes (Scheme 4) (McCaskill & Croteau, 1997; Fraga, 1998). This enzyme-bound carbocation can undergo electrophilic cyclizations, rearrangements, hydride shifts, and deprotonation to yield cyclic sesquiterpenoid constituents such as the cadinanes, bergamotanes, and bisabolanes (McCaskill & Croteau, 1997). Sesquiterpenes are unsaturated compounds and may be acyclic, monocyclic, bicyclic or tricyclic hydrocarbons and have many oxygenated derivatives (Nakanishi *et al*., 1980). Few acyclic sesquiterpenes occur naturally.

The sesquiterpene hydrocarbons with farnesane skeletone include; α-farnesene (**152**) (from oils of Grammy Smith apple) and β-farnesene (**153**) (Fraga, 1998) while the oxygenated derivatives are represented by: farnesol (**154**) (from ambrette seeds, citronella, rose, acacia and sevile orange oils), geranyl acetone (**155**), nerolidol (**156**) (from neroli and flowers of bitter orange), farnesal (**157**) and β-sinensal (from Chinese orange) (**158**) (Fraga, 1998).



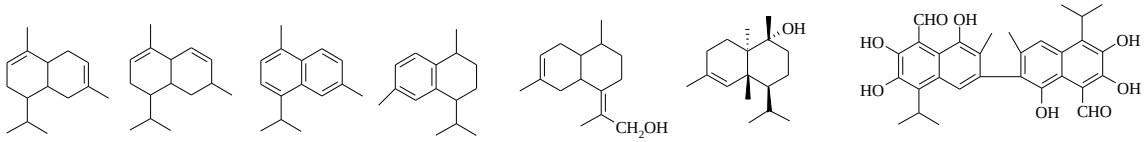
152 **153** **154** **155** **156** **157** **158**

The monocylic hydrocarbon sesquiterpenes with bisabolane skeletone are represented by: α-bisabolene (**159**) β-bisabolene (**160**), γ-bisabolene (**161**). The bisabolenes are got from bergamot, bisabol myrrh and carrot oils. Others are: zingiberen (**162**) (from ginger oil) and β-curcumene (**163**) (Fraga, 1998). The oxygenated derivatives are represented by: α-bisabolol (**164**), lanceole (**165**) (Mann, 1978; Fraga, 1998).



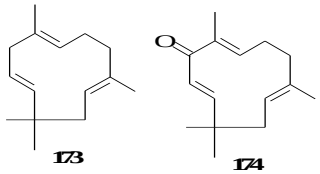
159 **160** **161** **162** **163** **164** **165**

The bicyclic sesquiterpene hydrocarbons of cadinane skeleton are represented by: α-cadinene (**166**), (from oleum, cubebs galbanum, *Angostura rind* and juniper wood oils), β-cadinene (**167**), cadalene (**168**) and calamene (**169**) (Fraga 1998) while the oxygenated derivatives incude: khusol (**170**), α-cadinol (**171**), and dimeric sesqiuterpene gossypol (**172**) (from cotton seed) amongst many others (Mann, 1978). Gossypol isolated from cotton (**172)** has been used as a stabilizer for vinyl compounds, as a reagent for analysis of some compounds and certain metals. It is an anti-fertilty spermicide for men (Fraga 1998).

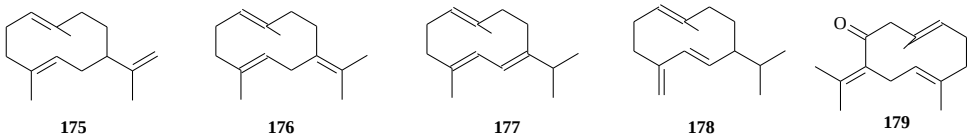


166 **167** **168** **169** **170** **171** **172**

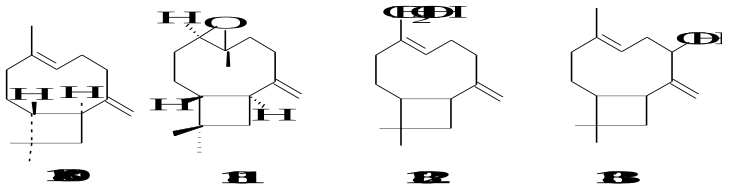
The hydrocarbon sesquiterpenes represented by humulane skeleton is humulene (**173**) while the oxygenated derivative is represented by zerumbone (**174**) (Fraga, 1998).



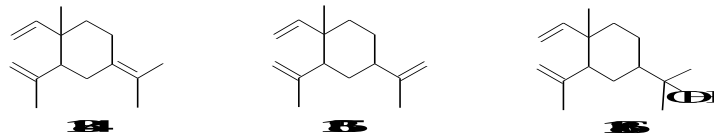
Germacrane is also another skeletal structure for the sesquiterpenes. The hydrocarbons consist of germacrene A (**175**), B (**176**) C (**177**) and D (**178**) while the oxygenated derivatives are represented by germacrone (**179**) (McCaskill & Croteau, 1997; Mann, 1978). Germacrene A (**175**) readily isomerizes to *β*-elemene (**185**).



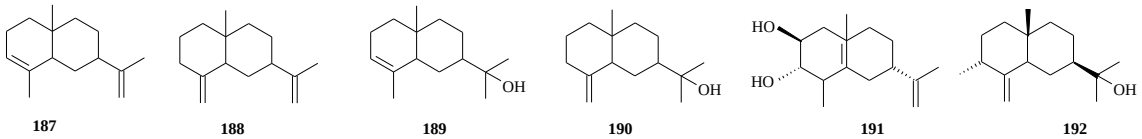
The hydrocarbons with caryophyllane skeleton sesquiterpene are represented by *β*-caryophyllene (**180**) (Mackaskill & Croteau, 1997) while the oxygenated derivatives include caryophyllene oxide (**181**), *α*-betulenol (**182**) and *β*-betulenol (**183**) (Dudareva & Pichersky, 2006; Fraga, 1998).



The sesquiterpene hydrocarbons with elemane skeleton are represented by *α*-elemene (**184**) and *β*-elemene (**185**) while the oxygenated derivatives are represented by elemol (**186**) (Dudareva & Pichersky, 2006; Fraga 1998).

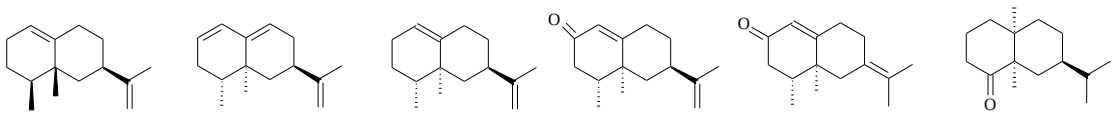


The sesquiterpene hydrocarbons with eudesmane skeleton are represented by *α*-selinene (**187**) and *β*-selinene (**188**) while the oxygenated derivatives are represented by *α*-eudesmol (**189**), *β*-eudesmol (**190**), rishitin (from oil of *Solanum tuberosum*) (**191**) and chrysanthemol (from oil of *Chrysanthemum indicum*) (**192**) (Fraga, 1998).



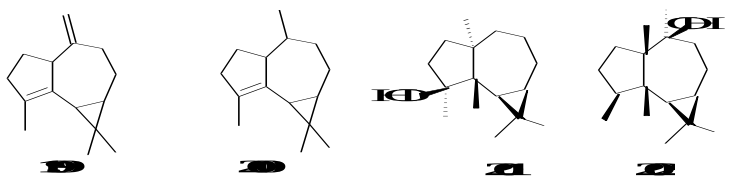
187 **188** **189** **190** **191** **192**

The hydrocarbon sesquiterpenes with eremophilane skeleton are represented by eremophilene (**193**), nootkatene (from oil of *Chameacypari nootkatensis*) (**194)** and valencene (**195**) while the oxygenated derivatives are represented by nootkatone (**196**), vetivone (**197**) and valeranone (**198**) (Fraga, 1998).

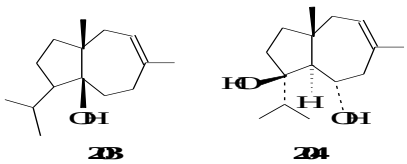


193 **194** **195** **196** **197** **198**

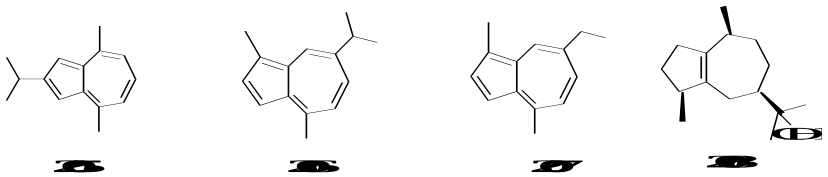
The aromandendrane-type sesquiterpene hydrocarbons are represented by aromandendrene (**199**) and *α*-gurjunene (**200**) (Mann, 1978) while the oxygenated derivatives are represented by maaliol (**201**) (from oil of *Valeriana officinalis*) and viridifloral (**202**) (Fraga, 1998).



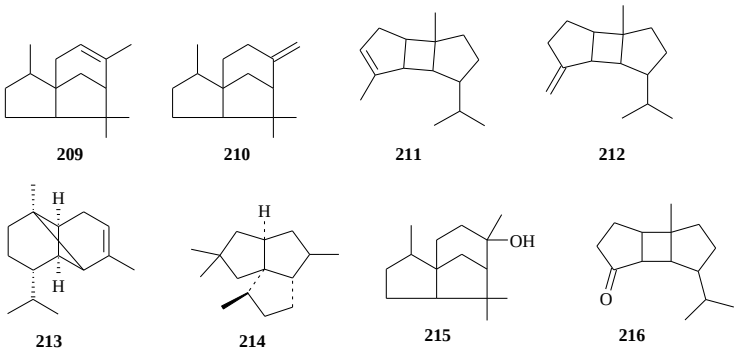
The sesquiterpenes with carotane skeletone are represented by caratol (**203**) and jaeschkeanadiol (**204**) (Mann, 1978).



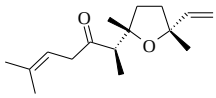
Essential oils may have a blue color due to the presence of azulenes which are beautifully colored aromatic sesquiterpenes (Pinder, 1960) with guianane skeleton (Fraga, 1998). Examples include: vetivazulene (**205**) (from vetivar oil), guaiazulene (**206**) (from geranium and guacum wood oils) and chamazulene (**207**) (from *Matricaria chamomilla*) (Fraga 1998) while the oxygenated derivatives are represented by guaiol (**208**).



The tricyclic sesqiterpene hydrocarbons are represented by *α*-cedrene (**209**), *β*-cedrene (**210**), *α*-bourbonene (**211**), *β*-bourbonene (**212**), α-copaene (**213**) and pantalanene (**214**) while the oxygenated ones include cedrol (**215**) and ketone 21 (bourbonone) (**216**) (Mann, 1978).



Most of known acyclic sesquiterpenes contain furan rings or tetrahydrofuranyl groups: davanone (**217**), ipomearone (**218**) and freelingyne (**219**) (Mann, 1978).



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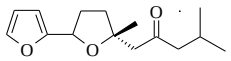

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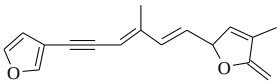


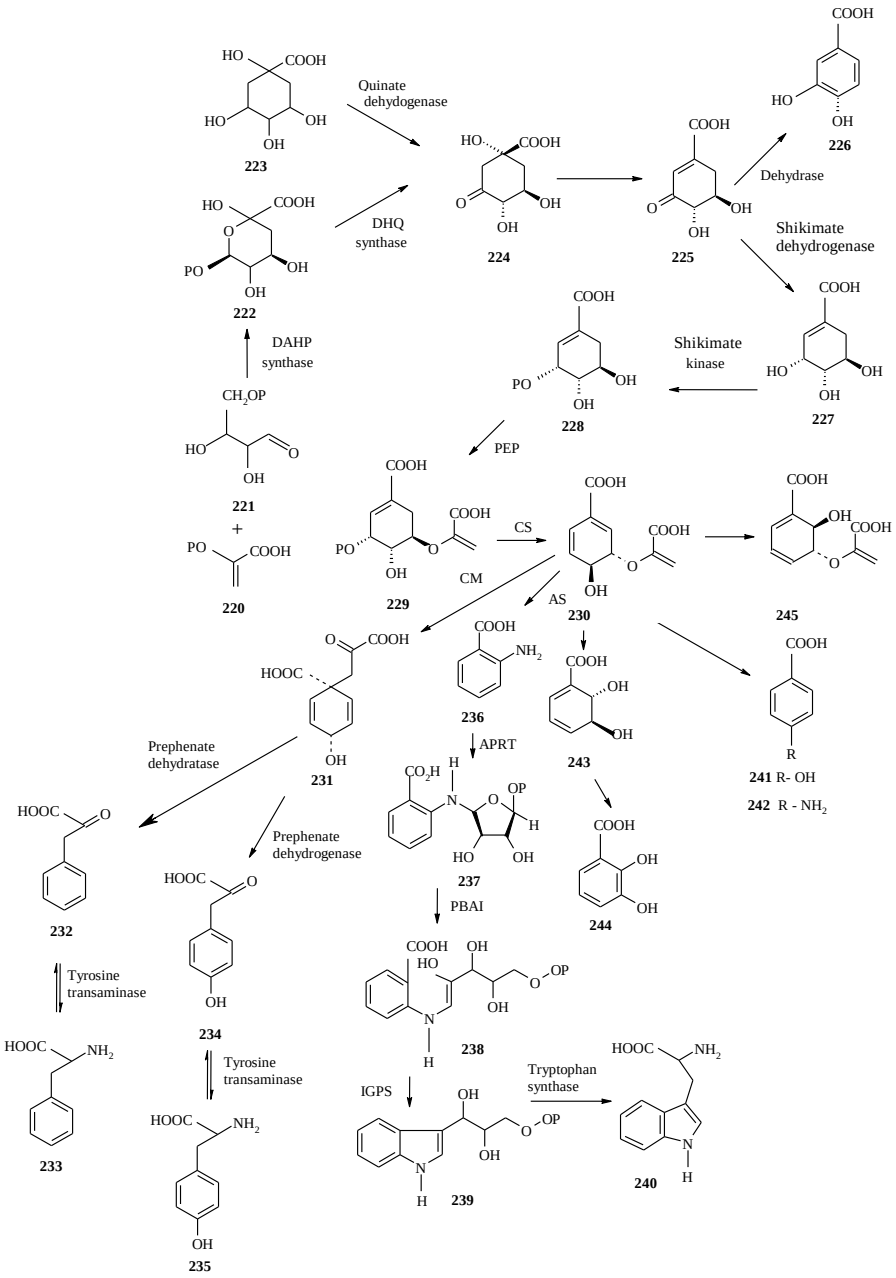
219

Terpenoid essential oil comprises the volatile steam distillate fraction responsible for the characteristic scent (odor) found in many plants and their flowers. They are commercially important as the basis of natural perfumes, spices and flavoring agents in the food and pharmaceutical industry. Plant families particularly, rich in essential oils include the Compositae, Matricaceae, Labiatae, Pinaceae, Rutaceae and Mrytaceae (Harbone, 1998). The C_{10} and C_{15} compounds are often referred to as lower terpenoids or essential oils (volatile terpenoids). C_{20} compounds and above are often referred to as higher terpenoids (non-volatile terpenoids) (Mann, 1978**).**

2.7.3 Aromatic constituents of floral volatiles

The shikimate pathway (Scheme 5) is an essential metabolic route by which micro-organisms and plants synthesize aromatic amino acids and many other aromatic compounds. Other compounds such as vitamin K, ubiquinone, anti-tumour anti-biotics and enterochelin are also synthesized through this pathway (Coggins *et al*., 2003; Mansfield, 2000; Heinz, 1997; Bu'lock, 1965). The starting materials in the shikimate pathway are phosphoenol pyruvate (PEP) (**220**) and erythrose-4-phosphate (**221**) which are involved in primary metabolism of sugars (Bu'lock, 1965). In the presence of enzyme 3-deoxy-D-arabino-hept-2-ulosonate-7-phosphate (DAHP) synthase these starting materials yield DAHP (**222**) Quinic acid (**223**) from quinate pahway is converted to 3-deydroquinic acid (**224**) in the presence of quinate dehydrogenase enzyme. 3-Dehydroquinic acid (**224**) also arises from DAHP (**222**) a reaction catalysed by dehydroquinate synthase. 3-Dehydroquinic acid (**224**) is converted to 3-dehydrishikimic

acid (DHSA) (**225**) by loosing water, a reaction catalysed by dehydroquinase enzyme. The (DHSA) (**225**) enters back into the quinate pathway as protocatchute (**226**), a reaction catalysed by dehydrase enzyme while shikimic acid (**227**) is formed from DHSA (**225**) under the influence of shikimate dehydrogenase.Shikimic acid (**227**) is phosphorylated to form 3-phosphoshikimic acid/3-shikimate-3-phosphate (**228**), a reaction catalysed by shikimic kinase. Phosphoenol pyruvate (PEP) (**220**) reacts with 3-phosphoshikimic acid/3-shikimate-3- phosphate (**228**), to produce 5-enol pyruvate 3-shikimate-3-phosphate (EPSP) (**229**). This reaction is catalysed by EPSP synthase. Chorismic acid (**230**) is formed from EPSP by the action of chorismate synthase. There are numerous products that are synthesized from chorismic acid (**230**) after loosing the pyruvate (Coggins *et al*., 2003; Dewick, 2002; Heinz, 1997).



Scheme 5: Shikimate pathway (Coggins *et al*., 2003; Mansfiled, 2000; Heinz, 1997; Bu'lock, 1965).

Chorismic acid (**230**) is used in the biosynthesis of aromatic amino acids. Intramolecular arrangement by the action of chorismate mutase (CM) forms prephenic acid (**231**). Decarboxylation and dehydration of prephenic acid (**231**) by the action of prephenate dehydratase forms phenyl pyruvate (**232**). Reductive amination (introduction of ammonia under reducing conditions) of phenyl pyruvate (**232**) produces *L*-phenylalanine

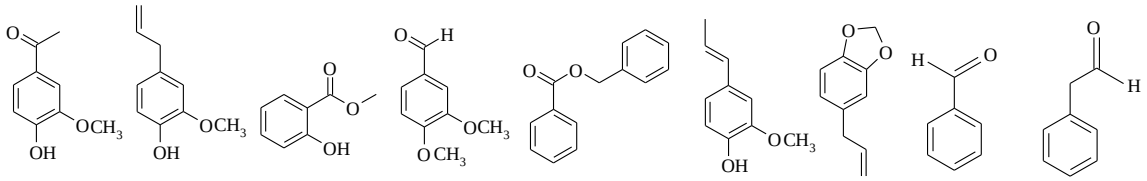
(**233**). On the other hand; prephenate dehydrogenase produces *p*-hydroxyphenyl pyruvate (**234**) which leads to *L*-tyrosine (**235**). Formation of these amino acids is catalysed by tyrosine transaminase.

Reduction of chorismic acid (**230**) and incorporation of ammonia from glutamine by the action of anthranilate synthase produces anthranilic acid (**236**). Phosphorylation and addition of ribose sugar to the anthranilic acid by action of anthranilate phosphribosyl trasferase (APRT) leads to N-(5'-phosphribosyl)-anthranilate (**237**). It isomerizes to produce 1-(*O*-carboxyphenylamino) - 1'-deoxyribulose-5'-phosphate (**238**) by the action of phosphribosyl-anthranilate isomerase (PRAI) enzyme. Decarboxylation and dehydration of 1-(*O*-carboxyphenylamino)-1'-deoxyribulose-5'-phosphate (**238**) by the action of indole-3-glycerol phosphate synthase forms indole-3-glycerol phosphate (**239**). Reductive amination leads to *L*-tryptophan (**240**) by the action of tryptophan synthase.

Decarboxylation of chorismic acid (**230**) and subsequent amination prouces *p*-hydroxybenzoic acid (**241**) and *p*-aminobenzoic acid (**242**). Removal of pyruvate group from chorismic acid (**230**) leads to (*2S, 3S*)-2, 3-dihydroxy-2, 3-dihydrobenzoic acid (**243**) which gives 2, 3-dihydroxybenzoic acid (**244**). Rearrangement of chorismic acid (**230**) leads to isochorismic (**245**) by action isochorismatase enzyme (Dewick, 2002).

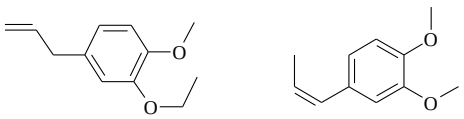
Examples of aromatic volatile constituents include vanillin (**246**), (from vanilla beans), eugenol (**247**) (from oil of cloves), methyl salicylate (**248**), veratraldehyde (**249**), benzyl benzoate (**250**), isoeugenol (**251**) and safrole (**252**) (from sassafras) (Bu'lock, 1965). Their role in perfumery is well established while the same cannot be said for insect-plant interaction (Morrison & Boyd, 1991). However, the roles of some of these compounds

in plant insect interactions have been identified. Benzaldehyde (**253**) and phenylacetylaldehyde (**254**) attract nocturnal Lepidoptera species (Dotterl *et al*., 2005).



246 **247** **248** **249** **250** **251** **252** **253** **254**

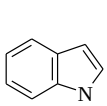
The young leaves of African tree (*Leonardoxa africana*) emit high levels of methyl salicylate (**248**). *Petalomyrmex phylax*, ants patrol the young leaves, which emit high levels of methyl salicylate (**248**) that attracts them. They make use of it as a kairomone or as an anti-septic for their nests (Brouat *et al*., 2000). Methyl salicylate (**248**) is also released by *Arabidiopsis thaliana* in the floral scent. Feeding by *Pieris rapae* larvae triggers production of methyl salicylate (**248**) which is attractive to the larval parasitoid. A novel gene for the scent producing enzyme, isoeugenol-*O*-methyltransferase (IEMT), that catalyses the production of methyleugenol (**255**) and methylisoeugenol (**256**) formation using the donor methyl group of *S*-adenosyl-*L*-methionine (SAM), has been characterized in *Clarkie breweri*. The gene has high levels of sequence similarity to caffeic acid-*O*-methyltransferase (COMT), which is useful in the biosynthesis of lignin converting caffeic acid to ferullic (**21**) acid in plants (Mansfield, 2000; & Pichersky, 1998).



255 **256**

2.7.4 Amine floral volatile compounds

Among nitrogen containing compounds found in floral fragrances, indole (**257**) and skatole (methylindole) (**258**) are the most common. Skatole is a potent attractant of male euglosine bees. The foul smell of many fly and beetle pollinated flowers is usually due to amines (Williams & Whitten, 1983).



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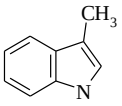


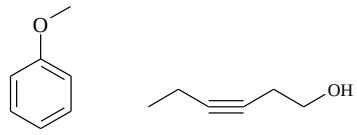
258

The precursor of indole alkaloids is tryptophan (**240**) produced in the shikimic acid pathway (Heinz, 1997; Mann, 1978). Metabolism of the amino acid tryptophan provides the indole C_2N sub-unit. They may be formed either as simple alkaloids from minor chemical modification of tryptophan or by incor-poration of a C_2 sub-unit (from pyruvate) into the structure. Skatole (**258)** belongs to another group of alkaloids formed as a result of mixed metabolism of tryptophan and mevalonate by incorporation of a C_5 or C_9/C_{10} sub-unit (Bu'lock, 1965).

2.7.5 Factors affecting the release and emission of plant volatiles

Composition of the volatile blend emitted by plant varies within plant species depending upon the taxonomic group to which the plant belongs (Lui *et al*., 1988; Tollsten & Bergstrom, 1989; Cole, 1980a), plant cultivar (Cole, 1980b), plant age (Matile & Alttenburger, 1988), defense (Brouat, 2000), plant wounding (Dicke *et al*., 1990), light intensity (Turlings *et al*., 1991), season and geographical location (Whiteman & Aller, 1990). The emission of other volatiles from foliage is thought to help protect plants against herbivores directly. For example, two species of trees belonging to the Brazil nut

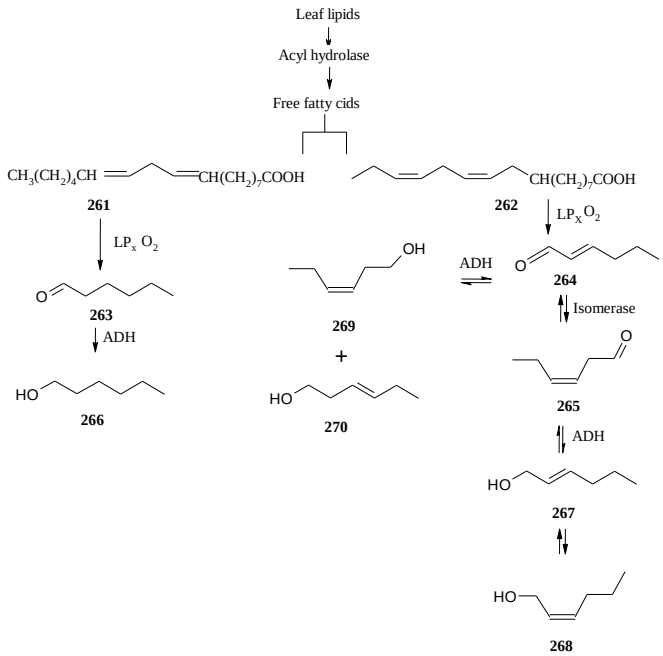
family (Lecithydaceae) that emit high levels of *S*-methylmethionine (SMM) are colonized by wood boring beetles at much lower frequency than other tree species in the same family that emit minute amounts (Berkov *et al*., 2000). Plant volatiles can also promote indirect defenses, like the protection of the *Leonardoxa Africana* (family (Leguminosae) from herbivores (Pare & Tumlinson, 1997; 1999). *Petalomyrmex phylax* ants attracted by methyl salicylate patrol young leaves of the *L. africana* predating on any phytophagus insects (Brouat, 2000). Wounding crushes plant cells and releases some degradative enzymes resulting in the emission of higher concentrations of volatiles and sometimes changes in the composition of the components as demonstrated in mechanically damaged maize (*Zea mais*) that produce anisole (**259**), 3-hexenyl-1-ol (**260**) and (*E*)-α-farnesene (**152**) in addition to its normal volatile profile (Brouat, 2000). They have been found to deter herbivores (Gershenzon & Croteau, 1991; Agrawal *et al*., 2000). They also act as airborne signals that activate diseases resistance via expression of defense related genes in neighbouring plants and in the healthy tissues of infected plants (Shulaev *et al*, 1997).



259 **260**

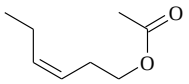
Emission of volatiles can also take place from the foliage after herbivore damage. Terpenoids, fatty acid derivatives and other volatiles such as indole (**257**) and methyl salicylate (**248**), germacrene D (**179**), *α*-copaene (**213**), guaiol (**208**) and *α*-caryophllene are released from plants after bouts of herbivory (Gang *et al*., 2001; Knudsen *et al*., 2004). Some volatiles are released when feeding ruptures pre-existing internal or external excretory structures in which the volatiles are synthesized and stored (Gang *et*

al., 2001). Volatiles may also be formed at the moment of damage. The C_6 aldehydes, alcohols and esters, generally known as "green-leaf volatiles' (GLVs), are metabolites of the oxidative degradation of fatty acids (Scheme 6). Lipase acyl hydrolases liberate free fatty acids from membrane lipids. The polyunsaturated fatty acids involved are linoleic (**261**) and linolenic (**262**). Linoleic acid (**261**) is converted to hexanal (**263**). The acids are oxidized by the action of lipoxygenase to 2*E*-hexenal (**264**) and 3*Z*-hexenal (**265**). Alcohol dehydrogenase converts hexanal (**263**) to 1-hexanol (**266**). The unsaturated aldehydes (2*E*-hexenal) (**264**) are converted to 2*E*-hexen-1-ol (**267**) and 2Z-hexen-1-ol (**268**) while 3*Z*-hexenal (**265**) is converted to 3*Z*-hexen-1-ol (**269**) and 3*E*-hexen-1-ol (**270**) (Mansfield, 2000). The biosynthetic pathway (Scheme 6) is operative in several plant species such as potatoes, tomatoes, tea, peas, apples and legumes like soybeans among others (Dudareva & Pichersky, 2006; Mansfield, 2000). More volatiles are released from dark flowers than from light coloured flowers (Pacetti & Tava, 2000).



Scheme 6: Biosynthesis of GLVs (Mansfield, 2000).

The GLVs embody the typical odor of damaged leaves and are derived via lipoxygenase catalysed cleavage of fatty acids shortly after injury (Pare & Tumlinson, 1997; 1999; Agrawal *et al*., 2000). Indirectly, volatiles attract insects that prey upon herbivores thereby reducing further damage to the plant (*Nicotiana alata*). Herbivore-induced volatiles from tobacco plant include the 3*Z*-hexenyl acetate (**271**) and hexen-1-ol (**272**) which deters female moths (*Heliothis virescens*) from laying eggs on the injured plants (Van Poecke *et al*., 2001).



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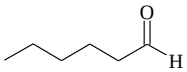


272

Light also affects the composition of plant volatiles. Plants under high light intensity are characterized by relatively high amounts of volatiles than those under low light intensity. This could be due to the fact that plants under high light intensity receive more photolytic energy input to produce volatiles (Dudareva & Pichersky, 2006; Gershenzon & Croteau; Heinz, 1997). Inverse correlation sometimes exists between age of plants and the amount of volatiles released, such that young plants produce more volatiles than old ones (Matile & Altenburger, 1988; Hatanaka, 1993). Data on the effect of the plant cultivar on the composition of volatile has shown marked difference in the composition of volatile blends emitted by cultivars (Cole, 1980b).

2.7.6 Floral volatiles and pollination biology

In angiosperms that rely on insect pollination, reproductive fitness partially depends on the ability of the plants to produce flowers that are very attractive (easily visible and overwhelming olfactory hints) to insects. Vision and olfactory cues are indeed the main

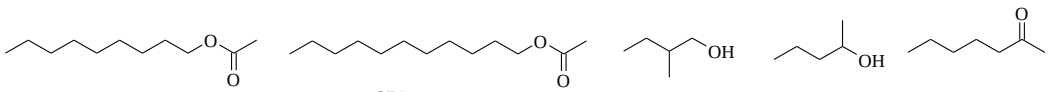
stimuli that attract insects to flowers although gustatory and tactile hints may be important once the flower has been located (Dobson, 1994). Odor is learned fast and used in preference to colour and shape. From afar, insects probably select their target by flower colour while at close range aroma of individual plants becomes the main guide. After alighting, the insect may use close range olfactory and gustatory cues such as nectar volume and sugar concentration (Dobson, 1994).

Flower volatiles may induce pollination by deceit (Pichersky & Gershenzon, 2002). Floral scent is an important factor in deceiving bees (a male of the solitary bee species) that pollinate some species of orchid flowers. The compounds responsible include *ß*-caryophyllene (**180**), citronellol (**49**), benzyl acetate, linalool (**46**), methyl cinnamate (**304**) and 1, 8-cineol (**72**) (Pichersky & Gershenzon, 2002; Eltz & Lunau, 2005; Eltz *et al*., 2006; Elza *et al*., 2008). After pollination, the *Ophris sphegodes* flowers have been found to emit farnesyl hexanoate, a compound that is normally released by non-receptive female bees depending on the stage of floral development. The compound is thought to deter floral visitors after pollination and and may help to minimize damage to the developing seed and direct polinators to adjacent unpollinated flowers (Pichersky & Gershenzon, 2002). Alfalfa is almost certainly the most important forage species worldwide. It is significant in trade of seed that parallels the use of hay and fodder. The pollination of alfalfa (*Medicago sativa L*.) and subsequent seed production requires 'tripping' of its flowers by insects (Viands *et al.*, 1988).

Honeybees (*Apis mellifera* L.) are used as pollinators for the production of alfalfa (*Medicago sativa* L.) seeds in South West United States of America. The vast majority

of honeybees visiting alfalfa seed fields are nectar collectors and not efficient pollinators. They accidentally trip 2% of all the florets visited out of which 63.5% of the available bloom remain untripped. Foraging honeybees (*Apis mellifera* L.) collect nectar and pollen from the flowers that they visit to provide the nutrient necessary for colony maintenance. It is therefore necessary to increase visitation by pollen and nectar collecting bees (Heinrich & Raven, 1972).

Composition of alfalfa flower volatiles has been investigated in regard to their attractiveness to honeybees. Flower volatiles can be an important factor in preferential bee visitation in lucerne cloves (Buttery *et al.,* 1982). A potential method of increasing visitation is the genetic alteration of alfalfa floral volatile components to amplify the plant attractiveness to honey bees (Henning & Teùber, 1992). Thirty-three floral components have been identified in 3 different alfalfa cultivars (Buttery *et al.,* 1984). (*Z*)-Ocimene (**44**) accounts for > 25% of the total floral volatiles in alfalfa (Loper *et al*., 1971; Buttery *et al*., 1982). Presence of *β*-myrcene **(41**), linalool **(46**), limonene (**65**), methyl salicylate (**248**), nonylacetate (**273**), and undecyl acetate (**274**): *cis*-3-hexenyl acetate (**271**), 2-methylbutanol (**275**), 2-pentanol (**276**), and heptan-2-one (**277**) have also been reported by the same group.

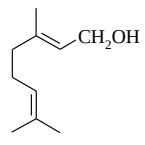


273 **274** **275** **276** **277**

Waller *et al.* (1972) analyzed honeybee olfactory training recognition of four volatile compounds including *β*-myrcene (**41**) (*Z*)-ocimene (**44**), linalool (**46**), limonene (**65**). Honeybees trained on the *β*-myrcene (**41**), (*Z*)-ocimene (**44**) limonene (**65**), and could

not differentiate between them. However, they could differentiate linalool (**46**) from all the other compounds. Later, the honeybees were trained on the scent of alfalfa from specific clones containing *β*-myrcene (**37**) (6.2%), (*Z*)-ocimene (**44**) (83.4%), linalool (**46**) (0.3%) and limonene (**65**) (3.5%). The bees chose to forage on the essential oil of the first three and not the linalool (**46**) clone and also identified alfalfa clones for visitation. However, the experiment could not demonstrate honeybee attraction or repellency towards floral volatile compounds.

During electro-antennographic assay of individual compounds, linalool (**46**) and methylsalicylate (**236**) elicited 'sticky' antennal signals (slow recovery towards baseline physio-electrical output) (Henning & Tueber, 1992). The 'sticky' response suggests that it is biologically active. Coincidentally, linalool (**46**) has a structural similarity to honeybee aggregation pheromone, geraniol (Nasonov) (**278**) which is also present in many plants (rose, lemon grass, geranium, lavender citronella oils) (Henning & Teuber, 1992). Chemical structure-activity relationship (insect antennal response) is dependent upon the compounds structural 'fit' in pheromone chemoreceptor cells (Struble & Arns, 1984). The closer the structural 'fit' the greater the antennal response. Non-preference for linalool (**46**) by honeybees trained to alfalfa flowers shows low concentration and possible masking by more prevalent volatile components (Waller *et al.*, 1972).



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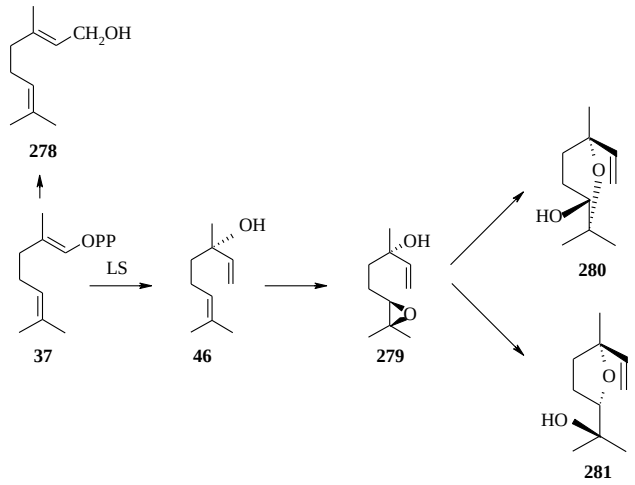
Linalool (**46**) is largely emitted by pistils and the stamens but to a lesser extent by petals (Pichersky *et al*., 1994). Three linalool oxides: 6, 7-epoxy linalool (**279**), linalool oxide (pyranoid) (**280**) and linalool oxide (furanoid) (**281**) are produced and emitted

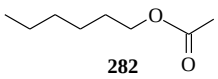
exclusively by the pistil (Pichersky *et al*., 1994). Linalool synthase is responsible for converting the C_{10} isoprenoid precursor GPP (**37**) to linalool (**46**) as shown in scheme 7.



Scheme 7: Biosynthesis of linalool and its oxides (Pichersky *et al*., 1994)

β-Myrecene (**41**), (*Z*)-ocimene (**44**) and limonene (**65**) give 'non sticky' response (faster recovery towards baseline physio-electrical output). *β*-Myrcene (**41**), (*Z*)-ocimene (**44**) and limonene (**65**), are chemically identical, but not structural 'fits' for Nasonov pheromone chemoreceptor (Henning & Teuber, 1992).

Another alfalfa floral volatile 3*Z*-hexenyl acetate (**271**), elicits a 'non sticky' response. It is structurally similar to the alarm pheromone, *n*-hexyl acetate (**282**) and exhibits a large antennal response for defense and so of no proper significance to pollination (Henning & Teuber, 1992). A similar response was observed from *Psila rosae* upon exposure to this compound.



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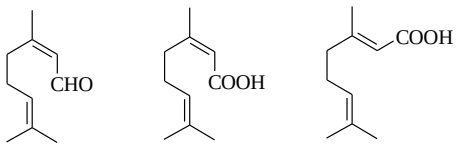
Loper *et al*., (1971) provided evidence that alfalfa floral volatile composition was under genetic control by comparing a limited number of parents and their F_1 offspring. For enhanced pollination and increased yields, plant breeders should select for populations with increased emission of these compounds and bee keepers should choose queens whose offsprings are more antennally sensitive to linalool (**46**) and methyl salicylate (**248**), but discriminates against 3*Z*-hexenyl acetate (**271**) (Henning & Teuber, 1992). Honeybee antennal non-discrimination ultimately results in behavioral non-discrimination. Genetic manipulation through selection for linalool (**46**) and methyl salicylate (**248**) against *n*-hexyl acetate (**282**) appears to offer great potential for increasing the attractiveness of alfalfa to honey bees.

It has also been suggested that knowledge of the flower volatiles from alfalfa would provide information on the compounds that act as attractants for pollinating insects (Buttery *et al.*, 1982; Dobson, 1994). It is possible that flower volatiles of other legumes like cowpea may also be an important factor in pollination due to attractancy to bees.

Common honeybee (*Apis mellifera)* emits a mixture of volatiles including 2-*Z*-citral (neral) (**283**) (a major constituent of lemon-grass oil), nerolic acid (**284**), geraniol (**278**) (from oil of roses) and geranic acid (**285**) in order to attract other bees to a prime source of nectar that it has discovered (Mann, 1978). They act as aggregation pheromone and therefore equally important in pollination. For increased pollination efficiency and increased yields, beekeepers should select queens whose offspring can produce enhanced

levels of aggregation components to attract more bees to the prime sources of pollen and nectar.



283 **284** **285**

2.7.7 Floral volatiles and agricultural biotechnology

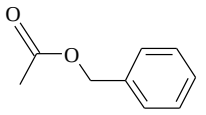
Agriculture involves the transformation of wild species of plants and animals into strains that are amenable to the provision of food, feed, fiber, and industrial uses through cultivation and husbandry. During 5,000-10,000 years of agricultural history, increase of the crop production came from taking additional lands into cultivation, an action that disturbs wild flora and fauna and may contribute to the loss of species and radical changes of landscape (Sharma *et al*., 2000). Further advances in agricultural productivity are needed to meet the food, fiber and industrial demands due to increasing populations, add nutritional value and ensure food security, and reduce use of marginal or fragile lands, by moving towards a more sustainable agriculture that will preserve and restore diversity of flora and fauna. Capability to identify and manipulate plant genes that increase productivity and nutritional values now exist. These biotechnologies enable scientists to conduct more informative research into the genetic and physiological basis of crop growth and environmental responses and interactions. New prospects have emerged for more effective and efficient improvement of crop performance across a range of environments. New techniques are available to transfer genes among species. These capabilities are being used to complement and enhance traditional crop breeding practices. Effective use of the new tools will require their integration into overall schemes of plant breeding and field evaluation. Effective stewardship of genetic

resources is a prerequisite to achieving the goal of a productive, sustainable, and environmentally harmonious agriculture (Sharma *et al*., 2000). New biotechnologies in agriculture and medicine will continue to play an increasing role in our daily lives. These include gene expression analysis to identify important genes and marker-assisted selection to efficiently select new varieties. Also included are transgenic products such as those that confer herbicide or insect resistance traits to crops. Varieties with enhanced nutritional values are on the horizon, and many other possibilities exist. It is important to discover which new approaches can improve sustainable crop productivity, human health, and environmental conservation.

The role of individual volatiles in pollinator attraction can be tested by genetic manipulation of floral emission using appropriate mutants and transformants (Pichersky & Gershenzon, 2002). (*S*)-Linalool synthase, the first floral volatile enzyme has been purified from *Clarkia brewerie* (Pichersky *et al*., 1994; Pichersky *et al*., 1995). Information on the amino acid sequence that facilitated the isolation of the corresponding gene expressed solely in flowers has been enabled by (*S*)-linalool synthase (Dudereva *et al*., 1996).

The characterization of additional genes that are involved in the biosynthesis of the phenylpropanoids: eugenol (**247**) and methylisoeugenol (**256**) (Wang *et al*., 1997), benzyl acetate (**286**) (Dudereva *et al*., 1998) and methyl salicylate (**248**) (Wang *et al*., 1997) has been done. Scent formation is regulated principally by transcriptional control of biosynthetic gene expression at the site of emission for example at epidermal cells of floral parts like petals (Pichersky & Gershenzon, 2002). A gene encoding the enzyme benzoic acid carboxyl methyl transferase (BAMT) (Dudareva & Pichersky, 2000) which

produces a principal floral volatile methyl benzoate (**287**) has also been isolated in snapdragon (*Antirrinium majus*) and shown to be flower specific (Dudereva *et al*., 2000; Murfitt *et al*., 2000). The expression of BAMT which belongs to the same family as SAMT (salicylic acid carboxyl methyl transferase), is correlated with the synthesis of methyl benzoate (**287**) (Dudereva *et al*., 2000; Kolosova *et al*., 2001a).



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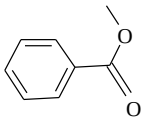

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The expression of (*S*)-linalool synthase is correlated with the synthesis of linalool (**46**) in *C. brewerie*. The gene encoding BAMT is expressed exclusively in epidermal cells of the petals some of which have a conical shape that increases their surface area (Kolosova *et al*., 2001b). There is a higher concentration of the enzyme in the petal near the path taken by bees to reach the nectar hence the scent may serve as guide for bees to find their way inside the flower. The identification of genes for the production of floral scent opens new opportunities to alter floral scent composition for research on enhancing pollination and increasing yields for commercial purposes (Pichersky & Gershenzon, 2002).

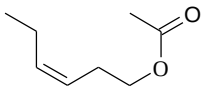
Biotechnology has provided several unique opportunities that include: access to novel molecules, ability to change the level of gene expression, capability to change the expression pattern of genes and develop transgenics with different insecticidal genes (Sharma *et al*., 2000).

For cowpea yield enhancement, the starting point would be the identification of wild, cultivated or inbred cultivars with the most attractive floral scents. The attractive components of the scents would be identified before genetic manipulations through

conventional plant breeding or biotechnological approaches are employed. It is with this in mind that, we chose to investigate the floral volatile composition of a pair of wild, inbred and cultivated cowpea cultivars with the aim of identifying molecular markers that would assist in enhancing flower attractiveness to pollinators.

2.7.8. Volatiles from vegetative parts of cowpea

Several compounds have been identified in intact cowpea plant volatiles (Lwande *et al*., 1989). The major constituents include α-cedrene (**209**) (42.4%), (*Z*)-3-hexen-1-ol acetate (**288**) (28.8%) and hexanal (**247**) (9.4%). The minor constituents include: 1-nonene (**289**), *α*-pinene (**94**), *β*-pinene (**95**), *n*-hexyl acetate (**282**), 1, 8-cineol (**72**), limonene (**65**), (*E)-β*-ocimene **(44)** and nonanal (**290**). Cowpea volatiles increase trap catches of the adult carrot fly, *Psila rosae* (Guerin *et al*., 1983). While *n*-hexyl acetate (**282**) is attractive to cabbage root fly, *Delia brassicae* (Wallbank & Wheatly, 1979). *α*-Pinene (**94**) and *β*-pinene (**95**) stimulate oviposition of the eastern spruce budworm, *Choristoneura fumiferana* (Staedler, 1974).



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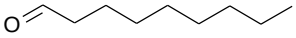

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Volatile compounds originating from the cowpea plant play a role in the orientation of insect pests towards the plant and recognition of the plant for feeding and oviposition (Visser, 1986). Knowledge of the volatile compounds associated with cowpea is useful in studies of insect pest-cowpea plant relationships (Lwande *et al*., 1989). Cowpea is a cross fertilized species relying on insects for pollination (tripping) and therefore genetic manipulation to select for cultivars that attract pollinators could lead to increased cowpea

yields. Floral volatile is one molecular marker that can be used to select bee attractive cowpea cultivars. No cowpea floral volatile studies have been reported to date.

Qualitative and quantitative investigations of the cowpea floral volatiles may lead to a better understanding of the factors that influence pollination of the various cultivars. Furthermore, comparison of the quality and quantity of nectar in the cowpea cultivars with the aim of understanding its influence on cowpea pollination is necessary. These may assist in cowpea breeding programmes, especially genetic manipulation to identify and enhance production of compounds which are behaviorally and nuitritionally important to honeybees, enhance crop yields and select for cultivars that are prefered by pollinators.

2.7.9 Isolation of plant volatiles

Generally plant volatiles occur in very small quantities in comparison to the main components of plant material (Dobson, 1994). Rapid advances in combined gas chromatography-mass spectrometry (GC-MS) technology during the past 40 years have accelerated the development of sensitive, reproducible chemical analyses of floral volatiles (Bergström *et al*., 1980; Williams, 1983; Bicchi & Joulain, 1990; Dobson, 1991; Kaiser 1993; Pecetti & Tava, 2000; Knudsen *et al*., 2004). The floral scent compounds of many plant species have been collected by different methods including, steam distillation, hydrodistillation, enfleurage, solvent extraction and headspace trapping: static, dynamic and solid phase micro extraction collection (SPME) usually followed by GC-MS analysis (Ameenah, 1994; Flamini *et al*., 2002; Fernando & Grun, 2001, Shang *et al*., 2001; Pecetti & Tava, 2000; Tava *et al*., 2000; Dobson, 1994; 1991; Bicchi & Joulain, 1990; Loper & Waller, 1970). Headspace trapping is the most prefered of these methods (Knudsen *et al*., 1993; Dobson, 1991; Hills & Schutzmann,

1990). It generates floral volatile profiles devoid of wound related compounds and extraction artifacts (Bergström *et al*., 1980; Mookherjee *et al*., 1990; Dobson, 1991). In dynamic headspace technique, scent compounds are concentrated in a small glass or plastic chamber enclosing the living floral tissues and are swept by a flow of filtered air over a cartridge packed with adsorbent particles (Dobson, 1991; Bicchi & Joulain, 1990; Williams, 1983). In static headspace method, the adsorbent satchets hang on the floral parts without passing air.

Trapped volatiles are removed from cartridge by solvent elution. The solvents that have been used for elution of trapped volatiles include hexane (Raguso & Pichersky, 1995; Loughrin *et al*., 1993; Haynes *et al*., 1991; Hills & Schutzmann, 1990; Hills, 1989), dichloromethane (DCM) (Ware *et al*., 1993; Heath *et al*., 1992; Patt *et al*., 1988), diethyl ether (Bergström *et al*., 1992; Bergström & Bergström, 1989; Dahl *et al*., 1990; Groth *et al*., 1987; Dobson, 1987) and pentane (Andersson, 2003; Dobson *et al*., 1990; (Bergström *et al*., 1995; Tollsten & Bergström, 1993; Borg-Karlson *et al*., 1985; Nilsson, 1983). DCM has also been used as an internal standard (Azuma *et al*., 2001).

The adsorbents that have been used for the trapping of volatiles include porapak Q (Raguso & Pellmyr, 1998; Bergström *et al*., 1995; Olesen & Knudsen, 1994; Dobson, 1987; Groth *et al*., 1987; Nilsson, 1983; Centelo & Jacobsen, 1979), tenax (Andersson & Dobson, 2003; Andersson *et al*., 2002; Tava *et al*., 2000; Bouwmester *et al*., 1999; Tava & Pecetti, 1997; Borg-Karlson *et al*., 1985; Robertson *et al*., 1994. Haynes *et al*., 1991; Loughrin *et al*., 1993; Hamilton-Kemp *et al*., 1990) and activated charcoal (Bartak *et al*., 2003; Raguso & Pichersky, 1995; Pichersky *et al*., 1994; Sazima *et al*., 1993). The absorbents have also been used as combinations: tenax with activated charcoal (Patt

et al., 1988) and tenax with carbotrap (Knudsen *et al*., 2004; Jurgens *et al*., 2002; Knudsen & Klitgard 1998).

Steam distillation and headspace trapping have been widely adopted in collection of volatiles in forage species. Classical steam distillation allows the investigation of higher boiling point compounds, which may be relevant for the odor characteristics and determination of the total concentration of the volatiles (Scheirer, 1984).

SPME, a fast and a technique that does not use solvent, is based on the adsorption of chemical compounds onto an extracting phase like polydimethylsiloxane (PDMS) on a fused silica fiber. The adsorbed volatiles are then thermally desorbed from the fiber in the injector port of GC. (Flamini *et al*., 2003; 2002; Shang *et al*., 2001; Fernando & Grun, 2001).

Volatiles that are solvent eluted or thermally desorbed from adsorbents are then analysed by GC-MS and comparisons done. The sampling method can influence the composition of volatiles significantly (Scheirer, 1984; Dobson, 1994). Steam distillation at atmospheric or reduced pressure has been used in alfalfa (Buttery & Kamm, 1980), tall fescue, red and white clover (Tava *et al.*, 1995) and compared to the dynamic headspace sampling of volatiles using tenax® (Buttery *et al.,* 1984 (Mayland *et al.,* 1997; Tava & Pacetti, 1997).

2.7.10 Ultra Violet (UV) Visible Spectroscopy (Spectrophotometry)

This technique involves the sspectroscopy of photons in the UV-Visible region. It uses light in the visible and adjacent near ultraviolet (UV) and near infrared (NIR) range.

Samples are placed in a transparent cell called cuvette. Fused silica and quartz are used as sample holders. Glass and plastic may be used but the disadvantage is that they also absorb light. It gives the UV-spectrum. This method is used to identify sugars. Sugars are organic compounds of high degree conjugation. It is based on the principles of Beer Lambert's law. It measures the intensity of light before it passes through the sample and after it passes through the sample. This is measured and is called trasmittance and is usually expressed as absorbance. If interfaced with personal computer they appear as peaks at different wavelengths and different times. This then characterizes the sugars. (Skoog *et al*., 2006; Clark *et al*., 1993).

CHAPTER THREE

METHODOLOGY

3.0 General experimental procedures

3.1 Reagents, apparatus and stardards

All solvents (acetonitrile, DCM, hexane and acetone) were obtained from Sigma-Aldrich Chemical Company (Dorset, England), Kobian Ltd. (Nairobi, Kenya) and Science-Scope Ltd (Nairobi, Kenya). Reference compounds were obtained from Sigma-Aldrich (Dorset, England) and Mayer & Baker (Birmingham, England). The adsorbents were acquired from chrompack (Middlebourg, Netherlands) and J.T. Baker (New Jersey, USA). The HPLC column for nectar sugar analysis was obtained from Supelco Chemical Co., (Pennslyvania, USA) through Sigma-Aldrich (Dorset, England). All glassware was obtained from Superior Company (Johnson city, Germany) and Drummond Scientific Company (Broomall, Pennslyvania, USA). Re-usable glassware, (collection chamber, glass tubing, columns and sample vials) were washed with hot water and soap, rinsed with cold water, acetone and finally distilled water then dried at 110 ºC for 1 h in a clean oven.

3.2 Cleaning of adsorbents

The adsorbents used were: porapack Q (80/100 mesh, Chrompack) (Raguso & Pellmyr, 1998; Bergström *et al*., 1995; Olesen & Knudsen, 1994; Dobson, 1987), reverse-phase C_{18} bondeded silica (40 µm), J.T. Baker, New Jersey, USA), and activated charcoal (80-100 mesh, Chrompack, New Jersey) (Bertak *et al*., 2003; Raguso & Pichersky, 1995; Pichersky *et al*., 1994; Sazima *et al*., 1993). Adsorbents were cleaned by soxhlet extraction for 48 h using DCM wrapped with aluminium foil, dried at 60 ºC and conditioned to activate it in readiness for trapping. Porapak Q traps were conditioned by heating under a stream of N_2 in a GC oven, at 110 ºC for 30 min., cooled under a stream

of N_2 and wrapped in a clean aluminium foil. Similarly, activated charcoal traps were conditioned by heating under N_2 at 250 ºC for 30 min., cooled under nitrogen and wrapped in a clean aluminium foil. Likewise, reverse-phase C_{18} bonded silica traps were dried under a stream of N_2 at room temperature and wrapped in a clean aluminium foil.

3.3 Preparation of traps

For static headspace volatile collection, traps were prepared by packing satchets of adsorbents (50 mg) in filter paper enclosed in wire gauze except for activated charcoal, which was packed in wire gauze alone. One of the packed sachets not used for trapping was extracted with DCM (4 ml) into a clean vial and the resulting solution concentrated to 10 µl using N_2 while cooling in ice (Jacobsen & Olesen, 1994; Porter *et al*., 1999; Miyake *et al*., 1998). The concentrated extract (2 µl) was injected into a gas chromatograph (GC) for analysis for any impurities in control traps.

3.4 Study location

The study was done in green houses (Dotterl *et al*., 2005; Porter *et al*., 1999; Sazima *et al*., 1993; Sutton *et al*., 1992) and laboratories at ICIPE, Nairobi (1º 14´ S, 36º 52´ E).

3.5 Plant material

Three cowpea lines with two cultivars in each line: wild inbred SP52, SP46; cultivated 524B, ICV12; wild out crossed 219 and 269 were used. The plants were propagated through seed (Raguso & Pichersky, 1995; Sazima *et al*., 1993) and vegetative cutting (Loper & Waller, 1970). Inbred lines were prepared by self-pollinating plants through "tripping" to minimize genetic variation. The plants were originally obtained from West Africa, Coastal parts of Kenya and USA. The wild inbred cultivars SP52 and SP46 were obtained from Cameroon in West Africa while 524B was from Califonia and ICV 12

aquired from Kakamega, Western Kenya and were cultivated in the green house at ICIPE. The wild outcrossed were collected from Coastal parts of Kenya. Cultivar 219 came from West of Msambweni and South West of Shimba Hills. Cultivar 269 was from Taita Hills and Mwatate (Figure 2).

Cultivars ICV12 and 524B, the seeds were planted directly in 2000 ml cylindrical pots (Plepys *et al*., 2002) containing sandy soil that was enriched with fertilizers. The seeds for the wild inbred varieties, SP52 and SP46, were cleaned by scrubbing, soaked in water for one day and planted in 250 ml pots with sandy soil enriched with synthetic fertilizer. After germination, the seedlings were transplanted into 2000 ml cylindrical pots with sandy soil and fertilizer.

The vegetative cuttings of SP52, SP46, 219 and 269 cultivars, obtained from plants already present in the green house and also from the field, were planted in 2000 ml cylindrical pots with sandy soil enriched with fertilizer. They were covered with plastic bottles or bags until new leaves appeared, and 2-5 week old flowering plants used in nectar and floral volatile collection. The plants were supported with sticks for climbing as they grew and sprayed with fenitrothion to control pests and viral infections (Jones, 1965; Erbaugh *et al*., 2002). Anatone, a flowering hormone, was applied to induce flower production in wild inbred (SP46 and SP52) and wild outcrossed (219 and 269) cultivars.

3.6 Flower sizes

The sizes of cowpea flowers were determined by width. The width (diameter) cowpea of open cowpea flowers for the extraction of nectar and volatiles were taken using 300 mm ruler and recorded in millimeters (Galetto & Bernardello, 2004; Azuma *et al*., 2001).

3.7 Nectar extraction

Nectar from each flower was extracted with, a 10 µl disposable micro-pipette (Drummond Scientific Co., Broomall Pennsylvania, USA) calibrated into 76 mm, hourly from 6.00 and 10.00 am without detaching the flowers from the plants (Langenberger & Davis, 2002; Silva & Dean, 2000; Bernardello *et al*. 1994). The nectar volume was determined by the following relationship:

Volume of nectar (µl) = (Height of nectar in pipette (mm)/76 mm) x 10 µl

3.8 Analysis of nectar

Same samples collected for nectar volume measurement were used to determine the sugar composition. The extracted nectar was spotted on Whatman No. 1 filter paper (Perret *et al*., 2001; Bernardello *et al*. 1994; Van Wyk, 1993). The filter paper labeled, air-dried and stored at -20 ºC in a clean dry vial until sugar analysis was done, in order to limit changes caused by nectar aging, until sugar analysis was done (Baker & Baker, 1983a). The nectar was eluted from the filter paper with 30 µl distilled water and analyzed by HPLC and LC-MS using amine column (Silva & Dean, 2000; Kearns & Inuoye, 1993).

3.9 High performance liquid chromatography (HPLC)

Analysis of nectar sugars was performed on the Beckmann HPLC System Gold with programmable solvent Module 126, and interfaced with IBM computer linked to Epson LQ-570 printer and Dessaga Sarstedt- Gruppe intergrator (Hewlett Packard, New York, USA). Detection was achieved using the Beckmann diode array UV detector Module 168 (Hewlett Packard, New York, USA). Separation was achieved using a Supelcosil ^{TM} LC-NH_2 (nucleosil amine) column (25 cm × 5 µm) with guard column (Supelco High Chrom, Bellefonte, Pennsylvania, USA). The column had the following characteristics:

silica stationary support; spherical particle shape; aminopropylsilyl bonded phase; 100 Å pore size, 170 m^2/g surface area; 0.6 ml/g pore volume, and pH 2-7.5. HPLC was performed isocratically at 1 µl/min. The sample (20 µl) was injected and the chromatograms analyzed for sugars. Freshly prepared standard sugar solutions (20 µl, 1 mg/ml of distilled water) (Van Wyk, 1993), were analyzed to confirm the sugars present by comparison of the retention times (R_t). The sugar standards: sucrose, glucose, fructose, (Nepi *et al*. 2003; Van Wyk, 1993) raffinose, mannose, maltose and lactose were used. Sugars present in nectar were characterized by comparing retention times (R_t) of standards, peak enhancement and LC-MS data. HPLC peak enhancement was achieved by analysis of 10 µl of standard sugar solution and an equal volume of nectar. The amounts of each sugar present in the nectar was quantified from calibration curves of HPLC peak areas or heights of standard solutions (Silva & Dean, 2000; Van Wyk, 1993).

3.10 Liquid chromatography-Mass Spectrometry (LC- MS)

The LC-MS was performed on a VG Platform 11 mass spectrometer (Fisons Insruments, Birmingham, UK) with electrospray (ES) ionization source using atmospheric pressure ionization (API). Control of the system was achieved by Mass Lynx for Windows NT 3.51, in Digital Celebris GL 5120 SL PC, using Microsoft Windows Graphical Environment. Ions from the mass analyzer were detected by dynode detector system; carrier gas used was white-spot N_2 gas. The cycle time was 3 sec., with scan duration of 2 sec. and interscan delay of 1 sec. The mass range was from 38 to 1000 amu. The ion source was maintained at atmospheric pressure and 120 $^{\circ}$C while the analyzer was at 2.5 x 10^{-5} mbar (Silva & Dean, 2000).

3.11 Collection and analysis of volatiles

3.11.1 Static headspace trapping

The diagrammatic representation of static headspace volatile collection is shown in figure 3.

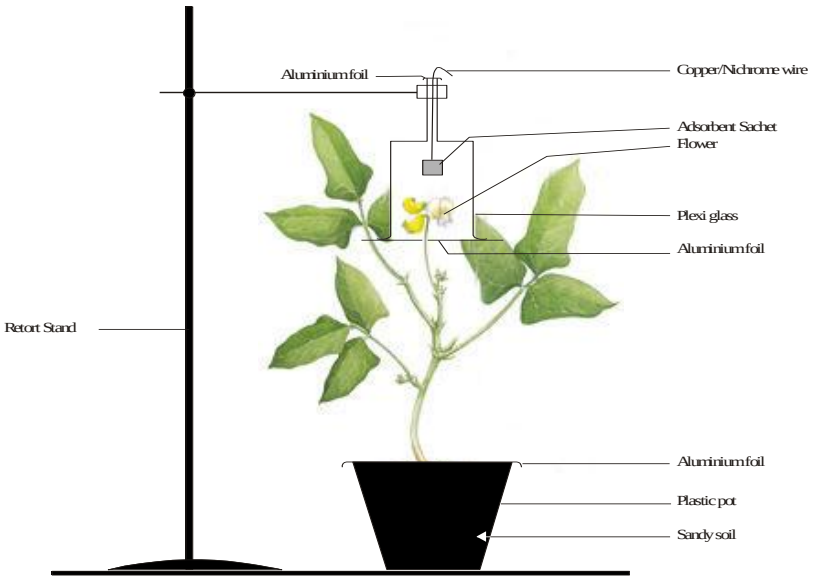


Figure 3: Diagramatic representation of static headspace trapping

The volatiles were collected from 2-5 week old plants between 6.00 and 10.00 am when the flowers are open (Anderson *et al*., 2002). The field collection was done using: activated charcoal (Raguso & Pichersky, 1995) reverse-phase C_{18} bonded silica and porapak Q (Raguso & Pellmyr, 1998; Bergström *et al*., 1995; Olesen & Knudsen, 1994; Dobson, 1987; Groth *et al*., 1987; Nilsson, 1983; Centelo & Jacobsen, 1979). The base of the flower and soil in the pot were covered with aluminium foil. The flower was enclosed in a cylindrical plexiglass vessel supported by retort stands and aluminium foil was also used to seal the space on the plexiglass and the soil on the pot was also covered with aluminum foil to prevent from the volatiles they release from being trapped. The scent was trapped in the adsorbent enclosed in a sachet hanging directly above the flower for 4 h until it closed (Loper & Waller, 1970). The sachets were removed from the

glasses, wrapped in aluminum foil, stored in bottles already dipped in ice. They were taken to the laboratory near the green house for further analysis.

3.11.2 Hydrodistillation

Varying amounts (200-300 g) of flowers, collected from the green house, were placed into distillation flask and 500 ml of water was added. The flask was directly heated and the distillate collected over hexane (4 ml) using clean Dean & Stark apparatus. The volatiles accumulated in hexane were dried using 5 mg anhydrous sodium sulphate and filtered into a clean vial. The hexane solution was concentrated to 20 µl using a gentle stream of N_2 gas and stored at $^{-}4$ºC when required for analysis (Ameenah, 1994).

3.11. 3. GC analysis

The adsorbent sachets used for trapping cowpea flower volatiles were eluted with 4 ml of HPLC grade CH_2Cl_2. The eluant was concentrated to 10 µl under a gentle stream of N_2 gas (Jacobsen & Olesen, 1994; Porter *et al*., 1999; Miyake *et al*., 1998) while cooling in ice and stored in teflon-capped glass vials at -20 ºC. The sample (2 µl) was subjected to GC analysis (Azuma *et al*., 2001; Olesen & Knudsen, 1994). Alternatively, the flowers were hydro distilled, concentrated and subjected to GC analysis. GC analysis of volatiles was performed on Hewlett Packard (HP) 5890 Series II capillary GC equipped with a splitless injector, a flame ionization detector (FID) and a HP 3396 Series II integrator (Hewlett Packard, Minnesota, USA). The separation was done on a HP cross-linked methyl silicone capillary column (50 m × 0.2 mm, d × 0.33 µm, film thickness) (Hewlett Packard, Minnesota USA). The carrier gas used was nitrogen while analytical grade hydrogen was used as fuel together with medical air (pure oxygen). The oven temperature was programmed from 40 (5 min.) to 280 °C (20 min.) at 5 °C/min. for trapped volatiles or 40 (5 min.) to 100 at 5 °C/ min., to 280 °C (35 min at 10 °C/ min.),

for hydro-distilled essential oils. The integrator was set at: attenuation of 2, chart speed 0.5, area rejection 200 and thresh hold 2.

3.11.4 GC-MS analysis

The GC-MS analysis was done on a HP 8060 Series II gas chromatograph coupled to VG Platform II mass spectrometer (Fisons, Birmingham, UK). The MS was operated in the electron ionization (EI) mode at 70 eV with emission current of 200 μA and a multiplier voltage of 300 V. The ion focus was held at 29 V. The temperature and pressure of the source was held at 180 ºC and 9.4 x 10^{-6} mBar, respectively. The scan speed was 112 to 1697 amu/sec while the scan duration was 1 sec. with a cycle time of 1.5 sec. and interscan delay of 0.50 sec. The solvent delay time was 0 sec while the analyzer mass range of was 38 to 650 amu. The mass spectral identification of the compounds was carried out by comparison with NIST (National Institute of Standards and Technology, Gauthersberg, MD) (Jurgens *et al*., 2003; Azuma *et al*., 2001) and Wiley 6.0 (Wiley, New York) mass spectral libraries. The identified compounds were confirmed by GC co-injection through peak enhancement. The retention time of the standards was also established to confirm identity (Jennings & Shibomoto, 1980; Davies, 1990). The standards were run both on the GC and GC-MS. The generated spectra of known standards were compared with those of the sample compounds (Azuma *et al*., 2001., Knudsen & Stahl., 1994) and the MS library data (Rodel & Petrzika, 1991; Adams, 1989; Stenhagen *et al*., 1974).

Samples from the six cowpea cultivars obtained by the different techniques were subjected to GC-MS.

3.11.5 Statistical analysis

The amount of sugars present in the nectar was quantified from the calibration curves of HPLC peak areasor heights of standard solutionsfrom regression analysis using SAS software (Hatcher, 2003). The nectar sugar concentrations in the six cultivars were subjected to two way factorial ANOVA and statistical analysis (Armstrong *et al*., 2000) using the LSD to obtain p values.

CHAPTER FOUR

RESULTS

4.1 Flowering and flower sizes

Although all cultivars gave flowers, the wild inbred cultivars (SP52 and SP46) and wild outcrossed (219 and 269) flowered with a lot of difficulty and required application of hormone (anatone) to induce flowering. The individual flowers bloomed for one day only. The bell-shaped cowpea flowers were coloured as follows: 524B yellow (Plate 1), ICV12 medium purple (Plate 2), SP46 orchid (Plate 3), SP52 violet (Plate 4), 219 psychedelic purple (Plate 5) and 269 electic purple (Plate 6).

Plate 1: Photograph of flowering cowpea cultivar 524B.

Plate 2: Photograph of flowering cowpea cultivar ICV12

Plate 3: Photograph of flowering cowpea cultivar SP46

Plate 4: Photograph of flowering cowpea cultivar SP52

Plate 5: Photograph of flowering cowpea cultivar 219

Plate 6: Photograph of flowering cowpea cultivar 269

The flowers opened before 6.00 a.m. and closed between 9.30 a.m. and 10.30 a.m.The flower sizes were measured and recorded. The data is summarized in fig. 4.

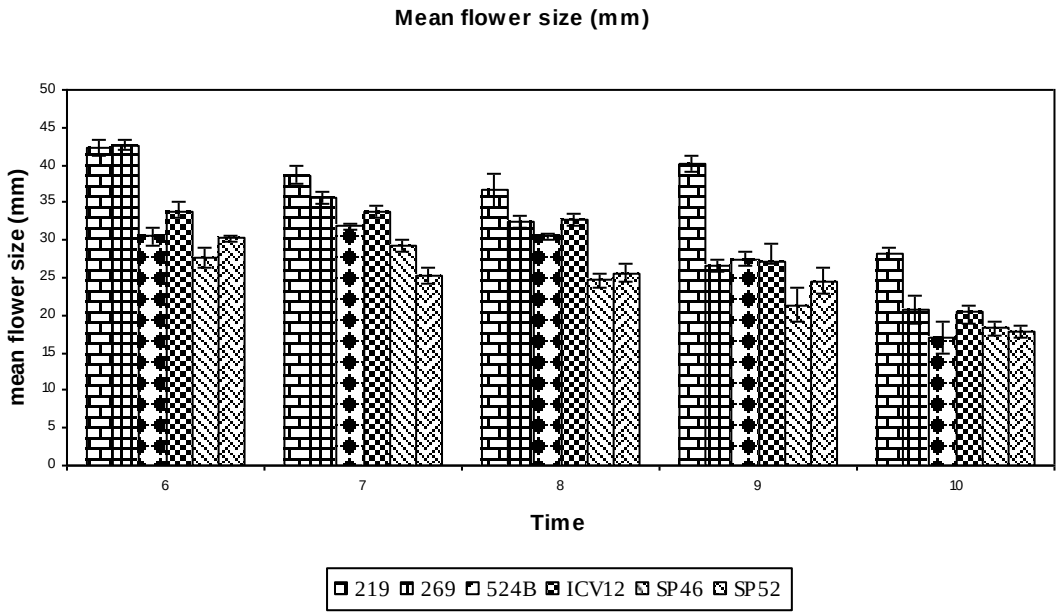


Figure 4: Mean sizes of cowpea flowers

The wild outcrossed cultivars (219 and 269) produced the biggest flowers, (42.62 ± 0.65 and 42.38 ± 1.10mm respectively), followed by cultivated cultivars (ICV 12 and 524B) (33.88 ± 0.93 and 32.87 ± 0.39 mm respectively) while the wild inbred cultivars (SP46 and SP52) produced the smallest flowers (17.13 ± 0.65 and 17.75 ± 0.79, respectively). There was no significant difference between the flower sizes of wild outcrossed cowpea cultivars (219 and 269). Similarly, there was no significant difference between the flower sizes of the cultivated cowpea cultivars. Likewise, there was no significant difference in the flower sizes of wild inbred cowpea cultivars (SP46 and SP52). However, significant difference was noted between the flower sizes of wild outcrossed, cultivated and wild inbred cowpea cultivars.

4.2 Nectar Production

The mean volume of nectar produced between 6.00 and 10.00 am for the six cowpea cultivars are summarized in figure.5

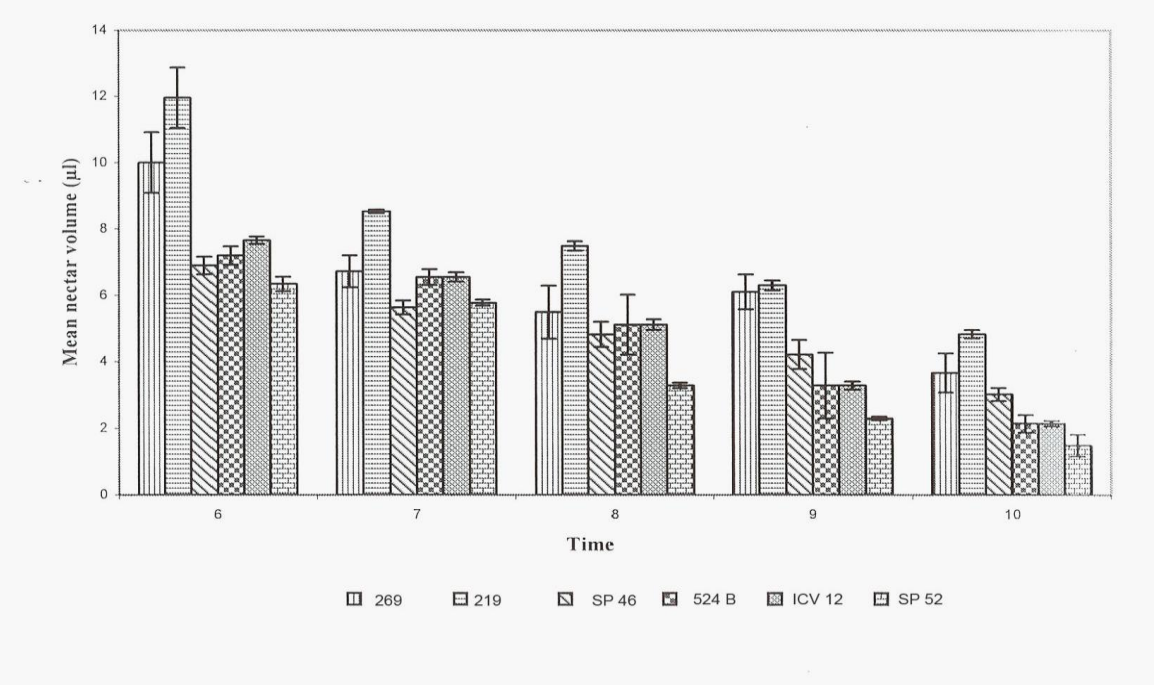


Figure 5: Mean volume of cowpea flower nectar

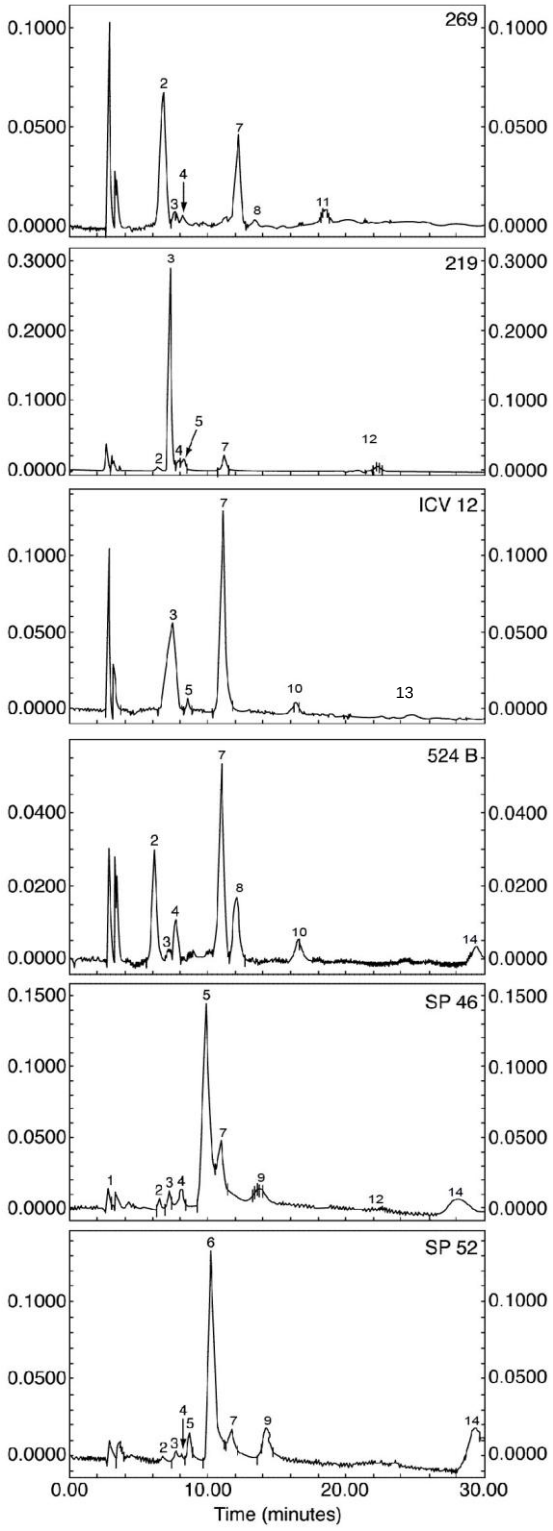
The pattern of nectar production was the same in all the six cultivars in the three lines. Nectar volume ranged from 0.2-18 µl with a mean of 2.28 ± 0.058 µl. Cultivars 219 and 269 produced big flowers flowers and more nectar. Cultivar 219 recorded the highest nectar volume (10.99 ± 0.78 µl) followed by 269 (10.29 ± 0.93 µl), 524 B (7.28 ± 0.82 µl), ICV 12 (6.99 ± 0.99 µl), SP 52 (3.49 ± 0.63 µl) and SP.46 (3.65 ± 0.59 µl). There was no significant difference in mean nectar volumes for the two wild outcrossed cowpea cultivars (219 and 269). Similarly, no significant difference was observed in mean nectar volumes between the two cultivated cowpea cultivars (524B and ICV12). Likewise, there was no significant difference in the nectar volume of wild inbred cultivars. The volume of nectar collected was generally high between 6.00 and 10.00 am

for all the six cultivars and correlates well with the activity of the pollinators suggesting that cowpea nectar is produced at night to facilitate bee visit in the morning. Nectar production occurred during the day before 8.00 am with the highest volume being collected at 6.00 am for all the six cultivars. The nectar volume reduced with time (6.00-10.00 am) with the lowest volume of nectar being recorded at 10.00 am. Reduced volumes of nectar were recorded after 9.00 am for all the lines.

However, significant differences were noted in nectar volume of wild outcrossed, cultivated and wild inbred.

4.3 Nectar sugar composition

Seven sugars were identified in nectar from the six cowpea cultivars (Table 4). Three sugars: glucose, fructose and sucrose, are found in varying amounts in the nectars from all the cowpea cultivars. Although all the nectars contained the three sugars in different combinations, they were not balanced, (equal quantities). The three most common nectar sugars did not occur singly in any cultivar but as a combination of three or in two. Necta from the six cowpea cultivars were sucrose dominant (S/G+F >0.999). (Mannose, maltose and raffinose, though rare, were detected in some cowpea nectars. Galactose was not detected in any of the cowpea nectars investigated. There was no significant difference in mannose and raffinose concentration in all the cultivars. For sucrose concentration, there was no significant difference in 524B and ICV 12; 219 and 269 and SP 46 and SP 52 cultivars. No significant difference was observed for maltose concentration in ICV 12 and 219 cultivars. For fructose, there was no significant difference detected in nectar of cultivars SP 46 and ICV 12 while the other cultivars were significantly different. The nectar from the 6 cowpea cultivars was subjected to HPLC analysis. The HPLC profiles of the nectar from 6 cultivars are presented in figure 6.



Key

Peak No.	Name
1	Allose
2	Xylose
3	Fructose
4	Mannose
5	Glucose
6	Unidentified
7	Sucrose
8	Maltose
9	Unidentified
10	Lactose
11	Unidentified
12	Raffinose
13	Unidentified
14	Unidentified

Figure.6: HPLC profiles for cowpea flower nectar

The UV analogue and LC-MS profiles are in appendix 1.

The peaks were identified by HPLC coinjection of cowpea nectar with sugar standards. The composition of the nectar from 6 cowpea cultivars is summarized in table 1.

Table 1: Sugar composition of nectar from six cowpea cultivars

			Relative peak areas (%)					
Peak No.	R_t (min)	Sugar	269	219	ICV 12	524 B	SP 46	SP 52
1	4.31	Allose[b]	-	-	-	-	1.67	-
2	6.78	Xylose[a]	46.29	0.85	-	22.90	1.30	6.01
3	7.61	Fructose[b]	1.48	75.54	26.29	tr	4.67	3.37
4	7.71	Mannose[b]	tr	0.99	-	5.63	-	0.18
5	8.33	Glucose[b]	-	2.83	1.58	-	-	22.9
6	9.85	Unidentified	-	-	-	-	-	50.47
7	12.18	Sucrose[b]	26	8.8	46.93	39.64	1.46	7.02
8	12.62	Maltose[b]	tr	-	-	1.59	-	-
9	14.31	Unidentified	-	-	-	-	4.61	8.32
10	16.40	Lactose[b]	-	-	1.63	2.52	-	-
11	18.48	Unidentified	2.74	-	-	-	-	-
12	22.26	Raffinose[b]	-	4.13	tr	-	tr	-
13	23.51	Unidentified	-	-	tr	-	-	-
14	28.72	Unidentified	-	-	-	tr	tr	12.32

Identification by: a -R_t only; b - R_t, coinjection and LC-MS

Standard solutions were subjected to HPLC analysis. The HPLC data for sugar stardards is summarized in table 2.

Table 2: HPLC data for sugar stardards

Peak No.	R_t (min.)	Sugar	Absorbance	Peak area (%)
1	4.31	Allose	0.43	77.10
3	7.61	Fructose	0.30	76.39
4	7.71	Mannose	0.05	100.00
5	8.33	Glucose	0.06	100.00
7	12.18	Sucrose	0.06	100.00
8	12.62	Maltose	0.18	35.62
10	16.40	Lactose	0.03	82.00
12	22.26	Raffinose	0.14	26.82

The UV absorbance data of the standard sugar solution were subjected to regressional analysis which yielded the corresponding calibration equations (Table 3).

Table 3: Calibration equations for sugar standards

Sugar	Regression equation	R^2	RMSE
Allose	y = - 0.035 + 0.001x	0.541	0.0337
Fructose	y = - 0.018 + 0.0019x	0.9696	0.0125
Glucose	y = 0.0032 + 0.0002x	0.9982	0.0004
Lactose	y = 0.0042 + 0.0001x	0.8885	0.0011
Maltose	y = 0.0028 + 0.0002x	0.8583	0.0028
Mannose	y = - 0.0436 + 0.002x	0.9451	0.0176
Raffinose	y = 0.0023 + 0.0001x	0.8666	0.0016
Sucrose	y = - 0.0075 + 0.0005x	0.9129	0.0053

The calibration equations were used to approximate the amount of sugars in cowpea nectar (Table 4).

Table 4: Mean (± SE) sugar concentration (mg/ml) in nectar from six cowpea cultivars

Peak	R_t	Sugar	219	269	524B	ICV 12	SP 46	SP 52
3	7.61	Fructose	1.26±0.2x a, b	4.70±1.2x a, b	2.15±0.6x a, b	6.80±0.1y a	5.00±2.0y a	2.95±1.3x b
4	7.71	Mannose	-	1.00±1.0y a	-	2.00±1.0y a	-	1.52±1.2y a
5	8.33	Glucose	1.10±0.6x a, b	1.62±0.1x a, b	3.20±1.1y a	3.20±1.1y a	1.52±0.5x	3.91±2.7x
7	12.18	Sucrose	4.40±2.3x b	1.098±0.3z b	1.94±0.09z a	2.26±0.37 a	5.30±1.0z c	3.34±0.25x c
8	12.62	Maltose	2.85±1.91z a	8.8±8.0y b	5.20±4.0x b	4.75±4.0x a, b	5.70±2.0y b	3.60±0.1y b
10	16.40	Lactose	2.30±2.0y a, b	1.20±1.0y b	5.70±1.0y b	-	-	1.50±0.1y b
12	22.26	Raffinose	4.00±2.0y a	5.50±1.0y a	1.00±1.0x a	2.80±2.0y a	4.10±1.0y a	4.40±1.0y a

N = 4; means with different letters are significantly different; x = $*10^{-2}$; y = $*10^{-3}$ and Z= $*10^{-1}$

The statistical characteristics for the sugar standards and in nectar are summarized in table 5.

Table 5: Statistical characteristics for sugar standards

Peak No.	R_t (min.)	Sugar	F value	Pr value	t_{conc}	P_{conc}	$t_{intercept}$	Pr $_{intercept}$
1	4.31	Allose	3.54	Pr > F = 0.1566	1.88	0.1566	-0.99	Pr > t = 0.396
3	7.61	Fructose	95.7	Pr > F = 0.0023	9.78	0.0023	-1.37	Pr > t = 0.2632
4	7.71	Mannose	51.62	Pr > F = 0.0056	7.19	0.0056	-2.37	Pr > t = 0.0988
5	8.33	Glucose	1691.62	Pr > F =< 0.0001	41.13	0.0001	8.46	Pr > t = 0.0036
7	12.18	Sucrose	31.42	Pr > F= 0.0112	5.61	0.0112	-1.35	Pr > t = 0.269
8	12.62	Maltose	18.17	Pr > F = 0.0237	4.26	0.0237	0.94	Pr > t = 0.414
10	16.40	Lactose	23.91	Pr > F = 0.0164	4.89	0.0164	3.63	Pr > t = 0.0359
12	22.26	Raffinose	19.42	Pr > F = 0.0216	4.42	0.0216	1.39	Pr > t = 0.2577

95 % confidence level

The statistical characteristics for the sugars in nectar are summarized in table 6

Table 6: The statistical characteristics of the sugars in nectar of six cowpea cultivars

Peak No.	R_t	Sugar	R^2	RMSE	F value	Pr value
3	7.61	Fructose	0.437	0.018	2.8	Pr > F = 0.0487
4	7.71	Mannose	0.205	0.013	0.93	Pr > F = 0.4862
5	8.33	Glucose	0.146	0.032	0.62	Pr > F = 0.6891
7	12.18	Sucrose	0.749	0.053	10.73	Pr > F = < 0.0001
8	12.62	Maltose	0.351	0.161	1.95	Pr > F = 0.1361
10	16.40	Lactose	0.461	0.002	3.08	Pr > F = 0.0351
12	22.26	Raffinose	0.204	0.003	0.92	Pr > F = 0.4882

95 confidence limit

4.4. Essential oils and floral volatiles

4.4.1 Essential oils

The floral essential oils sampled revealed great variation in the quality and quantity. The chemical composition was found to be dependent on the technique of volatile collection and the trapping material. Depending on the technique used for volatile extraction, the composition of the classes varied. The volatiles were classified into six categories on the basis of biosynthetic origin: aliphatics such as decanal, palmitic acid, *n*-hexanal, 1-octene-3-ol; aromatics including toluene, acetophenone, vanillin, methyl eugenol and xylene; monoterpenes like limonene, terpenyl acetate, menthol, linalool and geranyl nitrile); sesquiterpenes such as geranyl acetone, nerolidol, farnesol, bisabolene guaiol and cedrene; norisoprenoids like *β*-methyl ionone, phytol, *β*-cyclocitral and others and other miscellenious compounds that were present but did not have striking similarity (furfural and indole,). The composition of the floral volatiles varied greatly on the basis the categories. The number of compounds in the volatiles from each of the two techniques ranged from 43-109. The composition of the cowpea floral volatiles from hydrodistillation is summarized in table 7. The GC chromatograms are in appendix 2.

Table 7: Composition of cowpea floral volatiles from six cultivars by hydrodistillation

			Relative amounts (%)					
Peak No.	R_t (min)	Compounds	219	269	524B	ICV 12	SP 46	SP 52
		Aliphatic alcohols						
6	12.65	1- Hexanol[a]	-	**1.12**	-	0.04	-	0.3
7	12.7	2*E*-Hexenol[b]	-	-	-	-	**42.34**	-
13	16.63	3*Z*-Hexenol[a]	-	**2.36**	0.75	**1.56**	0.09	0.02
14	17.2	3*E*-Hexenol[a]	tr	tr	-	tr	-	tr
15	17.65	Heptanol[b]	-	**1.05**	0.31	0.57	-	-
26	21.6	1-Octen-3-ol[c]	0.47	**1**	0.13	**4.64**	**4.7**	**2.71**
31	22.1	1-Octanol[b]	0.2	-	-	-	-	-
36	22.53	3-Octanol[b]	-	**5.23**	-	-	-	**6.08**
		Aliphatic aldehydes						
8	13.5	2*E*-Hexenal[b]	-	-	0.12	**68.75**	**5.12**	-
11	14.57	*n*-Hexanal[a]	0.53	**0.98**	0.35	-	-	0.4
19	18.72	*n*-Heptanal[b]	0.12	0.25	0.3	-	-	-
50	24.88	Nonanal[b]	-	-	-	-	-	0.41
58	26.1	2E-nonenal[b]	-	0.84	**2.58**	0.19	-	0.04
71	29.18	2,4-decadienal[b]	-	**1.62**	-	-	-	-
96	35.03	Tridecanal[b]	**7.92**	**3.23**	**19.61**	-	0.28	0.58

		Aliphatic ketones						
1	9.7	3-Methyl butanone[b]	-	-	-	0.13	-	-
18	18.4	5-Methyl -2-hexanone[b]	-	0.06	-	-	-	0.01
25	21.33	4-Octen-3-one[b]	0.86	**1.36**	**2.17**	-	0.14	0.08
27	21.7	3-Octanone[a]	0.79	-	**18.61**	-	0.28	-
		Aliphatic esters						
10	14.17	*n*-Amyl isobutyrate[b]	-	0.08	-	-	-	-
55	22.4	*n*-Hexyl acetate[b]	-	-	0.15	-	0.05	0.01
100	36.32	Isopropyl myristate[b]	-	0.27	-	-	-	0.14
104	38.6	Ethyl palmitate[b]	-	-	-	0.41	-	-
105	38.88	Isopropyl palmitate[b]	-	0.62	**1.05**	-	-	-
108	40.5	Oleic acid propyl ester[b]	-	**2.37**	-	**2.67**	-	-
		Aliphatic acids						
88	32.95	Lauric acid[b]	-	0.18	-	-	-	0.33
102	37.82	Myristic acid[b]	-	-	**0.99**	**2.97**	-	0.17
103	38.23	Palmitic acid[b]	**63.83**	**43.52**	**18.21**	**7.41**	**11.5**	**8.34**
		Aliphatic hydrocarbons						
2	11.95	2,3,3 trimethyl pentane[b]	**1.45**	-	-	-	0.5	-
3	12.06	Cyclohexane[b]	-	-	-	-	6.3	-
4	12.1	2-methyl hexane	-	-	-	-	**10.62**	**72.33**
5	12.55	2-azido 2,3,3-trimethyl butane[b]	-	-	-	-	**16.19**	-
22	19.4	Nonane[a]	0.24	0.38	-	-	0.07	-
62	27.33	Dodecane[c]	-	-	-	-	-	0.07
84	31.82	*n*-Eicosane[a]	**1.43**	0.8	0.7	**1.29**	-	0.19
110	45.58	Octadecane[b]	-	-	-	-	-	0.07
111	48.13	*n*-Tricosane[b]	0.32	-	0.3	0.3	0.08	-
112	48.8	Pentacosane[b]	-	0.2	**1.22**	0.68	-	0.08
113	55.08	Hexacosane[b]	0.51	0.04	-	-	-	0.08
114	60	*n*-Heptacosane[b]	0.02	-	0.28	0.23	-	0.07
115	65.83	*n*-Octacosane[b]	0.8	0.96	0.45	0.14	0.007	0.06
		Aromatic hydrocarbons						
9	13.7	Toluene[a]	0.17	0.14	0.13	0.02	0.02	0.01
16	17.76	Ethyl benzene[b]	-	-	-	-	0.05	-
17	18.03	*p*-Xylene[b]	0.04	0.14	-	-	-	0.04
20	18.75	*o*-Xylene[b]	-	-	-	0.02	-	0.01
21	18.85	*m*-Xylene[b]	-	-	-	0.14	0.03	0.01
39	23.07	*p*-Cymene[a]	**5.36**	0.73	**8.47**	0.15	-	0.02
46	24.43	*m*-Cymene[a]	0.06	-	-	-	-	0.08
		Aromatic alcohols						
52	24.98	Benzene ethanol[b]	-	0.26	-	0.75	0.1	0.03
61	26.88	*p*-Cresol[a]	-	-	-	**2.01**	-	-
65	27.67	Benzene propanol[a]	-	-	-	0.65	0.05	0.02
68	28.92	Thymol[a]	-	0.52	-	-	-	-
		Aromatic ketones						
42	23.7	Acetophenone[a]	0.09	0.73	0.44	0.88	0.12	0.31
56	25.93	Phenylvinyl ketone[b]	**2.45**	-	-	-	-	0.6
70	29.15	*p*-Methoxy acetophenone[b]	-	**3.03**	**4.38**	-	-	0.4
		Aromatic esters						
98	35.75	Benzyl benzoate[c]	0.26	**9.46**	**1.24**	0.27	0.04	0.3
		Phenyl propanoids						
67	28.25	Cinnamaldehyde[a]	0.5	0.94	-	0.47	0.08	0.18
73	30.26	Methyl cinnamate[b]	0.41	**2.24**	**1.16**	0.66	0.05	0.2
99	35.78	Methyleugenol[a]	0.13	-	-	-	-	0.37
		Monoterpenoid hydrocarbon						
23	20.62	*α*-Ocimene[b]	0.12	0.25	-	-	-	-
29	21.85	*β*-Pinene[a]	0.02	-	0.06	-	-	-
32	22.18	*β*-Myrcene[a]	0.26	-	-	-	-	-
33	22.2	α-Pinene[a]	0.16	-	-	-	-	-
40	23.35	Limonene[c]	**2.18**	**1.51**	0.09	-	0.02	0.03
44	24	Camphene[b]	0.01	-	-	-	-	-
45	24.07	3-Carene[b]	-	0.30	0.57	-	-	0.05
53	25.38	*α*-Phellandrene[b]	-	0.14	-	0.05	-	-
		Monoterpenoid alcohols						
30	21.96	*trans*-α-Dihydroterpeneol[b]	-	-	-	-	0.3	-
41	23.6	1,8-Cineol[a]	0.54	0.10	-	-	-	0.04
51	24.92	Linalool[c]	0.70	**1.84**	**4.08**	0.55	-	0.4

59	26.4	Isopulegol[c]	0.24	-	-	-	-	0.07
72	29.23	Menthol[c]	-	-	-	-	-	0.18
		Monoterpenoid aldehydes						
28	21.8	Citral[c]	-	-	-	-	-	0.22
66	27.72	Isogeranial[b]	-	-	-	-	-	0.11
97	35.35	Tetrahydrogeranial[b]	-	-	-	-	-	0.22
56	25.52	Linalyl acetate[a]	-	-	-	-	-	0.12
		Monoterpenoid ketones						
48	24.7	Fenchone[a]	0.22	0.17	0.26	-	-	0.04
49	24.73	*cis*-Dihydrocarvone[b]	-	-	-	-	-	0.02
57	26.03	Camphor[a]	-	-	-	0.34	-	-
63	27.55	Pulegone[c]	0.22	-	-	-	-	0.07
		Monoterpenoid esters						
34	22.25	Dihydrocarvyl acetate[b]	-	0.36	-	-	-	-
60	26.55	4-Thujene acetate[b]	-	0.11	0.63	-	-	-
		Irregular terpenoids						
43	23.87	Artemisia ketone[a]	0.30	0.17	-	-	-	-
47	24.67	Chrysanthenyl acetate[b]	-	0.09	-	-	-	0.11
		Sesquiterpenoid hydrocarbons						
37	22.72	Germacrene D[b]	-	-	0.01	-	-	-
76	30.62	*trans*-Caryophyllene[b]	-	0.25	-	-	-	0.15
78	30.95	Aromandendrene[a]	-	0.18	-	-	-	0.09
80	31.15	Satevene	0.40	0.10	-	-	-	-
87	32.85	α-Cubebene[b]	-	-	-	-	-	0.02
90	33.35	β-Farnesene[b]	-	0.24	-	-	-	-
91	33.9	β-Bisabolene[b]	-	-	-	tr	tr	tr
106	38.97	Isocaryophyllene[b]	0.25	-	-	0.05	-	-
		Sesquiterpenoids alcohols						
89	33.12	Nerolidol[c]	**1.43**	0.50	**1.84**	-	0.04	0.13
94	34.25	α-Cedrol	-	-	-	-	-	0.02
95	34.98	β-Bisabolol[b]	-	-	0.12	0.1	-	-
101	36.6	Farnesol[c]	**1.43**	-	0.07	-	-	-
		Sesquiterpenoid ketones						
81	31.37	Neryl acetone[a]	-	-	-	-	0.01	0.04
82	31.41	Geranyl acetone[a]	0.15	0.32	0.89	-	-	-
86	32.7	Caryophyllene oxide[a]	-	-	0.14	-	-	-
		Azulenes						
78	30.94	Chamazulene[b]	-	0.05	-	-	-	-
		Norisoprenoids						
64	27.57	β-Cyclocitral[b]	-	0.90	**1.06**	-	-	0.11
74	30.55	Damascenone[b]	-	-	0.49	-	-	-
75	30.6	*cis*-Jasmone[b]	0.05	0.80	**1.53**	0.25	0.04	0.15
77	30.8	*β*-Methyl ionone[b]	-	-	0.36	-	-	-
83	31.43	α-Ionone[c]	0.05	0.22	0.15	-	-	-
85	32.08	β-Ionone[b]	0.45	**1.11**	0.73	0.25	0.03	0.09
90	33.42	Megastigmatrienone 4[b]	-	0.39	-	-	-	0.15
92	33.92	Megastigmatrienone 1[b]	-	0.19	**1.97**	-	0.02	-
93	34.05	Megastigmatrienone 2[b]	-	0.66	-	-	-	0.10
107	40.3	Phytol[c]	0.80	**1.2**	**1.29**	-	0.42	0.8
109	42.62	β-Allylionone[b]	-	-	0.24	-	0.07	-
		Others						
64	27.57	Indole[b]	0.19	-	-	-	-	0.04

Components > 1 % in bold; superscript refer to method of identification: a-retention time, mass spectrometry and co elution used to confirm identity of compounds, b-mass spectrometry used to identity of compounds, c-mass spectrometry and retention time used to confirm identity, - not detected and tr-trace amounts.

Aliphatics were the most abundant compounds in the hydrodistillation, followed by aromatics, monoterpenes, sesquiterpenes, norisoprenoids and other miscellaneous compounds.

4.4.2 Floral volatiles

The volatiles were also trapped from intact cowpea flowers using activated charcoal, reverse phase C_{18} bonded silica and porapak Q. The eluants from different adsorbents were run on GC and GC-MS and the results are summarized in tables 8-10. The GC chromatograms are in appendix 4.

Table 8: Composition of cowpea floral volatiles trapped in activated charcoal

			Relative amounts (%)					
Peak No.	R_t (min)	Compound	219	269	524 B	ICV 12	SP 46	SP 52
		Aliphatic alcohols						
2	8.8	Butanol[b]	**5.66**	**47.04**	0.03	-	-	**3.27**
4	8.08	2-Methyl butanol[b]	0.03	**8.72**	**14.66**	-	0.49	**16.12**
7	9.43	2,2-Dimethyl butanol[b]	-	-	-	-	-	**0.96**
11	10.65	2-Butanone-3-hydroxy[b]	0.23	0.84	0.64	-	**3.47**	0.44
12	13.23	2-Methyl pentanol[b]	0.04	0.18	0.03	0.20	0.25	0.03
16	15.03	2-Hexanol[b]	**1.22**	**1.29**	0.24	-	**3.11**	**2.49**
18	16.43	3-Z-Hexenol[b]	0.10	0.12	**7.79**	-	0.25	**7.82**
19	16.72	4-Hydroxy-2,5-dimethyl-3-hexanone[b]	**3.65**	**3.82**	-	-	**12.70**	**7.82**
24	19.13	2-Nonanol[b]	—	0.24	0.09	0.03	0.42	0.04
27	21.82	3-Octanol[b]	0.24	0.51	0.49	**1.08**	0.73	-
28	22.28	1-Octen-3-ol[c]	0.10	0.52	**3.53**	0.18	**3.24**	0.12
49	36.6	*trans*-Undecene- 1-ol[b]	0.24	0.08	0.46	0.22	0.71	0.15
		Aliphatic aldehydes						
5	9.13	3-methyl butanal[b]	**9.54**	**2.47**	**24.45**	**11.42**	**22.39**	0.48
15	14.55	Hexanal[b]	0.10	0.46	0.36	0.16	0.59	0.10
42	30.3	Decanal[b]	0.23	0.56	0.90	0.41	**1.36**	0.17
52	39.45	Tridecanal[b]	0.06	0.04	**1.16**	0.59	0.03	0.02
		Aliphatic ketones						
1	7.65	2-Butanone[b]	0.22	0.35	-	-	-	0.22
6	9.23	3-Methyl -2-butanone[b]	0.31	-	**11.81**	**61.26**	**28.55**	-
8	9.58	Cyclopentanone[b]	-	-	-	-	0.37	**9.65**
9	9.7	3-Methyl -3-butanone[b]	**6.94**	**8.62**	-	-	0.62	0.56
10	10.17	2-Pentanone[b]	**9.82**	**1.08**	**18.95**	**8.48**	0.64	**14.57**
14	14.30	Ethylvinylketone[b]	0.03	0.36	0.06	0.02	0.15	0.04
23	18.38	2-Heptanone[b]	0.03	**1.60**	0.49	**1.08**	**1.46**	0.05
29	22.38	3-Octanone[b]	-	0.34	0.27	0.34	**1.27**	-
35	26.30	2-Nonanone[b]	0.05	**3.01**	0.14	0.03	0.18	-
		Aliphatic esters						
3	8.52	2-Methyl-2,3-epoxy butane[b]	**1.82**	0.11	**2.50**	0.12	0.18	**12.86**
21	17.75	Hexyl acetate[b]	0.01	0.09	0.09	-	0.11	0.80
30	23.15	*cis*-3- Hexenyl acetate[c]	0.18	0.29	0.41	0.03	0.11	0.28
41	30.00	Ethyl octanoate[b]	0.05	0.81	0.06	0.02	**1.50**	0.27
45	33.18	Ethyl nonanoate[b]	-	0.33	0.16	0.07	-	0.24
47	36.18	Ethyl decanoate[b]	0.02	0.54	0.07	0.03	0.50	0.07
53	41.60	Dodecanoic acid ethyl ester[b]	-	0.19	-	0.31	0.38	0.09
54	46.43	Ethyl undecanoate[b]	0.05	0.43	**1.32**	**1.41**	0.57	-
		Aliphatic hydrocarbons						
43	30.65	Dodecane[c]	0.10	0.19	0.15	0.06	0.89	0.12
55	50.83	Tricosane[b]	**2.35**	**1.16**	**1.53**	**1.05**	0.82	**3.59**
56	53.08	Pentacosane[b]	0.61	0.27	0.16	0.06	0.10	0.03
57	55.28	Hexacosane[b]	-	0.18	-	0.14	0.06	-
58	59.90	Heptacosane[b]	0.06	0.18	0.29	0.16	-	-
59	62.70	Octacosane[b]	-	0.13	-	0.17	-	-
		Monoterpenoid alcohols						
17	16.03	Tetrahydromyrcenol[b]	-	0.28	0.79	0.59	0.17	0.01
25	20.48	Dihydromyrcenol[b]	0.20	0.51	0.15	0.06	0.21	0.77

36	26.63	Tetrahydrogeraniol[b]	0.05	0.49	0.70	0.14	0.18	**1.38**
37	26.83	Linalool[a]	0.45	0.90	0.78	0.63	**1.66**	0.53
46	34.92	3,6,6-Trimethyl-2-norpinanol[b]	0.23	0.09	-	-	-	-
		Monoterpenoid ketone						
39	28.4	Camphor[a]	**2.48**	**1.12**	0.81	**6.54**	-	**3.40**
		Irregular terpenoid						
32	24.95	Artemisia alcohol[b]	0.28	0.05	0.10	0.04	0.15	0.07
		Other monoterpene						
38	27.53	Geranyl nitrile[b]	-	0.08	-	-	-	0.15
		Sesquiterpenoid hydrocarbons						
50	37.18	α-Copaene[b]	-	0.15	-	0.02	0.26	0.03
51	37.97	*trans*-Caryophyllene[b]	0.04	0.34	-	0.04	0.02	0.31
		Sesquiterpenoid aldehyde						
48	36.45	α-Sinensal	0.08	0.06	0.07	-	0.27	0.03
		Norisoprenoid						
40	28.67	*cis*-Jasmone[b]	0.03	0.17	0.81	0.08	0.15	**4.05**
		Aromatic hydrocarbons						
13	13.55	Toluene[b]	0.42	0.76	0.42	0.36	0.56	0.88
20	17.55	*o*-Xylene[b]	0.44	0.25	0.20	0.12		0.69
22	17.90	Ethylbenzene[b]	-	0.11	-	0.10	0.32	0.08
		Aromatic aldehydes						
26	21.13	Benzaldehyde[b]	0.03	0.22	0.36	0.41	-	-
31	24.23	2-Methyl benzaldehyde[b]	0.28	0.87	0.67	0.37	**2.73**	0.53
		Aromatic alcohol						
33	25.15	Benzenemethanol[b]	0.15	0.08	0.37	0.41	-	-
		Aromatic ketone						
34	25.28	Acetophenone[b]	0.75	**1.14**	0.37	0.85	0.81	**3.61**
		Aromatic ester						
44	32.48	Methyl benzoate[b]	**49.64**	**5.17**	0.12	0.08	**4.36**	-

Components > 1 % in bold; superscript refer to method of identification: a-retention time, mass spectrometry and co injection used to confirm identity of compounds, b-mass spectrometry used to identity of compounds, c-mass spectrometry and retention time used to confirm identity, - not detected and tr-trace amounts.

Table 9: Composition of cowpea floral volatiles trapped in C_{18} bonded reverse phase silica

			Relative amounts (%)					
Peak No.	R_t (min)	Compound	219	269	524 B	ICV 12	SP 46	SP 52
		Aliphatic alcohols						
1	7.75	2,2-Dimethyl propanol[b]	**41.83**	**9.25**	**3.09**	0.32	-	-
2	8.18	Butanol[b]	0.16	0.01	0.04	-	0.21	0.01
3	8.38	2-Methyl-2-butanol[b]	0.08	0.21	**4.64**	**2.88**	0.17	0.94
10	12.67	1-Pentanol[b]	-	0.51	0.18	-	0.11	0.41
12	13.23	2-Methylpentanol[b]	0.58	0.28	0.88	-	0.66	0.63
17	14.73	2-Hexanol[b]	0.19	0.08	-	**1.64**	**1.30**	0.57
32	21.8	Octanol[b]	**6.51**	**1.25**	0.20	**1.35**	**3.17**	0.10
55	38.48	Dodecanol	0.05	0.13	0.07	**40.39**	**1.02**	0.85
56	38.5	Tridecanol[b]	-	0.10	0.61	-	tr	**2.42**
61	43.75	Undecanol[b]	0.08	0.04	0.04	0.15	**1.01**	0.04
		Aliphatic aldehydes						
5	9.15	3-Methylbutanal[b]	0.26	**14.43**	0.58	**2.73**	0.28	0.09
7	10.13	Pentanal[b]	0.13	**15.83**	**38.74**	**2.84**	**14.29**	0.18
8	10.9	4-Pentanal[c]	-	0.35	-	**2.52**	**9.37**	0.11
16	14.35	2-Hexanal[b]	0.34	0.05	0.18	0.66	0.32	**1.00**
27	18.85	*Cis*-Heptenal[b]	0.30	0.11	tr	tr	0.12	0.11
43	26.72	Nonanal[b]	-	0.12	0.62	0.50	tr	0.92
		Aliphatic ketones						
4	8.85	3-Methyl-2-butanone[b]	**10.06**	**1.21**	**12.65**	**6.69**	**2.17**	**1.32**
6	9.9	3-Methyl-2-butenone[b]	-	0.33	**10.27**	0.11	0.81	0.97
9	12.3	3-Buten-2-one-3-methyl[b]	tr	0.12	0.64	0.93	0.45	0.06
15	14.08	2-Hexanone[b]	0.04	0.86	0.56	0.64	**3.92**	0.01
20	15.78	*n*-Heptan-3-one[b]	0.30	-	0.07	-	**1.68**	0.20

		Aliphatic hydrocarbons						
11	12.97	2,3-dimethylbut-1-ene[b]	-	0.04	0.07	-	-	0.01
36	23.55	Decane[b]	**1.27**	0.16	0.02	0.26	**1.03**	0.46
44	26.85	Undecane[b]	-	0.85	-	-	**6.38**	**1.01**
49	30.50	Dodecane[c]	-	0.08	0.72	-	-	**1.15**
58	39.28	Eicosane[b]	0.03	tr	**0.98**	0.23	0.30	tr
66	55.15	Pentacosane[b]	0.67	0.68	0.24	0.78	0.15	0.01
67	57.53	Hexacosane[b]	0.78	tr	0.21	0.42	-	0.02
68	59.95	Heptacosane[b]	0.72	tr	0.16	0.29	-	0.01
69	62.78	Octacosane[b]	0.69	tr	0.17	**1.03**	-	0.01
		Aliphatic esters						
19	15.65	Methylpentanoate [b]	-	-	-	0.34	0.87	0.13
21	16.43	1,2,3-Propanetriol acetate[b]	-	**5.16**	**1.27**	**7.31**	-	0.76
22	16.90	4-hexenoic acid-3-methyl ester[b]	-	0.03	-	-	0.07	0.02
35	23.37	Hexyl acetate[b]	0.64	0.14	0.03	0.23	0.44	0.94
37	23.83	Methyl hexanoate[b]	0.51	0.15	0.03	tr	0.46	0.40
46	27.25	Heptyl acetate[b]	0.30	0.40	**3.96**	**5.45**	**1.25**	**1.41**
50	30.88	Methyl nonanoate[b]	0.34	0.10	0.51	tr	**2.10**	0.56
		Aliphatic acids						
34	22.93	Heptanoic acid[b]	0.01	0.21	0.10	tr	**2.93**	**1.45**
63	47.80	Palmitic acid[b]	0.08	0.29	**5.03**	tr	**8.17**	tr
		Monoterpenoid alcohols						
38	24.53	Tetrahydrogeraniol[b]	0.21	0.07	0.11	**0.97**	**1.95**	0.49
45	26.97	Isopulegol[C]	**3.36**	0.03	-	0.92	0.28	0.09
48	30.23	Menthol[b]	**1.50**	0.06	**1.15**	0.74	**5.93**	**11.20**
51	35.1	3,6,6-Trimethyl-2-norpinanol[b]	0.15	0.22	-	-	-	0.15
54	37.75	Citronellol[c]	0.55	0.04	0.11	0.97	**1.95**	0.49
		Monoterpenoid ketone						
31	21.55	Carvatanoacetone[b]	0.07	0.09	0.03	-	0.49	**1.50**
		Monoterpenoid ester						
57	38.65	5,6-Dioxobornyl acetate[b]	tr	0.61	0.23	-	-	**6.94**
		Irregular terpenoid						
42	26.55	Artemisia alcohol[b]	0.34	0.07	0.07	0.50	**2.09**	**15.96**
		Aromatic hydrocarbons						
13	13.55	Toluene[b]	0.34	0.52	0.71	**4.99**	**1.30**	0.26
24	17.45	*o*-Xylene[b]	-	-	-	-	0.25	0.05
		Aromatic alcohol						
47	28.46	Benzenemethanol[b]	0.05	0.06	0.03	0.20	**1.31**	0.09
		Aromatic aldehydes						
30	21.03	Benzaldehyde[c]	0.07	0.05	0.27	**5.06**	0.08	0.91
39		4-Hydroxy benzaldehyde[b]	0.20	0.05	tr	tr	tr	0.08
		Aromatic ketone						
40	25.76	Acetophenone[b]	**13.76**	0.05	0.69	0.62	**3.00**	**1.68**
		Aromatic esters						
29	19.10	Methyl-1,2,4-benzenetricarboxylate[b]	0.20	0.15	0.08	-	-	0.88
41	26.43	Methylbenzoate[b]	0.71	0.12	-	-	-	**11.78**
		Others						
14	13.48	Tetrahydrofurfurol[b]	0.58	0.28	0.21	0.64	0.73	0.31
18	15.48	Furfural[b]	0.07	-	0.07	-	**1.67**	0.32
23	17.25	Tetrahydrofurfuryl acetate[b]	-	0.04	-	-	0.15	0.89
25	18.45	1,2-butanolide[b]	-	0.09	0.21	-	**1.72**	**9.29**
26	18.75	2-Butylfuran[b]	-	-	-	-	-	**9.63**
28	18.98	Cyclohexyl-3-furanylmethanone[b]	-	0.03	0.02	-	0.79	0.50
33	21.97	Tetrahydro-2-methylfuranol[b]	-	-	0.34	-	0.38	0.53

Components > 1 % in bold; superscript refer to method of identification: a-retention time, mass spectrometry and co injection used to confirm identity of compounds, b-mass spectrometry used to identity of compounds, c-mass spectrometry and retention time used to confirm identity, - not detected and tr-trace amounts.

Table 10: Composition of cowpea floral volatiles trapped in porapak Q

			Relative amounts (%)					
Peak No.	Rt (min)	Compound	219	269	524 B	ICV 12	SP 46	SP 52
		Aliphatic alcohols						
1	7.75	2,2-Dimethylpropanol[b]	**1.82**	0.69	0.03	0.10	0.40	0.24
2	8.18	Butanol[b]	0.02	0.08	0.03	0.04	**1.48**	0.08

3	8.57	2-Methylbutanol[b]	**9.62**	**13.06**	**10.34**	**6.90**	0.18	**4.54**
4	8.87	2-methyl butan-2-ol[b]	**15.47**	**14.31**	**10.36**	**6.93**	**6.63**	**5.94**
14	14.85	Hexen-2- ol[b]	0.67	**2.90**	**1.32**	0.90	0.48	0.03
15	14.9	1-Hexanol[b]	**2.05**	**1.33**	0.66	0.90	0.77	0.87
16	15.05	2-Hexanol[b]	**0.95**	**2.23**	0.13	0.18	**2.15**	0.17
17	16.45	3*E*-Hexen-1-ol[b]	**11.04**	**7.26**	**3.63**	**12.61**	**4.54**	**4.85**
18	16.75	3*Z*-Hexen-1-ol[b]	0.35	0.32	0.36	0.39	0.08	0.02
19	17.17	2-Heptanol[b]	0.07	0.10	-	-	0.78	-
24	19.23	2-Nonanol[b]	-	0.23	-	-	-	0.14
29	22.25	1-Octen-3-ol[c]	0.01	0.13	0.14	0.01	0.18	0.02
38	25.67	1-Octanol[b]	**15.20**	0.62	0.32	0.34	0.81	0.24
72	38.48	Dodecanol[b]	0.12	0.07	0.11	**10.02**	0.26	**2.10**
75	43.75	Undecanol[b]	0.19	0.02	0.07	0.04	0.26	0.11
		Aliphatic aldehydes						
5	9.15	3-Methylbutanal[b]	0.49	0.36	0.32	0.29	0.67	0.33
13	14.58	Hexanal[b]	0.03	0.30	0.03	0.01	0.66	0.31
23	18.85	*n*-Heptanal[b]	-	-	0.01	0.07	0.55	0.09
31	22.95	*n*-Octanal[b]	0.09	0.17	-	-	**2.74**	0.57
53	30.28	Decanal[b]	0.55	0.12	**2.17**	**1.07**	**2.13**	0.94
70	36.57	Dodecanal[b]	0.30	0.37	**2.36**	2.40	0.80	0.36
74	42.1	Octadecanal[b]	0.11	0.02	0.10	-	0.02	0.04.
		Aliphatic ketones						
6	9.3	3-Methyl-2-butanone[b]	**6.41**	**8.89**	**5.59**	**4.60**	**10.01**	**3.76**
7	10.23	3,3-dimethylbutanone [b]	**10.98**	**4.97**	**9.83**	**6.01**	0.37	0.69
9	11.85	3-Methoxy-3-methyl butanone[b]	0.29	0.02	0.02	0.02	0.14	0.07
12	14.15	4-methyl-2-pentanone[b]	0.12	**1.01**	0.19	0.20	0.60	0.06
21	18.45	2-Heptanone[b]	0.14	0.20	**2.01**	**2.27**	0.33	0.05
28	22.01	4-Octen-3-one[b]	0.01	0.13	0.14	0.05	0.18	-
30	22.37	3-Octanone[b]	0.21	0.17	0.01	0.02	0.17	0.04
49	29.57	4-Octanone[b]	-	0.08	0.08	0.06	0.18	0.07
69	36.15	2-Decanone[b]	-	-	-	-	0.16	0.11
		Aliphatic ester						
32	23.15	3*Z*-Hexenyl acetate[b]	0.07	0.02	0.02	0.02	0.25	0.03
		Aliphatic hydrocarbons						
8	11.35	2,2-Dimethyl butane[b]	0.02	0.40	0.03	0.04	0.41	0.01
10	12.18	Methyl cyclohexane[b]	0.09	0.58	-	0.02	**1.24**	0.30
25	19.63	Octane[b]	0.01	0.06	0.52	0.33	-	-
43	26.78	Decane[b]	0.73	0.29	0.28	0.22	**8.88**	**2.86**
73	39.28	Eicosane[b]	0.02	0.02	0.01	0.06	0.08	0.02
76	55.15	Pentacosane[b]	0.12	0.04	-	0.19	0.25	0.13
77	57.53	Hexacosane[b]	0.02	**1.97**	-	0.11	0.02	0.08
78	59.95	Heptacosane[b]	0.50	-	-	0.07	0.03	0.03
79	62.78	Octacosane[b]	0.34	**1.34**	-	0.26	-	-
		Monoterpenoid alcohols						
40	26.42	Linalool[c]	**1.07**	0.22	0.15	0.39	0.01	0.07
41	26.63	Tetrahydrogeraniol[b]	-	0.48	-	-	0.15	**2.02**
45	28.53	Citronellol[b]	-	0.32	-	-	-	0.53
		Monoterpenoid ester						
65	34.40	Terpeneol hydrate[b]	-	-	-	-	0.41	0.23
		Monoterpenoid ketone						
35	26.63	Linalool oxide[b]	0.09	0.25	-	0.01	0.10	0.09
		Irregular monoterpene						
26	20.48	Chrysanthemyl alcohol[b]	-	-	-	0.02	0.16	0.45
		Sesquiterpenoid hydrocarbon						
58	31.62	*trans*-caryophyllene[b]	0.08	0.09	0.02	0.01	0.03	0.04
60	31.98	Aromandendrene[b]	0.32	0.01	0.07	0.01	0.10	0.01
		Sesquiterpenoid alcohols						
55	30.57	Guaiol[b]	0.57	-	0.02	0.03	**1.21**	0.35
71	36.90	Farnesol[c]	0.29	0.15	0.18	0.06	0.24	0.34
		Aromatic hydrocarbons						
11	13.58	Toluene[b]	0.45	0.07	0.36	**1.42**	0.12	tr
20	17.58	Ethylbenene[b]	-	-	-	0.01	0.03	0.25
22	18.70	Vinylbenzene[b]	-	0.10	-	-	0.09	0.07
39	26.35	2-(2-Propenyl) methyl benzene[b]	-	0.13	-	0.39	0.34	**1.29**
42	26.67	1-Ethenyl-4-ethylbenzene[b]	-	0.48	-	-	0.61	**2.02**
56	30.98	*p*-Cymene[b]	0.35	-	0.02	0.11	0.51	0.84

59	31.90	*o*-Allyltoluene[b]	-	-	-	-	-	**3.73**
61	32.45	4-Isopropylbenzene[b]	-	-	-	-	**34.59**	0.34
		Aromatic alcohol						
33	24.08	Benzenemethanol[b]	**1.01**	0.01	0.18	0.04	0.10	0.61
36	24.85	Benzeneethanol[b]	0.09	0.24	-	-	-	0.03
47	29.10	3-Ethylphenol[b]	0.10	0.05	0.04	0.52	0.10	0.73
57	31.48	Carvacrol[b]	0.14	-	-	-	-	0.79
		Aromatic aldehydes						
27	21.05	Benzaldehyde[b]	0.01	0.26	0.14	**2.27**	0.28	**1.94**
44	28.2	2,6-Dimethylbenzaldehyde[b]	-	-	-	-	0.04	-
46	28.83	Ethylbenzaldehyde[b]	-	-	-	0.06	0.69	**20.86**
48	29.33	Ethenylbenzaldehyde [b]	-	-	-	-	**4.38**	**2.76**
53	30.40	1,4-Benzenecarboxhaldehyde[b]	-	-	-	-	0.06	0.08
		Aromatic ketones						
34	24.23	Methylacetophenone[b]	-	-	0.09	0.04	-	**3.21**
37	25.18	Acetophenone[b]	**15.20**	**27.50**	**14.31**	**25.56**	**1.33**	**5.90**
52	30.15	3-Ethylacetophenone[b]	-	0.03	-	-	**1.33**	**4.33**
63	33.38	4-Phenyl-3-buten-2-one[b]	-	-	**32.60**	-	0.73	0.30
		Aromatic esters						
50	29.65	Thymylmethyl ether[b]	-	-	-	-	0.07	0.18
62	33.3	Carvyl acetate[b]	0.15	0.06	-	-	-	**7.78**
		Phenylpropanoids						
66	35.78	Methyl cinnamate[b]	0.15	**4.31**	0.52	**10.02**	0.45	**1.95**
51	29.93	Cinnamaldehyde[b]	0.55	0.04	0.06	0.03	0.77	**2.76**
67	36.00	Vanillin[b]	0.01	-	-	**1.24**	0.13	**1.95**
68	36.05	Methyl eugenol[b]	0.13	0.37	-	0.01	0.12	0.16

Components > 1 % in bold; superscript refer to method of identification: a-retention time, mass spectrometry and co injection used to confirm identity of compounds, b-mass spectrometry used to identity of compounds, c- mass spectrometry and retention time used to confirm identity, - not detected and tr-trace amounts.

The cowpea floral volatiles trapped by the three adsorbents were composed of short-chain fatty acid derivatives as the main class of compounds. Porapak Q trapped the highest number of compounds (47-75) in all the six cowpea cultivars followed by reverse phase C_{18} bonded silica (43-66) and activated charcoal (44-58). Benzenoids (21 compounds) were mostly trapped by porapak Q. This could be due to its polymeric nature resulting in the largest surface area per unit mass (Withycombe *et al*., 1978; Wyllie *et al*., 1978). The number of compounds trapped by porapak Q conforms with the findings in *C. brewerie* (Raguso & Pellmyr, 1998).

Reverse phase C_{18} bonded silica trapped the highest number of aliphatics. Many furan derivatives were obtained from reverse phase C_{18} bonded silica traps, in which

sesquiterpenes and nor-isoprenoids were not trapped. Activated charcoal and porapak Q did not trap any furan derivatives.

Due to the large variation in the composition of cowpea floral volatiles trapped by the three adsorbents, they were connected in series and used to trap the volatiles to give a complete profile of the flower volatiles. The composition of cowpea floral volatiles trapped in combined adsorbents is summarized in table 11. The GC chromatograms are in appendix 3.

Table 11: Composition of cowpea floral volatile trapped in combined adsorbents.

			Relative amounts (%)					
Peak No.	R_t (min)	Compound	219	269	524 B	ICV 12	SP 46	SP 52
		Aliphatic alcohols						
8	9.16	1-Butanol[b]	**16.41**	0.72	**25.55**	0.74	0.4	**6.72**
14	14.88	Hexanol[b]	0.67	**2.45**	**1.42**	**3.31**	0.53	0.78
26	23.52	Nonanol[b]	0.13	**4.31**	0.07	**3.10**	0.22	0.11
39	26.4	3-Octanol 2,3 dimethyl[b]	0.35	-	-	0.72	0.04	0.13
		Aliphatic aldehydes						
1	7.8	Propanal 2,2 dimethyl[b]	**1.97**	-	0.03	-	**2.57**	0.08
3	8.38	3-Methyl butanal[b]	**1.12**	0.64	0.2	0.09	**3.64**	-
6	9.35	4-Pentenal[b]	0.4	**9.77**	**0.97**	**6.5**	**3.48**	0.15
13	14.45	*n*-Hexanal[b]	0.1	0.13	0.18	0.15	0.14	0.13
20	18.85	Heptanal[b]	0.13	0.46	0.14	0.12	0.06	0.06
53	30.28	Decanal[b]	**1.54**	0.64	0.39	0.46	0.56	0.14
		Aliphatic ketones						
4	8.9	3-Methyl-2-butanone[b]	**10.53**	**17.71**	**32.08**	**17.28**	**5.44**	**3.43**
10	11.23	4-Pentene-2-one[b]	0.08	0.05	0.49	0.06	0.02	-
16	16.35	3-Hexanone[b]	**4.12**	**8.54**	-	**7.09**	**1.41**	**2.54**
19	18.45	2-Heptanone[b]	0.45	**1.55**	0.34	0.23	0.05	0.35
		Aliphatic esters						
9	10.95	2-Propenoic acid methyl ester[b]	**1.67**	0.09	-	**1.63**	0.08	-
24	23.15	cis-Hexenyl acetate[b]	0.06	0.08	tr	0.002	0.12	0.06
51	29.85	1-Octen-2-ol acetate[b]	0.16	-	-	0.2	0.07	**28.10**
		Aliphatic hydrocarbons						
11	12	2-Hexene[b]	0.11	0.13	0.33	0.12	0.14	-
27	23.61	Decane[b]	0.03	0.07	-	0.05	0.02	-
55	30.68	Dodecane[c]	**4.51**	**2.23**	-	0.61	0.07	0.03
65	35.68	Tetradecane[b]	-	-	0.29	0.62	-	-
80	49.8	*n*-Tricosane[b]	**4.2**	0.04	-	0.5	**1.33**	0.57
81	50.08	Pentacosane[b]	**3.82**	0.02	-	**1.20**	0.07	0.28
82	55.28	Hexacosane[b]	**1.03**	0.03	-	0.02	-	0.05
63	59.93	*n*-Heptacosane[b]	0.56	0.02	-	-	-	0.05
115	62.7	*n*-Octacosane[b]	0.01	0.01	-	-	-	0.01
		Other aliphatics						
2	8.13	Methanamine N butanone[b]	0.02	0.02	-	-	-	-
7	9.95	2-Methoxy-2-methylbutane[b]	**10.46**	**11.46**	**0.96**	**11.71**	**4.95**	**4.29**
8	10.35	3-Chloro-3-methylbutene[b]	0.6	-	-	0.19	-	-
17	17.23	1-Chloro hexane[b]	0.01	0.05	0.01	0.05	-	-
		Monoterpenoid alcohols						
21	20.45	Dihydromyrcenol[b]	0.4	0.5	0.66	0.64	0.04	0.13
25	21.35	Dihydrolinalool[b]	0.03	0.05	**1.09**	0.02	0.12	0.04
31	24.53	Tetrahydrogeraniol[b]	**1.06**	0.12	0.15	0.2	-	0.03

33	24.63	1,8-Cineol[a]	0.08	0.18	0.05	0.21	0.76	-
41	26.83	Linalool[a]	**1.76**	0.87	-	-	-	0.41
48	28.65	Isopulegol[c]	0.07	0.01	-	-	-	-
52	30.25	Menthol[b]	-	-	-	-	-	0.14
56	31.1	β-citronellol[b]	-	-	-	0.27	0.03	0.3
58	31.63	Isopinocampheol[b]	-	0.78	-	0.17	-	-
64	35.3	Isocitronellol[b]	-	-	-	-	0.21	-
		Monoterpenoid esters						
37	25.83	Linalyl acetate[b]	0.07	0.59	-	0.11	-	0.67
63	34.4	3,8-terpeneol hydrate[b]	**1.76**	0.2	0.1	0.81	-	-
64	35.13	Terpenyl acetate[b]	0.95	0.11	-	0.74	0.41	-
76	38.65	Dihydrobornyl acetate[b]	-	-	-	-	-	0.17
		Monoterpenoid hydrocarbons						
32	24.6	Limonene[a]	0.08	0.18	0.05	0.21	0.76	-
41	26.83	3-carene[a]	0.07	0.59	-	0.11	-	0.67
43	27.53	Fenchene[a]	0.07	0.59	-	0.11	-	0.67
		Monoterpenoid aldehyde						
45	28	2,3-Epoxyneral[b]	-	-	-	0.02	-	-
63	34.25	Citronellal[b]	-	0.21	-	0.003	-	-
		Monoterpenoid ketones						
46	28.4	Camphor[a]	0.38	**3.42**	0.04	**4.06**	**5.24**	**1.39**
59	31.9	Linalool oxide[b]	0.06	0.13	0.1	0.05	**4.21**	-
69	36.6	Limonene dioxide	0.07	-	0.21	0.01	-	-
		Others						
30	24.35	Sorbic acid[b]	-	-	-	0.08	-	-
43	27.53	Geranyl nitrile[b]	0.04	0.46	-	-	-	0.41
		Irregular terpenes						
22	20.48	Chrysanthenyl alcohol[b]	-	-	-	-	0.04	-
34	24.95	Artemisia alcohol[b]	-	-	-	-	-	0.30
43	27.53	Lavandulol[b]	0.07	0.59	-	0.11	-	0.67
60	31.91	Yomogi alcohol[b]	0.06	0.13	0.1	0.05	**4.21**	-
		Sesquiterpenoid hydrocarbons						
71	37.16	Germacrene D[b]	-	0.3	-	-	**1.63**	-
73	37.88	*trans*-Caryophyllene[b]	0.21	0.09	-	-	-	-
72	37.8	α-Cedrene[b]	-	-	-	0.04	-	-
74	38.03	*cis*-Bisabolene[b]	0.21	-	0.03	0.1	-	-
		Sesquiterpenoid aldehyde						
69	36.4	α-Sinensal[b]	0.32	0.31	-	-	-	-
		Sesquiterpenoid ketone						
70	36.47	Geranyl acetone[b]	0.60	-	0.43	tr	**3.16**	0.13
		Sesquiterpenoid alcohol						
77	41.4	Nerolidol[c]	0.05	-	-	-	0.84	0.18
		Azulene						
54	30.58	Guaiol[b]	**4.51**	0.13	0.11	0.61	0.09	0.03
		Norisoprenoids						
48	28.65	*cis*-Jasmone[b]	-	**1.14**	-	-	-	-
61	32.08	β-Methyl ionone[b]	0.50	-	-	0.24	-	-
		Aromatic hydrocarbons						
12	13.35	Toluene[b]	0.02	0.8	0.04	**2.00**	0.14	0.13
18	17.55	*p*-Xylene[b]	0.03	0.05	-	-	-	-
28	24.1	1,3,5-Trimethylbenzene[b]	**1.06**	-	-	0.10	-	0.10
35	25.33	1,4-Diethylbenzene[b]	-	-	-	0.24	-	-
36	25.58	*p*-Cymene[b]	0.12	-	-	0.14	-	0.04
		Aromatic alcohols						
42	27	Benzene methanol[b]	0.38	0.09	-	-	0.04	**1.39**
47	28.46	Benzene ethanol[b]	0.47	0.16	**14.7**	**3.72**	0.21	0.02
57	31.49	Carvacrol[b]	-	-	**1**	-	-	**7.80**
		Aromatic ketones						
29	21.55	Carvatanoacetone[b]	-	-	-	-	-	0.05
34	25.15	Acetophenone[b]	**12.6**	**1.52**	**11.1**	**11.52**	0.09	**2.12**

49	28.78	Phenyl propanone	0.42	**17.03**	0.04	**4.12**	**5.24**	**28.1**
		Aromatic esters						
39	26.4	Methyl benzoate[b]	0.13	**4.31**	0.07	**3.10**	**2.86**	**11.76**
62	33.3	Carvyl acetate[c]	-	-	-	-	**1.46**	-
		Phenylpropanoids						
50	29.35	Cinnamaldehyde[b]	-	-	-	-	**3.39**	-
67	36.03	Vanillin[b]	**2.88**	0.09	0.13	**1.00**	0.16	0.37
68	36.08	Methyl eugenol[b]	0.32	-	-	0.62	0.09	-
66	35.73	Methyl cinnamate[b]	**2.24**	0.43	0.28	**1.46**	**1.39**	0.09
		Others						
15	15.58	Furfural[b]	0.2	0.14	0.31	-	0.06	0.05
38	25.93	Tetrahydrofurfuryl acetate[b]	-	-	-	0.02	**1.52**	**2.70**
40	26.25	2-Furan methanol[b]	-	-	0.13	0.69	-	-

Components > 1 % in bold; superscript refer to method of identification: a-retention time, mass spectrometry and co injection used to confirm identity of compounds, b-mass spectrometry used to identity of compounds, c- mass spectrometry and retention time used to confirm identity, - not detected and tr-trace amounts.

The mass spectra for some selected volatile and essential oil compounds are in appendix 5.

4.4.2.1 Monoterpenes

Monoterpenes, trapped in combined adsorbent constituted a larger portion than from hydrodistillation. Linalool (**46**), a common floral volatile in most angiosperms was trapped by activated charcoal and porapak Q in all cultivars. However it was not trapped by reverse phase C_{18} bonded silica in all the cultivars.

Camphor (**101**) was found in the steam distillate of ICV 12 in small amounts but is abundant in the headspace collection of volatiles by combined adsorbents. In the individual asorbents, it was trapped by all adsorbents except reverse-phase C_{18} bonded silica. Isopulegol was present more in the headspace of combined adsorbents than in the hydrodistillation, respectively.

A number of acyclic monoterpenes were identified in cowpea floral volatiles. These included nerol, geraniol (**278**), citronellol (**49**), citronellal (**48**) and citral (**278**) all of which have been reported in the floral volatiles of sweat pea (Porter *et al*., 1999). Small amount of linalyl acetate was detected in the oils of cowpea flowers.

Sorbic acid was present in the headspace volatiles of cowpea cultivar ICV 12 but absent in the steam distillate.

Limonene (**65**) was present in steam distillate and combined headspace volatiles of all the cultivars except ICV 12 and SP46, respectively. It has been reported in many floral fragrances and it coelutes with 1, 8-cineole (eucalyptol) (**72**) during gas chromatography (Sazima *et al*., 1993). 1, 8-Cineole (**72**) can also be formed from limonene or menthol (**71**) (Scheme 2).

Isocitronellol and epoxyneral among others were detected only in headspace volatiles of one cultivar, ICV 12. Similarly, α-ocimene (**43**), β-myrecene (**41**), pulegone (**69**), and camphene (**105**) were detected in the steam distillate of one cultivar only.

Irregular terpenenes were detected in both techniques especially headspace volatiles trapped by combined adsorbents compared to steam distillate. Interestingly, only artemisia alcohol, yomogi alcohol, lavandulol and chrysanthemyl alcohol were present in the headspace volatiles trapped in individual and combined adsorbents while artemisia ketone and chrysanthemyl acetate were present in the steam distillate. 3,6,6-Trimethylnorpinanol was trapped by activated charcoal and reverse-phase C_{18} bonded silica only.

4.4.2.2 Sesquiterpenes

Just like the monotepenes, sesquiterpenes were trapped in higher amounts by the combined adsorbents than in steam distillate though the latter yielded larger number of compounds. Guaiol (**208**) was exclusively present in the headspace volatiles of all the cultivars and has been reported previously (Knudsen *et al*., 2004). The highest amount

was recorded in cultivar 219. Sativene was found only in the steam distillate of wild outcrossed cultivars (219 and 269) and is being reported for the first time in floral volatiles. Nerolidol (**156**) was present and headspace volatiles and in the steam distillate while farnesol was present in steam distillate. In the headspace geranylinalool co-eluted with farnesol during analysis (Buouwmeester *et al*., 1999). Geranylacetone (**155**) was more abundant in the headspace volatiles than in steam distillates. α-Cedrol, *β*-caryophyllene (**180**), germacrene B (**176**) and D (**178**) were present in both the steam disillate and headspace volatiles Germacrene D (**178**) co-eluted with *α*-copaene and *γ*-cadinene during GC analysis Aromandrene (**199**), α-cubene, bisabolol (**164**), *β*-farnesene (**153**), *α*-farnesene (**152**) caryophyllene oxide (**181**), nerylacetone and *β*-sesquiphellandrene were only present in the steam distillates. Only present in the headspace were *α*-sinensal and *α*-cedrene.

4.4.2.3 Norisoprenoids

Norisoprenoid were more in the steam distillate than in headspace volatiles. *cis*-Jasmone was present in the steam distillate and headspace volatiles of all cultivars trapped in activated charcoal but was only found in headspace volatiles of cultivar 269 trapped in combined adsorbents. *β*-Methylionone was in the steam distillate and in the headspace volatiles of combined adsorbents. *β*-Ionone (**81**), *α*-ionone (**80**); *β*-cyclocitral (**79**) megastigmatrienone 4, megastigmatrienone 2, megastigmatrienone 1, phytol, damascenone and allyl-*β*-ionone were exclusively present in the steam distillates.

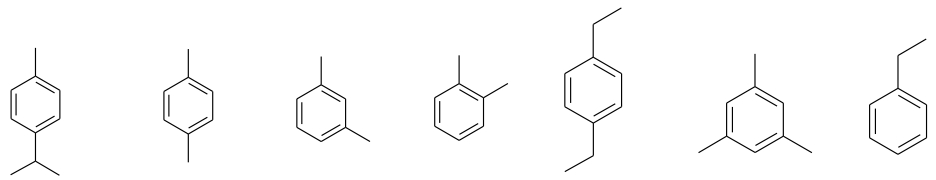
Steam distillate of cowpea flowers from cultivar 524B exclusively gave carotenenoids. In the individual adsorbents, only activated charcoal trapped *cis*-jasmone from floral

volatiles of all the six cowpea cultivars. No norisoprenoids were trapped in porapak Q and reverse-phase C_{18} bonded silica.

4.4 2.4 Aromatics

These were the second most abundant class of compounds in the steam distillate and headspace floral volatiles in all cowpea cultivars. The aromatics detected included hydrocarbons, oxygenated benzenoids (alcohols, aldehydes, ketones, esters plus phenyl propanoids) and nitrogen containing benzenoids.

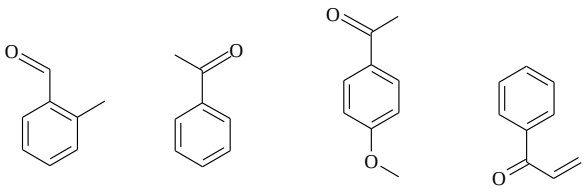
The hydrocarbon detected included *p*-cymene (**291**), which occurred in volatiles from all the cultivars in both the steam distillate and headspace. The two cultivars that registered the highest amounts were 524B and 219. Toluene was detected in steam distillate and headspace trapping with ICV 12 giving the highest amount while all the isomers of xylene: *p*-xylene (**292**), *m*-xylene (**293**) and *o*-xylene (**294**), were detected in the steam distillates, while only *m*-xylene (**292**) was in the headspace volatiles. 1, 4-Diethylbenzene (**295**) and 1,3,5-trimethylbenzene (**296**) were detected in the headspace volatiles only while ethylbenzene (**297**) was found in the steam distillates.



291 **292** **293** **294** **295** **296** **297**

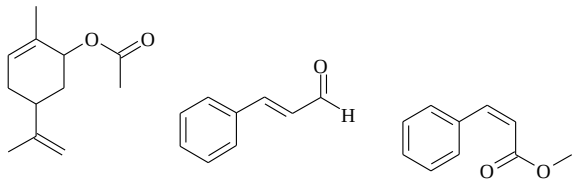
The aromatic aldehydes detected include: 2-methylbenzaldehyde (**298**), in the floral steam distillate of cowpea cultivar SP 46 and headspace volatiles; and benzaldehyde (**253**), in the steam distillate and headspace floral volatiles. The aromatic ketones were more prevalent with acetophenone (**299**) in the steam distillate and headspace volatiles

while *p*-methoxyacetophenone (**300**) and phenylvinylketone (**301**) were detected in the steam distillates only. Phenylacetaldehyde (**254**) was also found in cowpea floral volatiles. Phenylpropanone (**311**) was exclusively in the headspace volatiles.



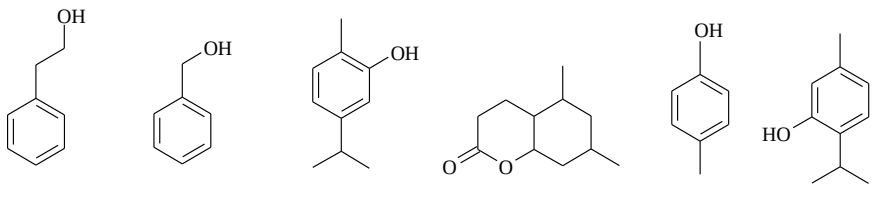
298 **299** **300** **301**

The esters detected included: benzyl benzoate (**250**), in the steam distillate; while methyl benzoate (**287**) and carvyl acetate (**302**) were in the headspace volatiles only. The phenylpropanoids included cinnamaldehyde (**303**), which was in both steam distillate and headspace. Methyleugenol (**255**) was present in the steam distillate and headspace volatiles while vanillin (**246**) was in the headspace volatiles only. Methyl cinnamate (**304**) was in the steam distillates and headspace volatiles.



302 **303** **304**

The alcoholic aromatics detected included; benzene ethanol (**305**), in both steam distillate and headspace volatiles; while benzyl alcohol (benzene methanol) (**306**), carvacrol (**308**), and 5, 7-dimethyloctahydrocoumarin (**308**) were present in the headspace volatiles only. *p*-Cresol (**309**) and thymol (**310**) were detected in the steam distillate only.



305 **306** **307** **308** **309** **310**

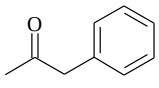
Higher amounts of phenylpropanoids were found in the headspace volatiles. Methyl cinnamate (**304**) was more abundant in the headspace volatiles than in the steam distillate contradicting earlier reports on floral volatiles of alfalfa where it was only found in traces (Tava *et al*., 2000). Vanillin (**246**) was one of the major components identified in cowpea floral volatiles. It has been reported in the floral volatiles of *Clarkie breweri* though as a minor constituent (Raguso & Pichersky, 1995). Methyleugenol (**255**) has been reported in floral scents of *C. breweri* (Raguso & Pichersky, 1995) and in floral scents of *Dianthus* and *Saponaria* species (Jurgens *et al*., 2003). Cinnamaldehyde (**303**) seems to be a rare floral volatile though it was abundant in cowpea floral volatiles. The floral scent of *Silene* species contains cinnamaldehyde (**303**) in trace amounts (Jurgens *et al*., 2002).

p-Cresol (**309**) was only detected in appreciable amounts in the steam distillate of ICV 12 cowpea cultivar. It was previously reported in abundance in floral oils of *Silene* species (Jurgens *et al*., 2002) and in one species of Theophrastaceae (Knudsen & Stahl, 1994). Thymol (**310**) was detected in the steam distillate of cowpea cultivar 269 while carvacrol (**307**) was present in large amounts in cowpea volatiles. Phenylmethanol (**306**) was detected in large amounts in the headspace volatiles of cowpea. Benzyl alcohol (**306**) has previously been reported in steam distillate and headspace volatiles of alfalfa (Tava *et al*., 2000).

Benzaldehyde (**253**) was found in higher amounts in the headspace volatiles than in the steam distillates in almost all the cowpea cultivars and was previously reported in abundance in alfalfa headspace volatiles (Tava *et al*., 2000), floral volatiles of *Silene* species (Jurgens *et al*., 2002), and several species of Theophrastaceae (Knudsen & Stahl,

1994). Other aromatic carbonyls like 2-methylbenzaldehyde (**298**), *p*-methoxyacetophenone (**300**), phenylvinylketone (**301**) and phenylpropanone (**311**) were abundant in headspace volatiles only. Acetophenone (**299**) is a major headspace volatile component though it also appeared in the steam distillate.

The only nitrogen containing benzenoid, methyl anthranilate (**312**) was present in the headspace trapped volatiles and the steam distillates of all the cowpea cultivars. It was previously reported in flowers and flower parts of mandarin, *Citrus deliciosa* (Flamini *et al*., 2003).



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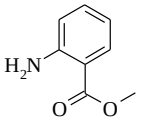

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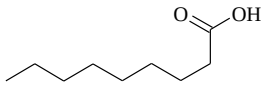
4.4.2.5. Aliphatic compounds

This class had the most abundant compounds both in the steam distillate and headspace. The steam distillate contained the largest number of compounds compared to the headspace volatiles. The groups of the aliphatic compounds included the aldehydes, ketones, esters, alcohols, hydrocarbon and carboxylic acids. The most abundant aldehydes: *trans*-2-hexenal, 2-methyl hexane, tridecanal, 2, 4-decadienal and *trans*-2-nonenal were only present in the steam distillate while 4-pentenal, 3-methyl butanal, decanal and 2, 2-dimethylpropanal were present in headspace volatiles. *n*-Heptanal and *n*-hexanal in the headspace volatiles and *n*-hexanal, *n*-heptanal, 2-heptenal, 2-octenal and nonanal in the steam distillates were present in low amounts.

The ketones present in high amounts in the headspace volatiles only included 4-octen-3-one, 3-octanone and 5-methylhexanone. The most abundant ketone in headspace

volatiles were short-chain hydrocarbons: 2-butanone-3-methyl 4-hydroxy-2, 5-dimethyl-3-hexanone and 2-heptanone while methanamine-*n*-butanone and 4-penten-2-one in traces. The major alcohols detected were *n*-butanol in the headspace volatiles only; *n*-hexanol, 1-octen-3-ol and nonanol in steam distillates and headspace volatiles. Several other alcohols: *Z*-3-hexen-1-ol (**269**), 3-octanol, *trans*-1-hexenol and 4-nonanol were detected in the steam distillates in small amounts.

Carboxylic acids were mostly found in the steam distillates although some were present in the headspace volatiles. Nonanoic acid (**313**) was detected in the headspace volatile. Lauric acid (**314**) and myristic acid (**315**) only appeared in the steam distillate. Palmitic acid (**316**) was present in headspace volatiles and steam distillates and was most abundant aliphatic compound.



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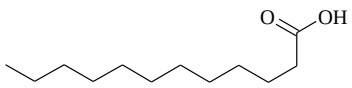

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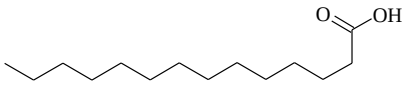

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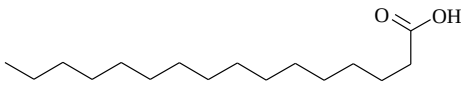

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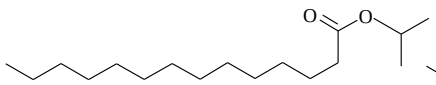
Several esters were detected: isopropyl myristate (**317**), isopropyl palmitate (**317**), oleic acid propyl ester (**319**), *n*-hexyl acetate (**282**) and ethyl palmitate (**320**) in the steam distillate while 2-propenoic acid methyl ester (**321)**, 1-octen-2-ol acetate (**322**) and *cis* -hexenyl acetate (**282**) were in the headpace volatiles.



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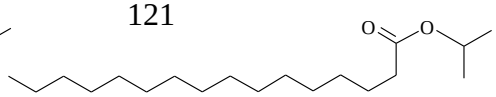

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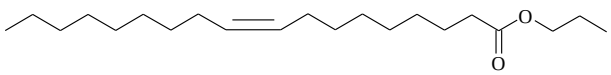

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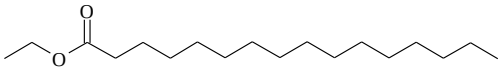

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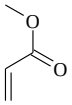

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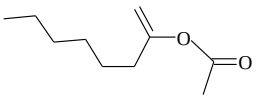

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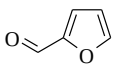
Several hydrocarbons ranging from 5-28 carbon atoms were detected in the steam distillate only: cyclohexane, 2-methyl hexane, 3-methylhexane, 2-hexene, *n*-nonane heptacosane, decane and tetradecane. Some were present in both headspace and steam distillates: *n*-eicosane, tricosane, pentacosane, octacosane and dodecane

4.4.2.6. Miscellaneous compounds

These compounds have no common biosynthetic origin. They had the lowest abundance in both steam distillate and headspace volatiles. Notably absent in the headspace but present in the steam distillate was indole. Exclusively present in the headspace volatiles were furanoids: furfural (**323**) in all cultivars and 2-pentylfuran (**324**), 2-furanmethanol (**325**) and tetrahydrofurfuryl acetate (**326**) all in low amounts. Furfural (**323)** was present in the headspace volatiles of cowpea flowers. Other derivatives of furan like (2-pentylfuran (**325**), tetrahydrofurfuryl acetate (**326**), 2-butylfuran (**328**) among others were found in the headspace volatiles only.



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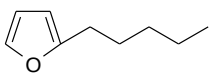

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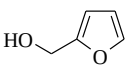

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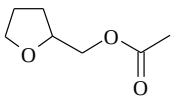

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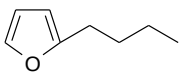

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CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Flowering and flower sizes

Flower colours are important signals to pollinators just like odours (Dobson, 1991Barth, 1985). Many pollinating insects are discriminatory to flower scent and colour and variation may have an important impact upon reproductive isolation and speciation (Dobson, 1991; Beker *et al*., 1989; Stanton, 1987). Just like other flowers cowpea produced colour morphs (different flower colours): family Theophrastacea (Knudsen & Stahl, 1994), *Coridalis cava* of Fumariaceae (Olesen & Knudsen, 1994), *Medicago sativa* flowers (Tava & Pecetti, 1997) and alfalfa flowers (Pecetti & Tava, 2000).

Pecetti & Tava (2000) found out that there is great emanation of volatiles from dark coloured flowers than from light coloured ones, all colours can be associatively learnt by bees (Menzel, 1990). The research findings of Odell *et al*., (1999) on the Snapdragon revealed that bumble bees discriminated against white flowers when the floral rewards were comparable. Reinhard *et al*., (2004) also found out that bees discriminated bright colours. Attractive onion flowers produced highest nectar volumes (Silva & Dean, 2000; Van Wyk, 1993). Bees are attracted to the bright saturated colours (Lunau, 1991; 1990; Kay, 1976). Wild cowpea cultivars (219, 269, SP 46 and SP 52) had bright colours and would then be preferred by honey bees. Genetic manipulation should be done to transfer genes from wild cowpea to the cultivated to pass the traits that would ensure and enhance efficient pollination and maximize production.

5.2 Nectar production

Pollinator visits are usually observed in the first hours of anthesis (Nepi *et al*., 1996b). The three nectar characteristics (volume, sectretion and composition) affect bee visits.

Bees visit flowers, which produce high nectar volume and have high rates of nectar production (Silva & Dean, 2000). Nectar secretion usually begins a few hours before pollinators start visiting the flowers (Cruden *et al*., 1983). Secretion of nectar by cowpea flowers in the early hours of the morning may be an adaptation to behaviour of bees, which are practically inactive in the heat of the day and the cold of the night (Gould & Gould, 1988). Inactivity of bees and low evaporation of nectar water at night may explain large volume of nectar in the morning. Environmental variations (temperature, soil moisture and humidity), may also have contributed to nectar production in the morning. Anthesis for cowpea flowers is very brief with flowers closing before noon, a feature that might favor re-absorption of nectar resulting in low nectar volumes collected after 9.00 am. The low nectar volume collected from wild inbred cultivars (SP52 and SP46) with small flowers and high volume collected from wild outcrossed cultivar (219 and 269) suggests a correlation between flower size, nectarines and nectar reserves. Galetto & Bernardello (2004), Barnes & Furgala (1978) and Silva & Dean (2000) found that flower length correlated with nectary size and total volume of nectar produced suggesting structural constraints playin a major role in the nectar traits. This agrees with results of the present study where 269 and 219 with the large flowers also produced more nectar that is a reward to the pollinators. Silva & Dean (2000) recorded variation in nectar volumes in the cultivars a phenomenon we also established. This may be due to genetic differences between plants hence variability (Silva & Dean 2000) though Southwick & Southwick (1983) in their research established that nectar volume varies as a function of flower age. It would be interesting to investigate the nectar volumes of the cross of the cultivated and wild species for the purpose of increasing the nectar production since the production of more nectar may help to increase pollinator visits.

5.3 Nectar sugar composition

Bees can effectively pollinate all the six cultivars since long tongued bees prefer sucrose dominant while short-tongued ones prefer sucrose rich nectar (Baker & Baker, 1983b). The hexoses (glucose and fructose) are similar to sucrose in density and energy content. Some nectar: SP 52 had glucose - fructose ratios of 1:1 suggesting that they could be products of hydrolysis of sucrose. This agrees with the results of Van Wyk (1993). Glucose – fructose was not exactly 1:1 in nectar from 219, 269, 524B and ICV 12 suggesting that they are secreted from the nectaries independently and are not products of hydrolysis (Nepi *et al*., 2001). The chemical composition, production dynamics and removal of nectar could not be related to the pollinator visits in these cowpea species. Flower size (width) was positively correlated to nectar volume, suggesting that structural constraints may play a major role in the determination of nectar traits in cowpea species (Galleto & Bernardello, 2004). Flower size, colour, shape and fragrance are all means by which plants advertise to foraging pollinators (Heinrich & Raven, 1972; Waser & Price, 1983).

5.4 Essential oils and floral volatiles

Most of the essential oils in floral volatiles are common components of many scented angiosperm flowers (Knudsen *et al*., 1993). Floral scents emitted during the life span of the flower vary both in total output and in specific composition (Dudareva & Pichersky, 2000). The terpenoids comprised of the oxygenated and the hydrocarbons. The flowers of *V. unguiculata* (cowpea) lines investigated varied quantitatively and qualitatively. The identified floral components like eucalyptol (**72**), α-pinene (**94**), octen-3-ol, limonene (**65**), linalool (**46**), bezaldehyde (**253**) and others have been found in flowers pollinated by bees (Williams & Whitten, 1983), beetles (Thien *et al*., 2000) and butterflies (Pellmyr, 1986). This compounds detected in cowpea should also enhance

pollinator attraction. Linalool is biologically active from previous studies usin honey bee.

5.4.1 Monoterpenes

The low levels of monoterpenes in the steam disillate volatiles may be due to their low boiling points and they may have been lost at high temperatures. The observation conforms to earlier reports on low levels of monoterpenes in the steam distillate of alfalfa floral volatiles (Tava *et al*., 2000).

Ocimene (**43**) has been reported in high amounts in volatiles of many plants by several authors (Loper *et. al*., 1971; Loper & Lapioli 1972; Buttery *et. al*., 1982; Miyake *et. al*., 1998; Jurgens *et. al*., 2003). Ocimene (**43**) is a predominant floral volatile in alfalfa though it has not been detected in some cases (Pecetti & Tava, 2000). Low levels of ocimene (**43**) (0.37 %) in cowpea volatiles confirm the findings of Tava & Pecetti (1997). Ocimene (**43**) is derived from linalool (**46**) via linalyl pyrophosphate and therefore the amounts of ocimene should be related to that of linalool (Raguso & Pichersky, 1995).

Linalool is often found in the floral volatile of moth-pollinated plants (Miyake *et al*., 1998; Raguso & Pichersky, 1995) and also commonly occurs in many diurnal flowers pollinated by bees (Borg-Karlson *et al*., 1996; Loper & Lapioli, 1972) as well as Faba bean foliage (Blight *et al*., 1985). It may be located in pollen since it was not isolated from the flower during steam distillation (Flamini *et al*., 2003). Linalool (**46**) is produced in plants from the universal monoterpene precursor, geranyl pyrophosphate (**37**), by a single enzymatic reaction (Pichersky *et al*., 1994). It was found in the steam

distillates of all cowpea culivars except SP46 and in all headspace voltiles except from reverse phase C_{18} silica.

Linalool oxides are presumably derived from linalool (**46**) via the 6, 7-epoxy intermediate. Destruction of linalool synthase during hydrodistillation implies that no oxides can be formed. However, during the trapping of headspace volatiles the flowers were intact and the enzymes available hence the presence of linalool oxide in headspace volatiles. The absence of linalool oxide (**280**) and terahydrolinalool in the steam distillate could be due to denaturing of enzymes since these two monoterpenes were detected in the headspace volatiles. Knudsen & Klitgard (1998) reported the presence linalool (**46**) together with its oxides in the moth pollinated species. Linalool oxide has been reported to occur in large amounts in moth pollinated species (Knudsen & Tollsten, 1993; Knudsen & Klitgard, 1998). It also occurs in hawkmoth pollinated flowers (Miyake *et al*., 1998), flower scent in *Silene latifolia* (Dotterl *et al*., 2005), anther volatiles of Ranunculaceae (Jurgens & Dotterl, 2004) and also in floral volatiles in *Clarkie breweri* (Raguso & Pichersky, 1995) supporting our findings. It was also absent in the steam distillate just like in our case. Linalool oxide (**280**) also elicits a very strong antennal response in butterflies (Andersson & Dobson, 2003). Consquently, electroantenography (EAG) and behavioral experiments should be done to find out the bee response to linalool oxide.

Linalyl acetate has been reported in the steam distillate of *Medicago sativa* (Pecetti & Tava, 2000) in trace amounts and is supported by our research findings. It has also been previously reported in floral volatiles of *Michelia alba* (Shang *et al*., 2000), *Laurus*

nobilis (Flamini *et al*., 2002) that also contained isobornyl acetate, and butterfly pollinated plants (Andersson *et al*., 2002).

Citronellol (**49**) and geraniol (**278**) have also been reported to elicit antennal responses in butterflies (Andersson & Dobson, 2003). Geranial (**51**) and neral attract insects but also serve as deterrents to grazing herbivores (Mann, 1978). Terahydrogeranial, neral and isogeranial were also identified in the cowpea floral volatiles. Their role in honey bee behavior should be investigated since they may be structural fits for Nasonov pheromone.

Limonene (**65**) and α-pinene (**95**) found in volatiles of *Anthonomus grandis* attract cotton boll weevil while β-pinene (**95**), β-myrcene (**41**) and car-3-ene (**93**) attract female bark beetles, *Dendroctonus brevicomis* (Mann, 1978). Limonene (**65**), β-pinene (**95**), β-myrcene (**41**), α-pinene (**94**), β- myrcene (**42**) and 1, 8-cineole (**72**) have been found in many floral volatiles (Bergström, 1978).

The biosynthetic pathway of irregular terpenes is enzyme controlled and may explain the reduced number in the steam distillate compared to the headspace volatiles. Chrysanthemyl skeletal derivatives are the most common because they are derived directly from chrysanthemyl pyrophosphate (**115**).

5.4.2 Sesquiterpenes

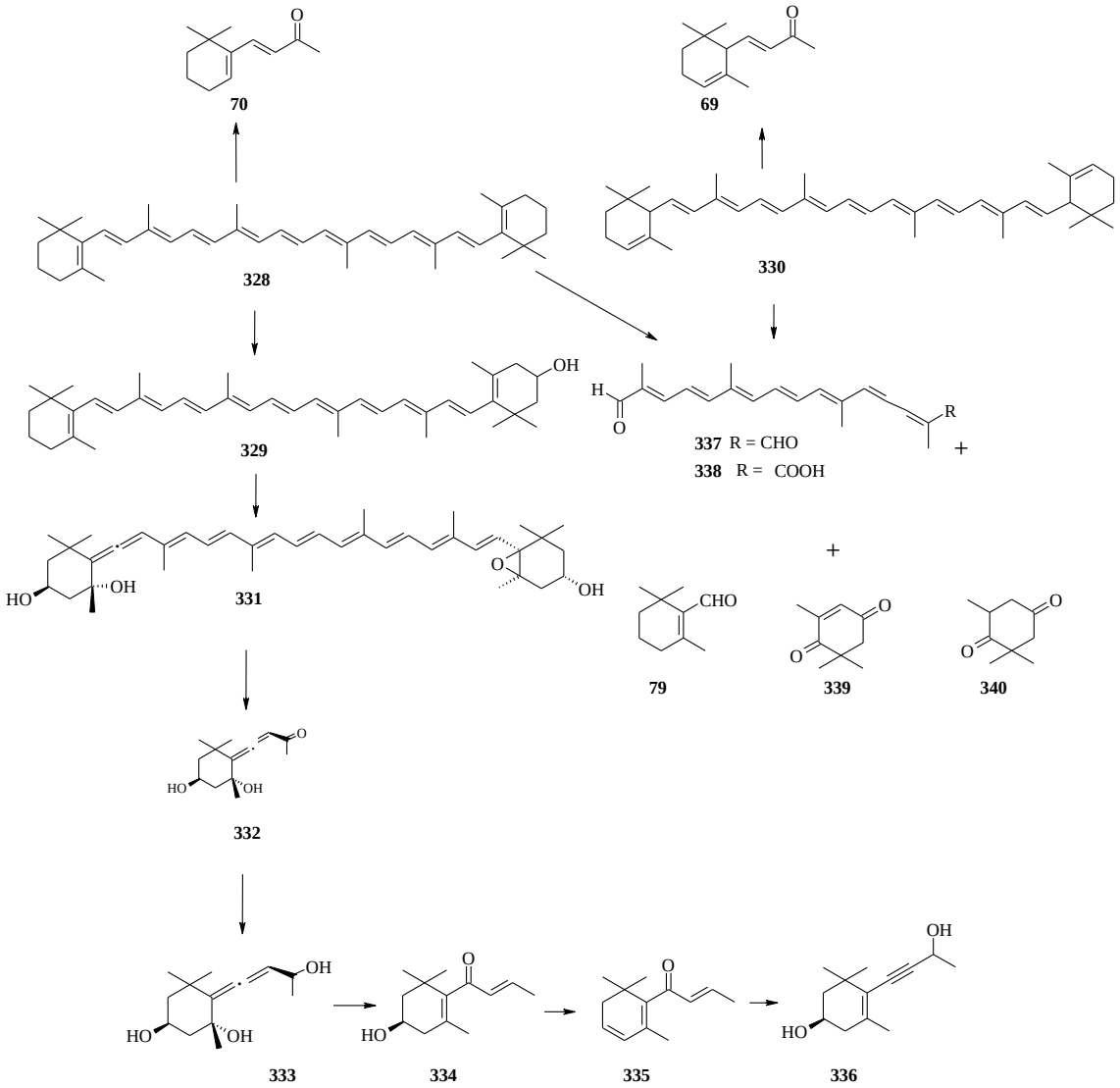
Nerolidol (**156**) is a constituent of many plant essential oils and headspace volatiles of several flowers (Knudsen & Stahl 1994; Miyake *et al*., 1998; Tava *et al*., 2000). Geranyl linalool is an isomer of nerolidol (**156**) (Gabler *et al*., 1991). Nerolidol (**156**) and farnesol (**154**) are isomeric sesquiterpenes derived from FPP (**134**) and NPP (**135**)

(Scheme 3). In the steam distillate of cultivar 219, nerolidol (**156**) was found in equal proportion with farnesol suggesting thermal isomerization. In 524B, nerolidol (**156**) was more than the farnesol (**154**) in the ratio 3:1. This could mean that NPP (**135**) was more than FPP (**129**), the later forming farnesyl cation (**231**) and the NPP (**135**) forms neryl cation (**232**) (Bouwmeester *et. al.*, 1999). Other sesquiterpenes whose production may have been enzymatically controlled from the universal precursors were also reported in both the steam distillate and headspace volatiles.

Cadinene (**166**), *β*-caryophyllene (**180**), germacrene B (**176**) and D (**178**) have been reported in floral scent composition in humming-bird pollinated taxa (Knudsen *et al*., 2004). From biosynthetic considerations, the presence of *β*-caryophyllene (**180**) and germacrene D (**178**) can be closely related to that of nerolidol (**156**) and farnesol (**157**) (Scheme 3). However, only germacrene D (**178**) was detected in the floral volatiles. This could be due to the fact that it may be the only stable germacrene isomer. Germacrene D (**178**) is produced by cotton plants and attracts cotton boll weevil (Fraga, 1998). Germacrene A (**175**) was not detected in the voaltiles. It is highly sensitive to heat and the acidic conditions in plant vacuoles and undergoes cope rearrangements to form *β*-elemene (**185**) (De Kraker *et. al*., 1998; Fraga, 1998). The sesquiterpenes identified in cowpea floral steam distillate and headspace volatiles have been reported in other floral oils (Shang *et. al*., 2001; Andersson *et al*., 2002). However, the presence of sesquiterpenes in floral volatiles has not been reported in many cases (Olesen & Knudsen, 1994; Azuma *et al*., 2001)

5.4.3 Norisoprenoids

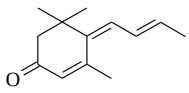
Norisoprenoids are derived from caroteniods present in flowers (Scheme 8). The *β*-norisoprenoid precursors, *β*-carotene (**328**) and *β*-cryptoxanthin (**329**) may have resulted in the formation of *β*-ionone (**81**) while *α*-carotene (**330**) may have undergone cleavage to yield *α*-ionone (**80**). *β*-Cyclocitral (**79**) may be a product of *β*-carotene (**328**) cleavage (Knudsen & Stahl, 1994). When acted upon by cleaving enymes, it forms *trans*-crocetindialdehyde (**337**) and *trans*-crocetinhalbaldehyde (**338**). 4-Oxoisophorone (**339**) and 2, 6, 6- trimethyl-2-cyclohexene-1, 4-dinone and 2, 6, 6-trimethyl-1, 4-cyclohexanedioxide (**340**) (Knudsen & Stahl, 1994). *Cis*-Jasmonic acid, a derivative *cis*-jasmone, is used in plant deterrence to herbivores and is produced when a plant is wounded (Bodenhausen & Phillippe 2007; Mueller, 1993; Zenk, 1993). The presence of *cis*-Jasmone points to the fact that cowpea plants may produce deterrent compounds to prevent herbivore feeding. It has previously been reported in the floral volatiles of butterfly pollinated plants (Andersson *et al*., 2002) and in floral oils of mandarin (Flamini *et al*., 2003).



Scheme 8: Biosynthesis of norisoprenoids from carotenoids (Puglisi *et al*., 2001; Knudsen & Stahl, 1994).

Cowpea cultivar 524B, with yellow flowers, gave a higher percentage of *β*-cyclocitral (**79**). This may be due to high amounts of carotenoids in the yellow flowers (Knudsen & Stahl, 1994). *β*-Cyclocitral (**79**) has been reported in the floral volatiles of *Lantana camara* (Anderson & Dobson, 2003) while in the flowers of alfalfa it is found together with *β*-ionone (**81**) and *α*-ionone (**80**) (Tava *et al*., 2000). *β*-Ionone has previously been reported in the floral volatiles of butterfly pollinated plants (Andersson *et al*., 2002). Damascenone (**334**) is one of the important flavour compounds (Pickenhagen, 1999). It

is found in many fruit and vegetable products and is also used in fragrance industry. It is formed from hydrolytic breakdown of complex secondary metabolites derived from secondary carotenoids such as neoxanthin (**331**) (Skouroumounis & Sefton, 2001). Neoxanthin (**331**) cleaves to produce grasshopper ketone (**332**), which leads to 3, 5, 9-trihydroxymegastigma-6, 7-diene (**333**). *β*-Damascenone (**335**) has been found together with 3-hydroxydamascenone (**334**) and megastigma-5-en-7-yne-3, 9-diol (**336**) (Puglisi *et al*., 2001; Woo *et al*., 1991). Megastigmatrienone (**341**) is a product of carotenoid cleavage (Puglisi *et al*. 2001) and a precursor of *β*-damascenone (**335**). It exists as isomers and has been isolated from tobacco, several plant flower oils and *Quercus petreae* Liebl. heartwood oil (Nonier *et al*., 2005).



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5.4.4 Aromatics

Anthers and pollens emit distinctive floral odors (Cook *et al*., 2002). Some components of volatiles from flowers elicit sticky antennal responses (Blight *et al*., 1995). Phenylacetyldehyde (**254**) elicits a strong antennal response and foraging behaviour (Omura, 1999a; 1999b) and it stongly attracts day and night active Lepidoptera species (Honda *et al*., 1998). Since it was also detected in the cowpea volatiles, it should be tested to investigate the effect on honey bee behaviour and antennae.

A number of aromatic compounds detected in cowpea floral volatiles have been reported in other plants. Their effect on foraging response of bumble bees has been reported (Odell *et al*., 1999). Aromatic compounds are present at high concentrations in the floral bouquets of other leguminous flowers such as white (Jacobsen & Olsen, 1994) and red

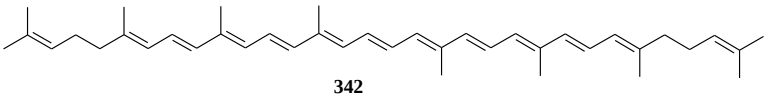
clover (Buttery *et al*., 1984). The presence of toluene in both steam distillates and headspace volatiles confirms earlier observations on alfalfa floral oils (Tava *et al*., 2000) where its existence in both techniques was also reported. The exclusive presence of 1, 3, 5-trimethylbenzene in the headspace volatiles of cowpea flowers confirms earlier observations in alfalfa volatiles (Tava *et al*., 2000). However, the presence of ethylbenzene only in the steam distillate in cowpea floral volatiles does not conform to previous observations on alfalfa where it was found in the headspace volatiles only (Tava *et al*., 2000). In *Medicago sativa* floral volatiles, 1,2-dimethylbenzene and 1,3-dimethylbenzene were both found in the steam distillate but the latter was only present in the headspace volatiles (Tava *et al*., 2000). In cowpea floral volatiles, 1,4-diethylbenzene was found in the headspace volatiles only. *p*-Cymene (**291)** was in cowpea floral volatiles while it has not been reported in alfalfa oil (Tava *et. al*., 2000). This confirms the earlier findings on butterfly pollinated plants (Andersson, 2003) and SPME floral volatiles (Flamini *et al*., 2003). *p*-Cymene (**291)** elicited large antennal responses in butterflies (Andersson *et al*., 2002) and was also present in the floral volatiles of *Dianthus* and *Saponaria* species (Jürgens *et al*., 2003). *p*-Xylene (**292)** has been reported in floral volatiles of *Lavandula* species (Mansfield, 2000).

Methyl cinnamate (**304**) has been reported in the floral odours in Theophrastaceae (Knudsen & Stahl, 1994). Interestingly, methyl cinnamate (**304**) has not been found to deter nor stimulate foraging bumble bees (Odell *et al*., 1999). Carvacrol (**307**) was previously reported in the floral oils of several species of Theophrastaceae (Knudsen & Stahl, 1994) and floral oils of plants pollinated by butterflies (Anderson *et al*., 2002). Similarly phenyl methanol (**306)** was previously reported in traces in floral oils of plants pollinated by butterflies (Anderson *et al*., 2002). Benzaldehyde (**253**), which is present

in floral volatiles of all cowpea cultivars, has been reported as a characteristic attractant in generalist flower. It also shows a significant antennal response in butterflies even when present in small amounts (Anderson & Dobson, 2003). Phenylvinyl ketone, (**301**) phenylpropanone (**311**), 2-methylbenzaldehyde (**298**), *p*-methoxyacetophenone (**309**) and acetophenone (**299**) have not previously been reported in floral volatiles and therefore behavioral and EAG studies should be done to investigate their significance on foraging by pollinators. Methyl anthranilate (**312**) has been reported in the floral oils of bitter gourd (Fernando & Grun, 2001). It is also found in the floral oils of *Plantantheria biflora* (Plepys *et al*., 2002b) and plants pollinated by butterflies and evoked EAG responses of silver Y moth (Anderson *et al*., 2002).

5.4.5 Aliphatic compounds

The numerous acids may have been formed from breakdown of carotenoids: lycopene (**342**), antheroxanthin (**343**) and flavoxanthin (**344**), found in the petals. Carotenoids are wideliy distributed in non-photosynthetic tissues of plants and are responsible for the yellow, red and orange color of flowers (Britton, 1991).



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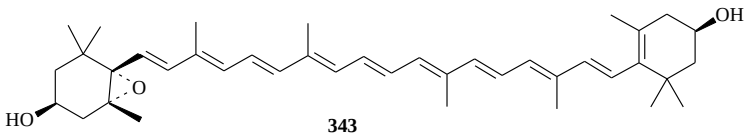

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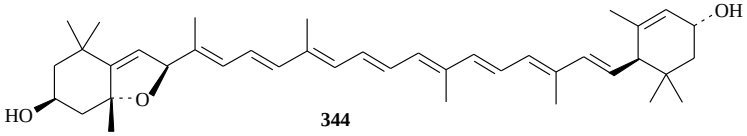

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Several aliphatic compounds like heptanal, eicosane, pentacosane and methyl esters among many others have been identified in nectar (Jakubska *et al*., 2005). Aliphatic compouds may be abundant in floral volatiles because appreciable quantitities of lipids can be present in the nectar and also on the surface of pollen (Ross & Murphy, 1986, Simpson & Neff, 1983). In hydro distillation, the intense heating (Buchmann, 1987) may have resulted in numerous straight chain compounds such as esters, ketones, acids, alcohols and aldehydes from pollen, nectar and floral oils. There were lower molecular weight compounds in the headspace volatiles than in the steam distillate as reported in floral oils of alfalfa (Tava *et al*., 2000) and volatiles of Faba beans (Porter *et al*., 1999). They are most likely produced by enzymatic activity of flower lipoxygenases and hydroperoxide lysases, which are active under normal conditions but are denatured by heat (Tava *et al*., 2000).

Very few long chain hydrocarbons and esters were detected in cowpea volatiles. The presence of tricosane, eicosane and pentacosane in cowpea volatiles confirms their presence in floral oils of flowers pollinated by bees as previously reported (Sazima *et al*., 1993) and in the SPME collected volatile constituents of *Mechelia alba* flowers (Shang *et al*., 2001). Like in the SPME collected *Mechelia alba* floral volatile (Shang *et al*., 2001) and flowers pollinated by bees (Sazima *et al*., 1993) tricosane was found in higher amounts in the headspace cowpea floral volatiles.

The long chain acids, myristic, lauric and palmitic acids were detected in the steam distillate only and have been reported in alfalfa floral volatiles together with oleic and stearic acids (Tava *et. al*., 2000). The presence of palmitic acid in headspace volatiles in small amounts conforms to earlier results in perfume flowers of *Cyphomandra species*

(Sazima *et al*., 1993). Palmitic acid is the preliminary end product of fatty acid biosynthesis through fatty acid synthase (FAS) and is ubiquitous. A part from producing nectar, the flowers of *Brysonima crassifolia* L. contains fatty acids (palmitic, stearic, oleic) together with their esters (Rezende & Fraga, 2003). The high temperatures used for steam distillation may have caused the thermal degradation of amino acids, sugars and fatty acids resulting in the accumulation of the many aliphatic compounds and facilitated extraction of the less volatile compounds such as long chain hydrocarbons and fatty acids.

Most of the alcohols found in the cowpea floral volatiles (octanol, hexanol, *cis*–3-hexenol, nonanol, 1-octen-3-ol amongst many others) have been previously reported in the floral oils (Andersson *et al*., 2002; Tava *et al*., 2000; Bergstrom *et al*., 1995; Jurgens *et al*., 2003; Flamini *et al*., 2003). Hexyl acetate (**282**) and *cis*-3-hexenyl acetate found in the cowpea floral volatiles have also been reported in floral oils of various plants (Anderson *et al*., 2002; Tava *et al*., 2000). 4-Octen-3-one present in the cowpea floral steam distillate is being reported for the first time in the floral oils. Nonanal (**290**), *trans*-2-hexenal, hexanal (**247**), decanal and heptanal found in cowpea floral volatiles have been reported in the floral oils of other plants (Anderson *et al*., 2002; Tava *et al*., 2000; Bergstrom *et al*., 1995; Jurgens *et al*., 2003; Flamini *et al*., 2003). Heptanal and nonanal have been reported in the nectar of Orchideceae plants (Jakubska *et al*., 2005). The many aliphatic compounds (alcohols; aldehydes and ketones) are most likely produced by enzymatic degradation of fatty acids during sampling in headspace trapping and thermal degradation of floral oils during distillation. 3*E*-Hexen-1-ol (**270**) and 3*Z*-hexen-1-ol (**269**) may have come from the green flower sepals (Hatanaka, 1993). In general, the aliphatic C_6 compounds (hexanal and 2*E*-hexenal, alcohols 3*Z*-hexen-1-ol

(**269**) and 2*E*-hexen-1-ol), contribute to the "green" notes of the aroma that is present in the majority of the petals of mums (Jakubska *et al*., 2005). The formation of these compounds in the plant is related to cell destruction or cellular breakdown due to maturation of the flowers. 1-Octen-3-ol has also been detected in several mums suggesting the activity of lipoxygenase and hydroperoxidase that produce C_8 compounds from linoleic acid (Jakubska *et al*., 2005). It is also an aglycone produced by acid hydrolysis of glycosidically bound volatiles (Radonic & Mastellic, 2008).

5.4.6 Miscellaneous compounds

Furfural (**323**) was reported in alfalfa steam distillate only (Tava *et al*., 2000). It is also found in the nectar of orchids (Jakubska *et al*., 2005). The presence of furfural in cowpea cultivars is of good value because of its anti-fungal and anti-bacterial properties (Jakubska *et al*., 2005).

Indole has been found in hawk moth pollinated flowers (Miyake *et al*., 1998), butterfly pollinated plants (Andersson *et al*., 2002) and perfume flowers of *Cyphomandra species* (Sazima *et al*., 1993). Indole (**257**) was found in cowpea floral volatiles as previously reported in alfalafa floral steam distillates (Tava *et al*., 2000). It has also been reported in the floral scents of *Dianthus* and *Saponaria* species (Jurgens *et al*., 2003), *Lantana camara* (Andersson & Dobson, 2003), *Magnola kobus* (Azuma *et al*., 2001), bitter and ridge gourd (Fernando & Grun, 2001) The presence of indole (**257**) in the floral steam distillate only may be due to degradation of nectar constituents. A related compound 3-{2-{3-{3-(benzyloxy) propyl}-3-indole has been found in the orchid nectar (Jakubska *et al*., 2005). Together with skatole (**258**), they attract insects (Williams & Whitten, 1983) that forage on the hawk moth pollinated flowers.

Ethers have been reported in the floral volatiles of cowpea. Jacobsen & Olesen (1994) also reported ethers like thymyl methyl ether, 2-methoxy-2, 3-epoxy butane and eugenol methyl ether in the floral volatiles of *Trifolium repens*. Radonic & Mastellic, (2008) have also reported thymol methyl ether, carvacrol methyl ether, and 4- hydroxyl-3-methoxy acetophenone in the essential oils of *Thymus pulegioides*.

5.5 Conclusion

Wild outcrossed (219 and 269) and cultivated (524B and ICV12) cowpea produced large flowers while wild inbred (SP46 and SP52) produced small flowers. Plants with large flowers gave higher yields of nectar. The three most common sugars in nectar were sucrose, glucose and fructose. The nectars were sucrose rich. Mannose and raffinose were rare in the nectar. Steam distillate produced more compounds than trapping using adsorbents (activated charcoal, reverse phase silica gel and porapak Q). However, in the two techniques aliphatic compounds were the most abundant. Porapak Q adsorbent trapped the largest number of volatile compounds especially aromatics. More acids were found in the steam distillate than headspace. Headspace technique had numerous short chain aliphatic cmpounds that were lacking in the steam distillate. Similarly, more isoprenoids were collected from the headspace technique. Norisoprenoids were mostly in the steam distillate.

5.6 Recommendations

Since the release of volatiles and nectar are controlled by a number of factors (climatic environmental and soil), it is therefore necessary to undertake further research to establish the best conditions for the optimum production of attractants and rewards for pollinators to maximize cowpea crop yields.

Reflectance spectrum of cowpea flowers should be done to establish if colour affects bee visitation (pollination), improves efficiency of pollination and enhances crop yield.

EAG and behavioral experiments should be conducted to establish the floral volatile compounds, which attract honeybee to cowpea flowers with a view of breeding cultivars rich in such compounds to enhance pollination and hence improve cowpea yields.

Further work should also be done to establish nectar sugar composition on hourly basis after withdrawing nectar.

The amino acids in the cowpea nectar also need to be analysed to establish their effect on foraging insects and birds.

The organic acids in the cowpea nectar need to be analyzed to establish their effect on foraging insects and birds.

During this work it was noted that the cultivated variety of cowpea was readily attacked by viral diseases. The chemical bases of resistance to pests and viral diseases by inbred and wild cowpea cultivars should be investigated.

Since some cowpea varieties may have medicinal value, the compounds responsible should be isolated and investigated for the therapeutic properties.

Dynamic headspace trapping and SPME should also be done on cowpea cultivars to compare the volatiles profile with static method.

Extraction of volatiles needs to be done using different solvents to establish qualitative and quantitave differences to establish the pollinator attractants

Cowpea pollen also needs to be analyzed for the volatiles they release and amino acid content to establish the amount of rewards in them for the honey bee visitation.

More work should also be done on the EFN to establish if they also affect pollination efficiency.

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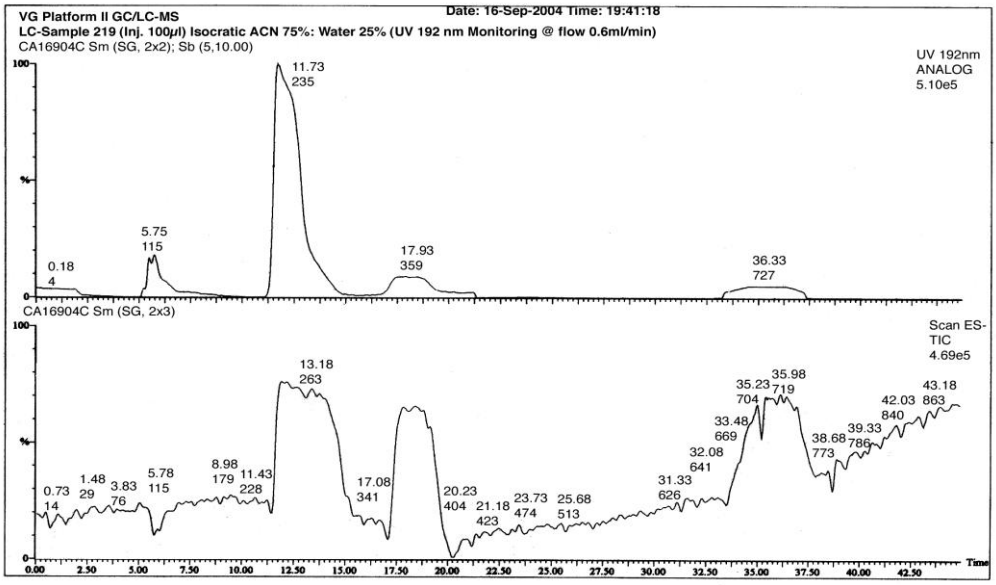
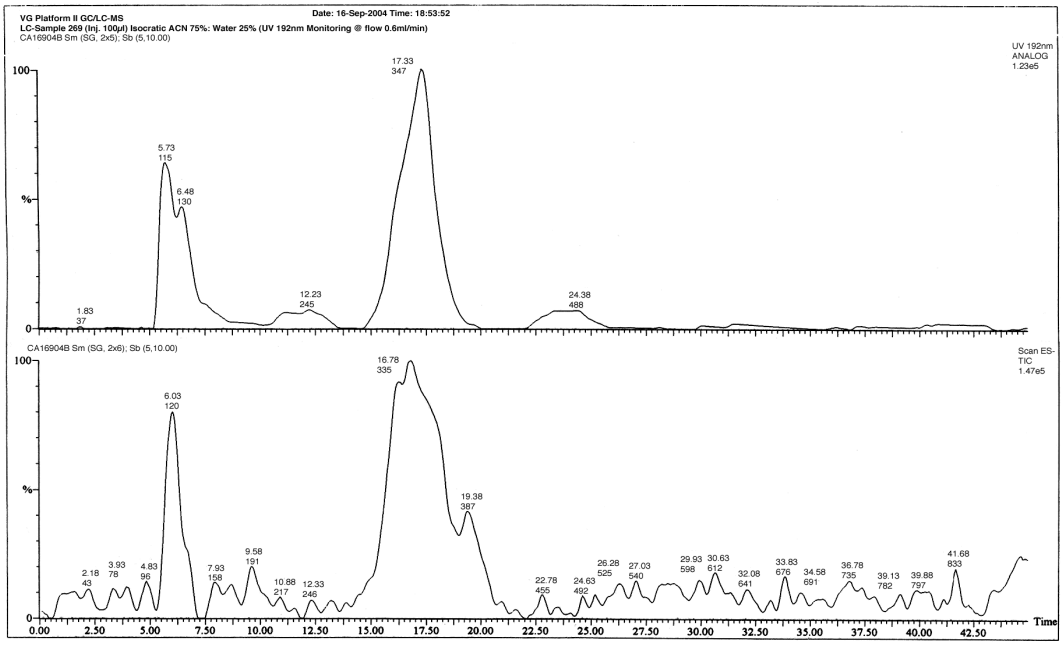
APPENDICES

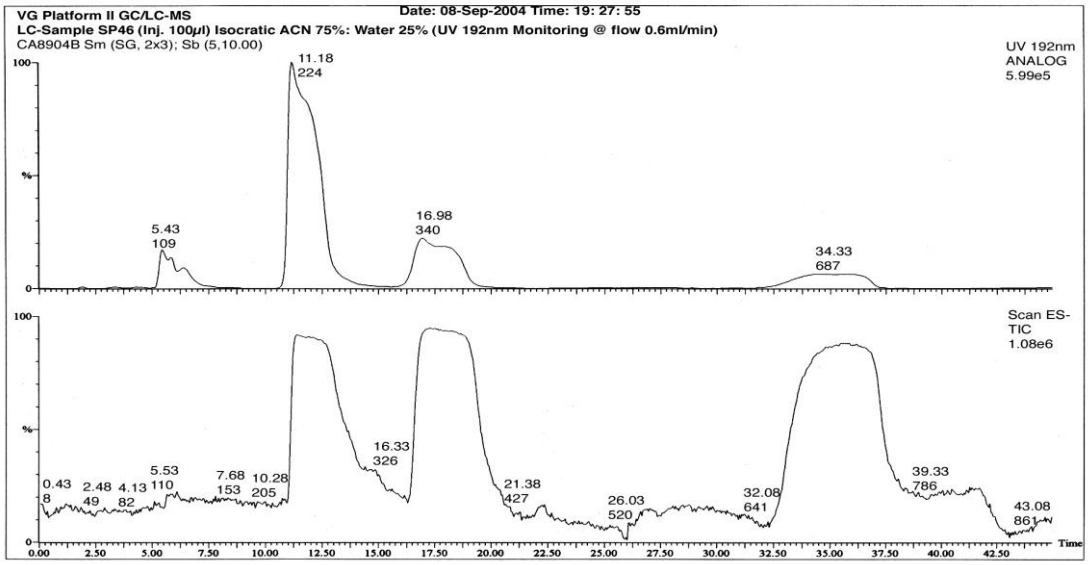
Appendix 1: Combined UV analogues and LC-MS profiles



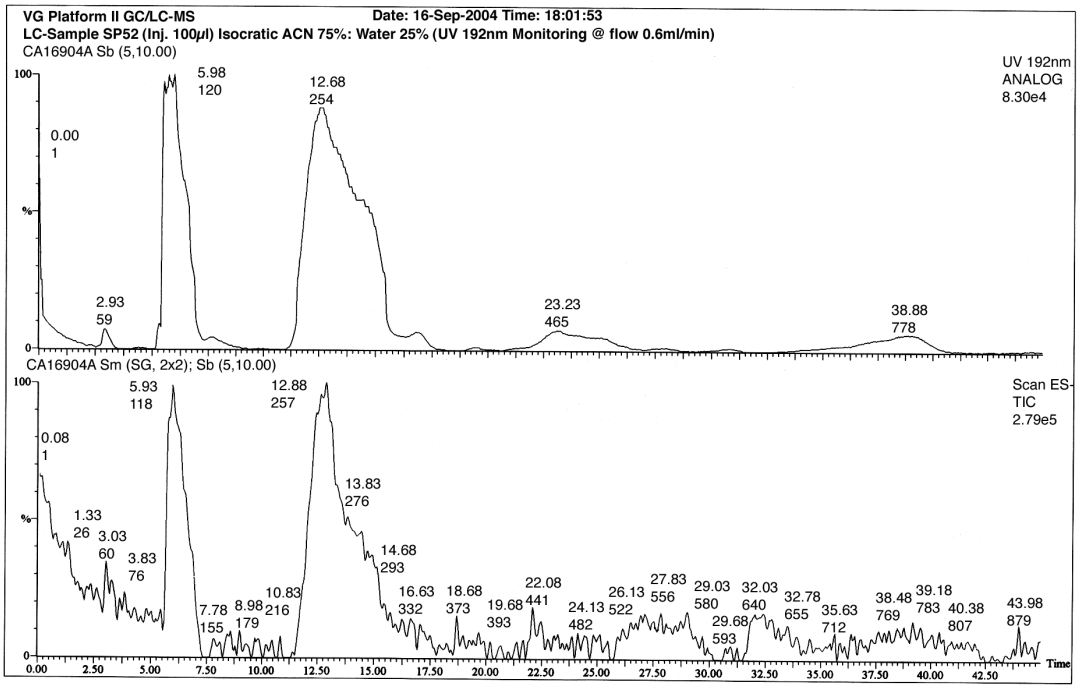
Appendix 1a: Combined UV analogue and LC-MS profile for cultivar 219



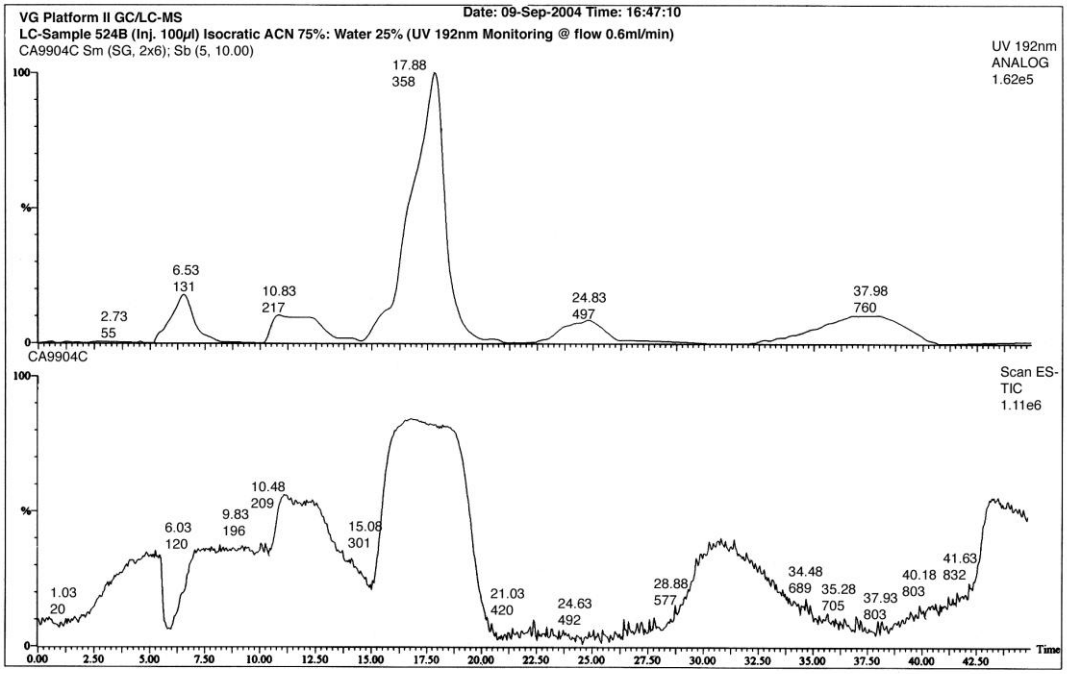
Appendix 1b: Combined UV analogue and LC-MS profile for cultivar 269



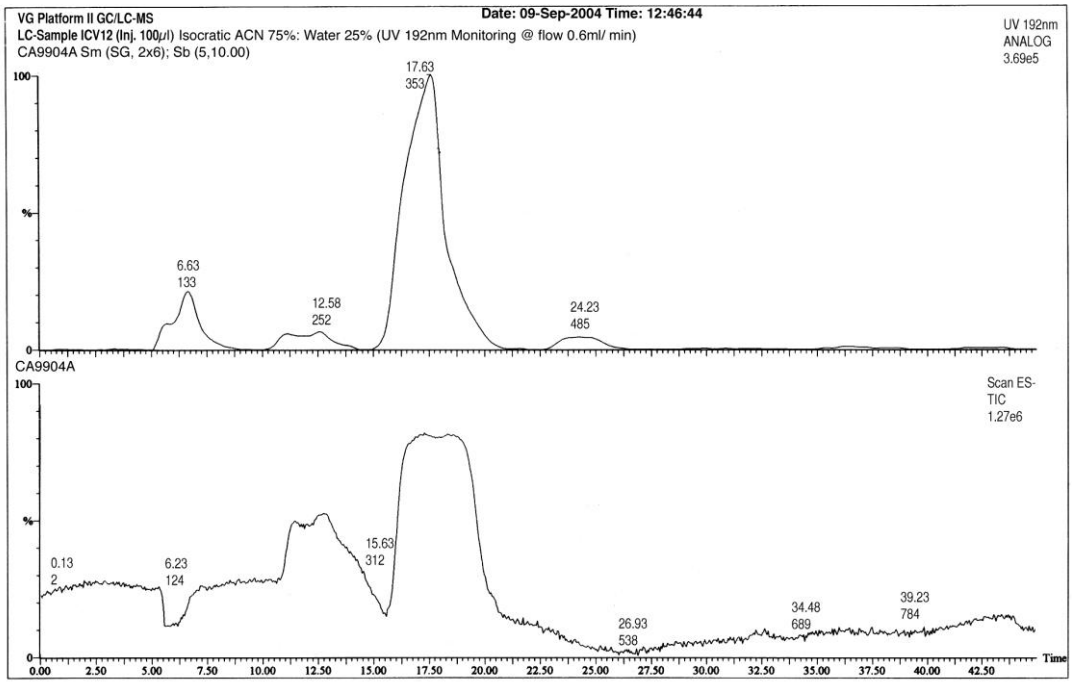
Appendix 1c: Combined UV analogue and LC-MS profile for cultivar SP46



Appendix 1d: Combined UV analogue and LC-MS profile for cultivar SP52

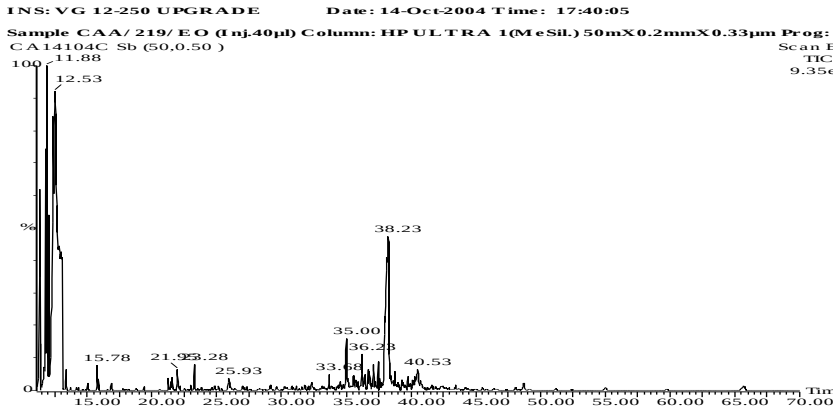


Appendix 1e: Combined UV analogue and LC-MS profile for cultivar 524B

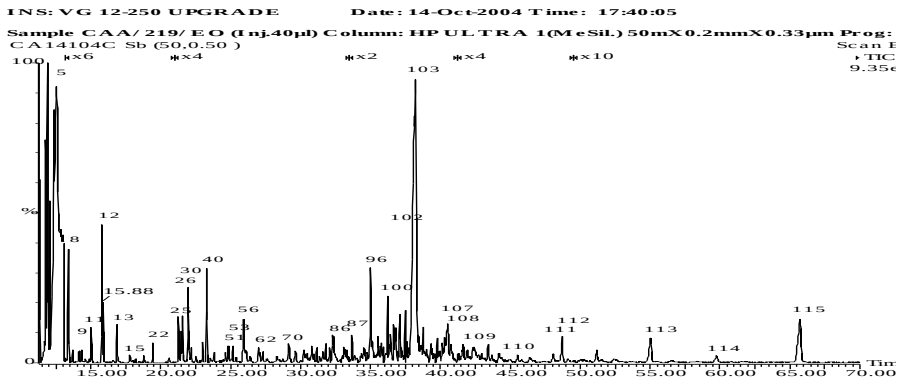


Appendix 1f: Combined UV analogue and LC-MS profile for cultivar ICV12

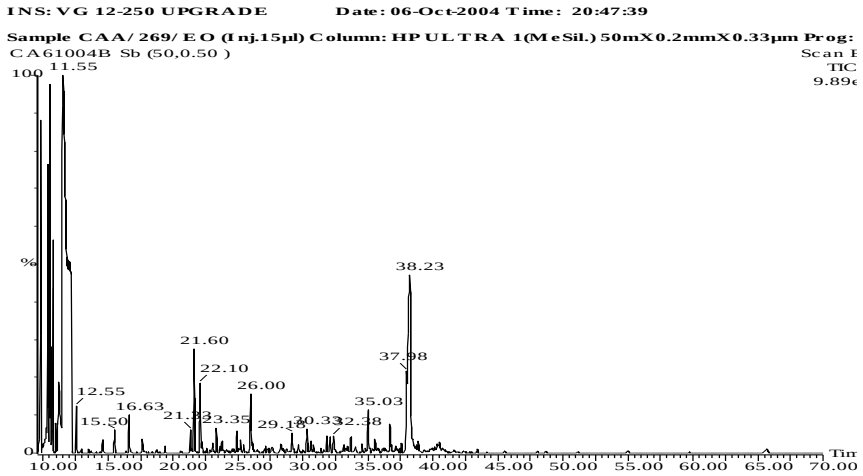
Appendix 2: GC profiles for essential oils



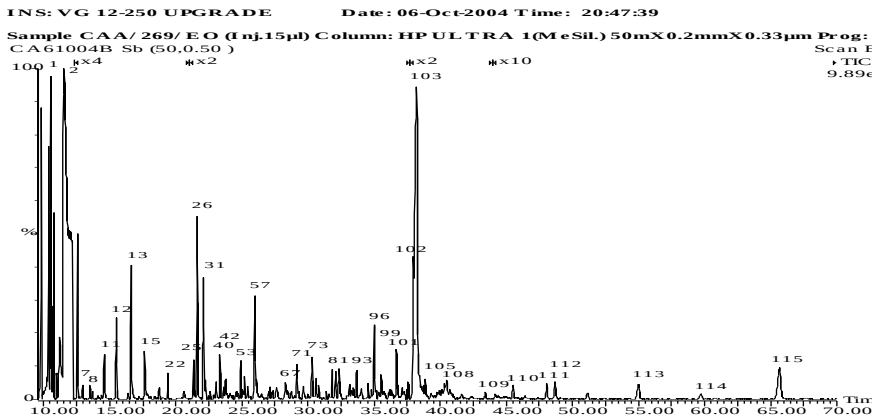
Appendix 2a: GC profile of essential oil of cowpea cultivar 219



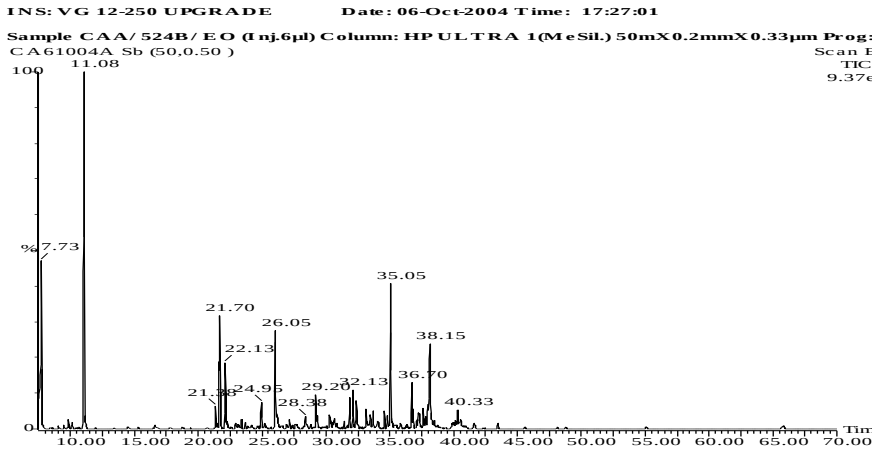
Appendix 2b: Magnified GC profile of essential oil of cowpea cultivar 219



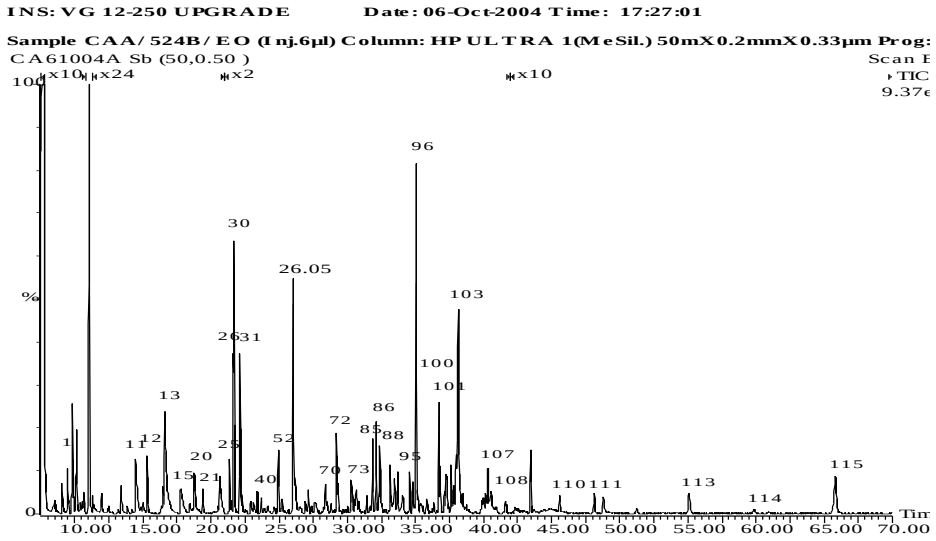
Appendix 2c: GC profile of essential oil of cowpea cultivar 269



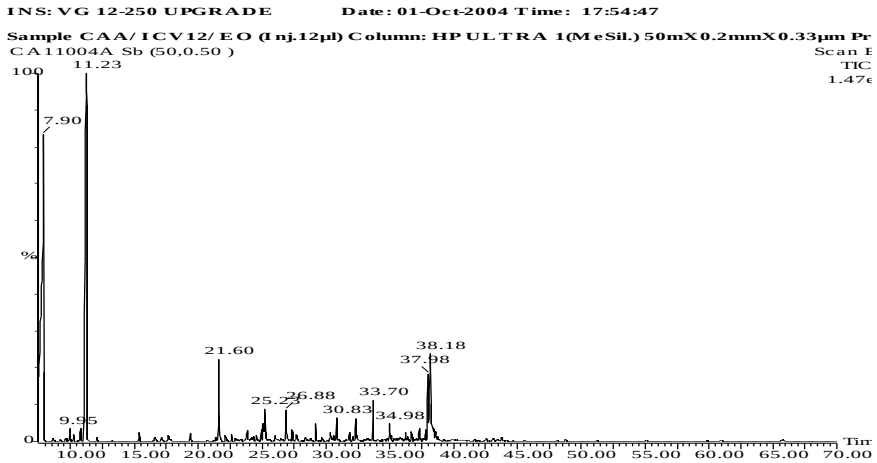
Appendix 2d: Magnified GC profile of essential oil of cowpea cultivar 269



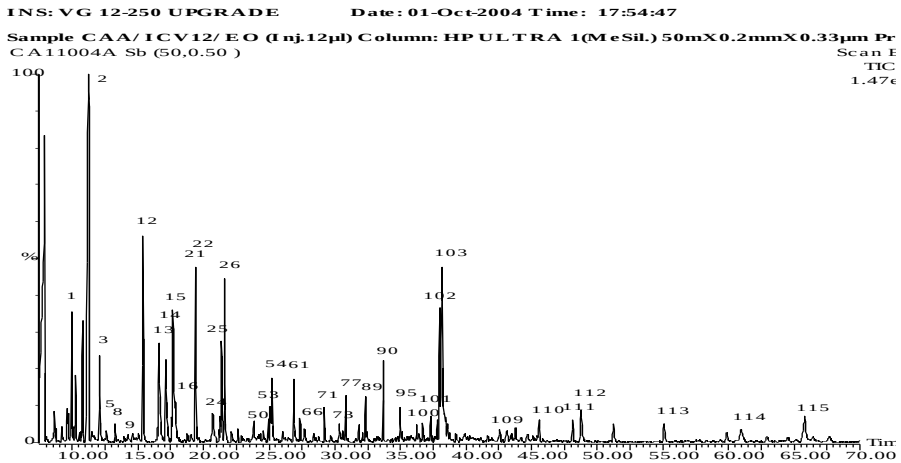
Appendix 2e: GC profile of essential oilof cowpea cultivar 524B



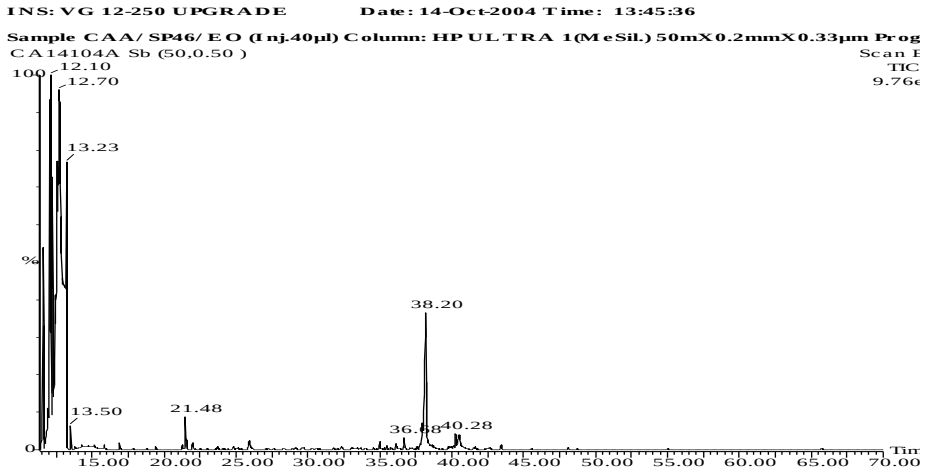
Appendix 2f: Magnified GC profile of essential oil of cowpea cultivar 524B



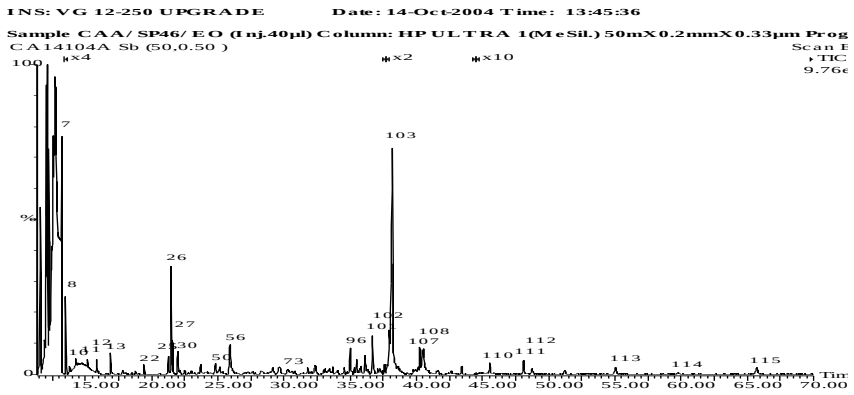
Appendix 2g: GC Chromatogram of essential oil of cowpea cultivar ICV12



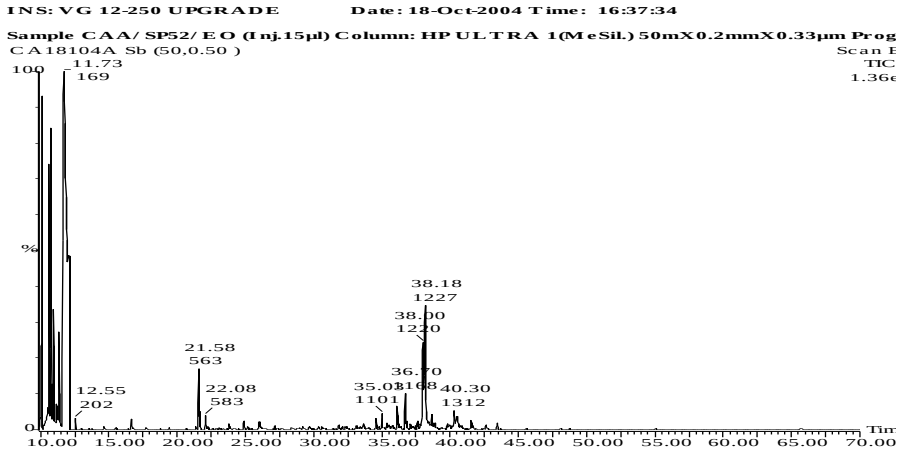
Appendix 2h: Magnified GC profile of essential oil of cowpea cultivar ICV12



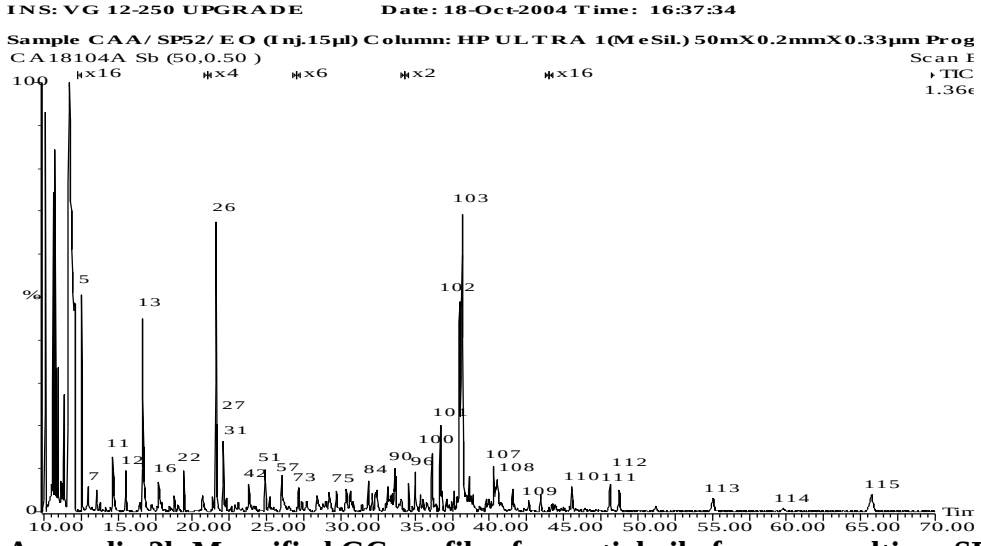
Appendix 2i: GC profile of essential oil of cowpea cultivar SP46



Appendix 2j: Magnified GC profile of essential oil of cowpea cultivar SP46

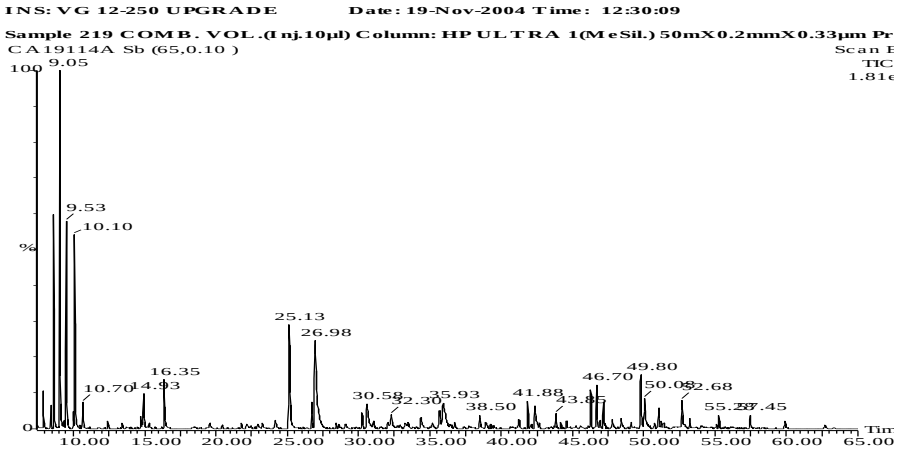


Appendix 2k: Normal GC profile of essential oil of cowpea cultivar SP52

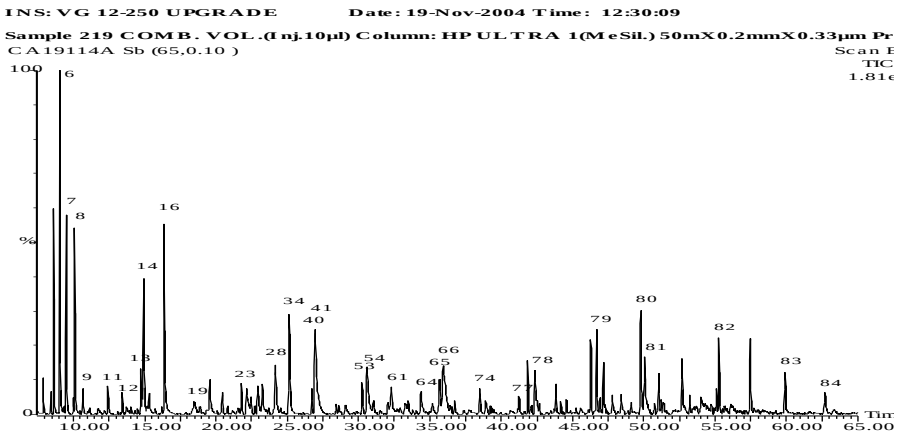


Appendix 2l: Magnified GC profile of essential oil of cowpea cultivar SP52

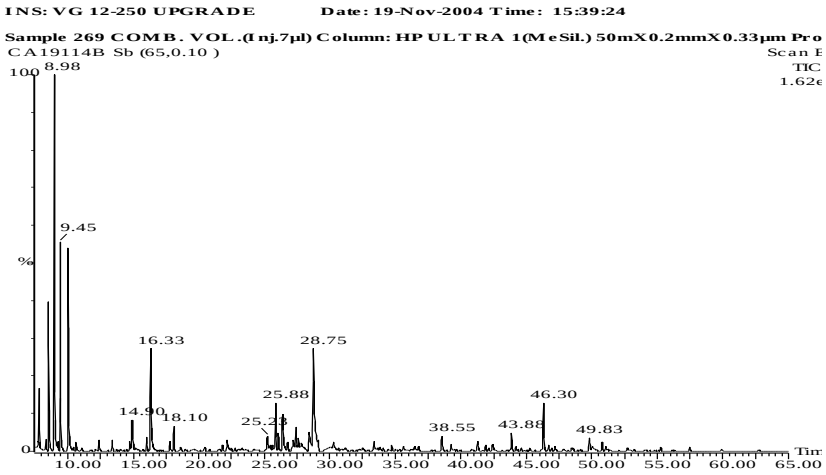
Appendix 3: GC profiles for floral volatiles trapped in combined adsorbents



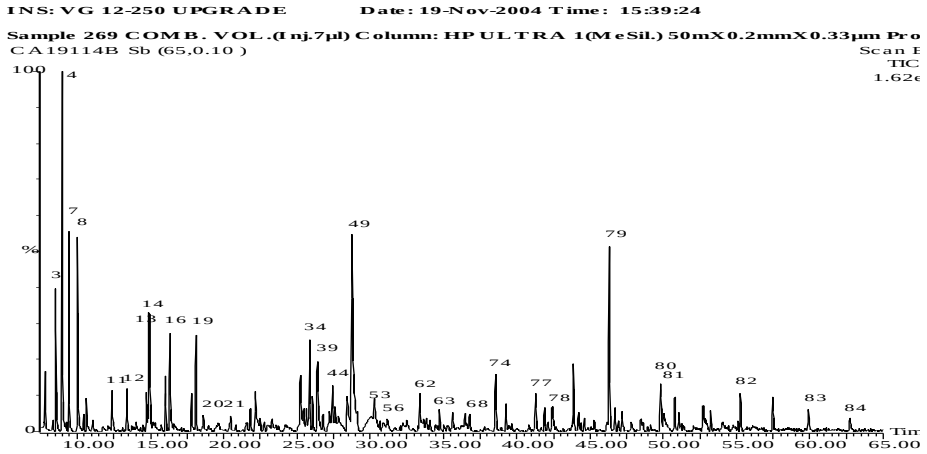
Appendix 3a: GC profile of floral volatile from cowpea cultivar 219



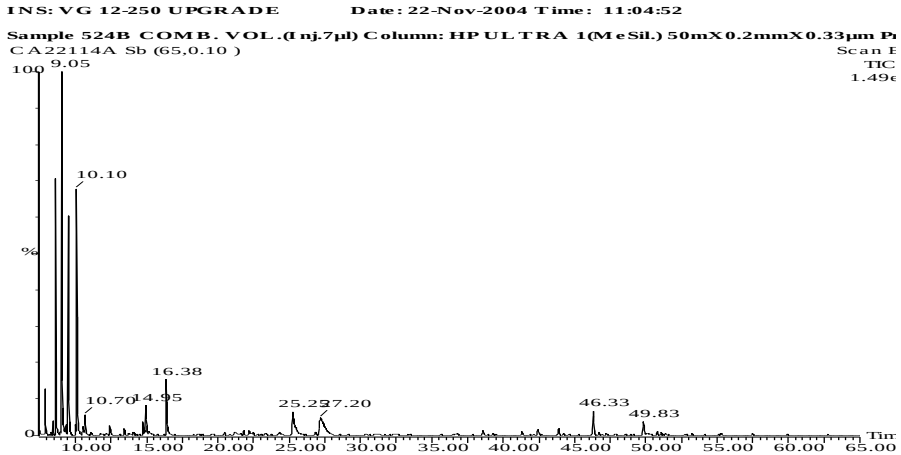
Appendix 3b: Magnified GC profile of floral volatile from cowpea cultivar 219



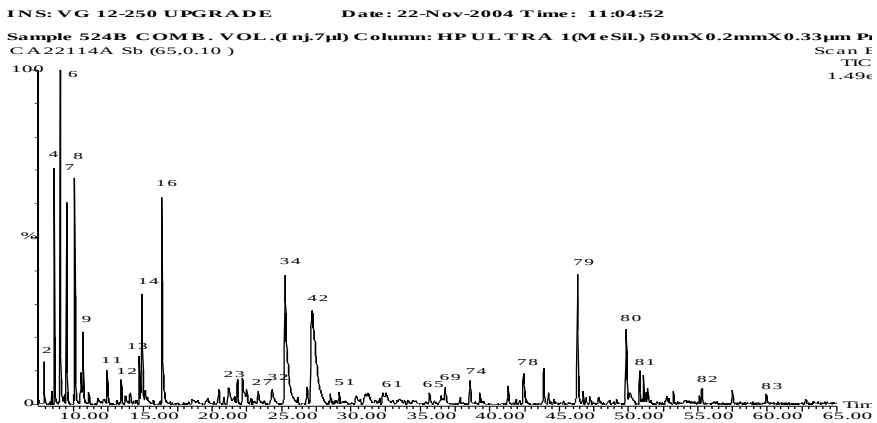
Appendix 3c: GC profile of floral volatile from cowpea cultivar 269



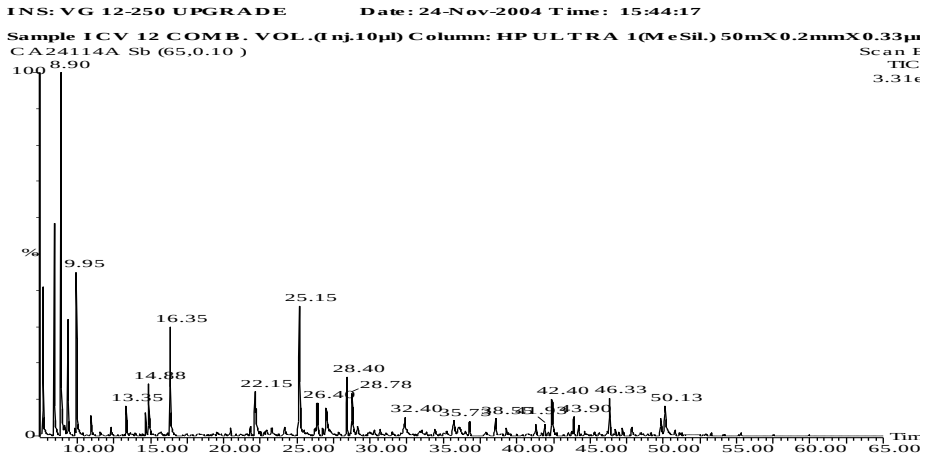
Appendix 3d: Magnified GC profile of floral volatile from cowpea cultivar 269



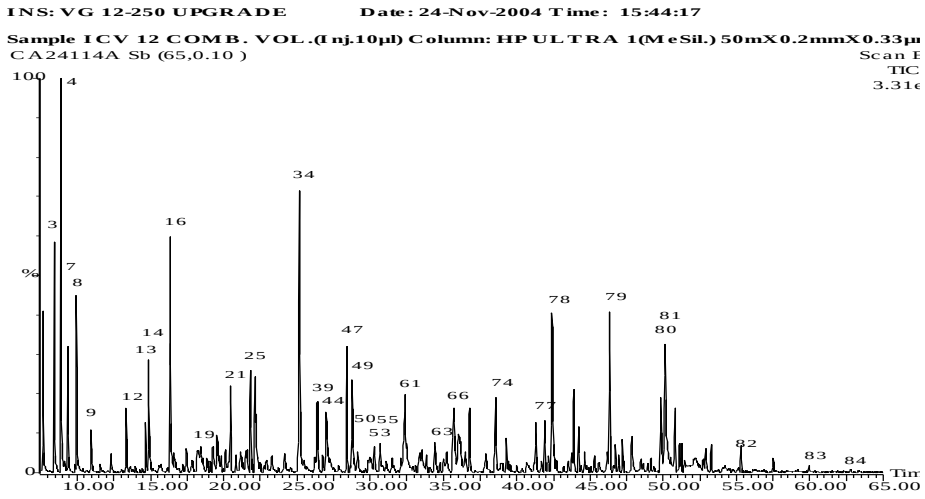
Appendix 3e: GC profile of floral volatile for cultivar 524B



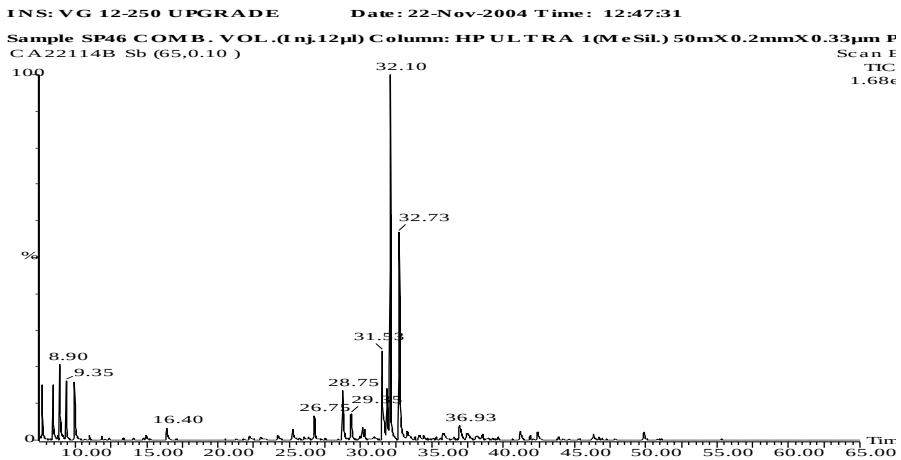
Appendix 3f: Magnified GC profile of floral volatile for cultivar 524B



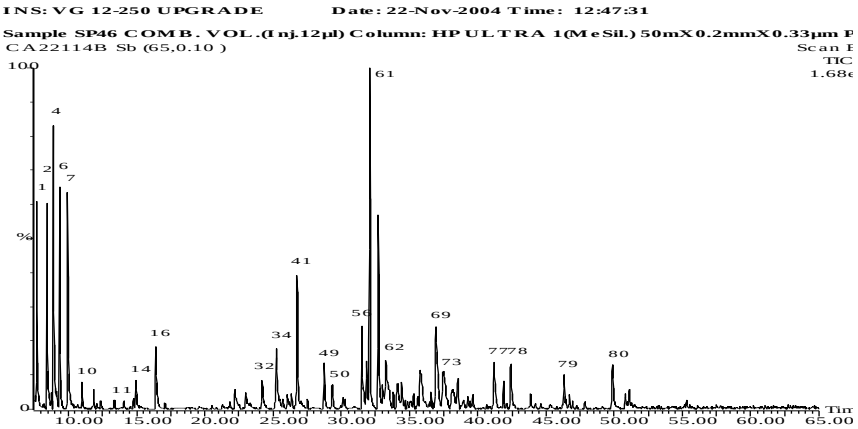
Appendix 3g: GC profile of floral volatile for cultivar ICV 12



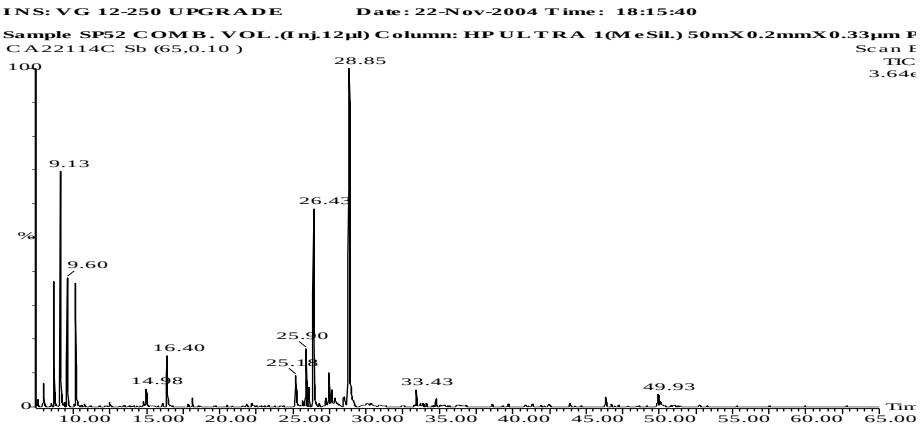
Appendix 3h: Magnified GC profile of floral volatile for cultivar ICV12



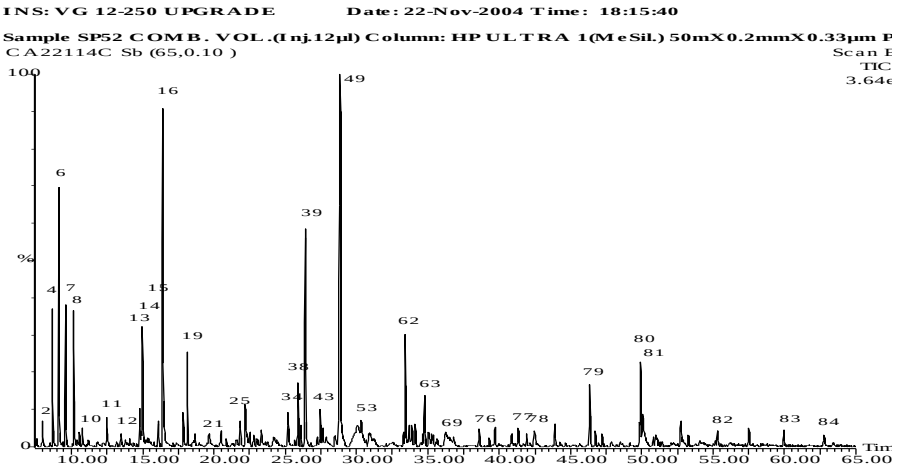
Appendix 3i: GC profile of floral volatile for cultivar SP46



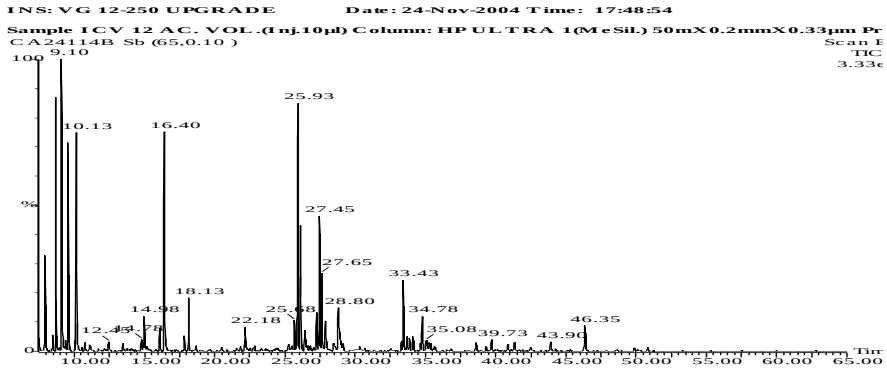
Appendix 3j: Magnified GC floral of floral volatile for cultivar SP46



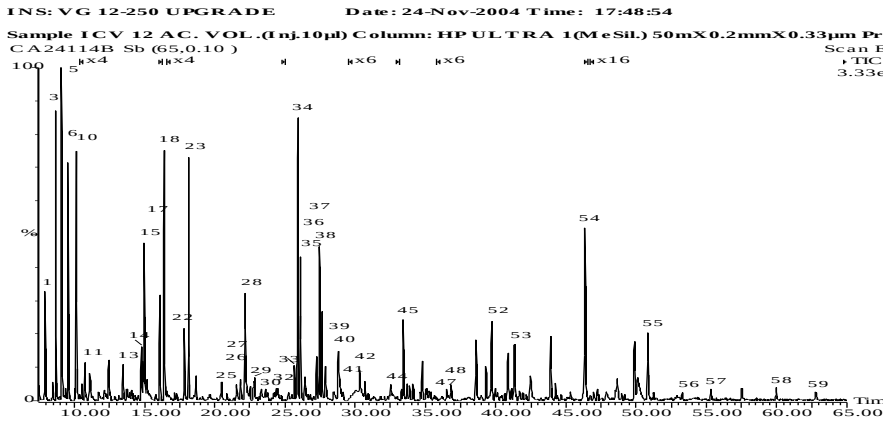
Appendix 3k: GC profile of floral volatile for cultivar SP 52



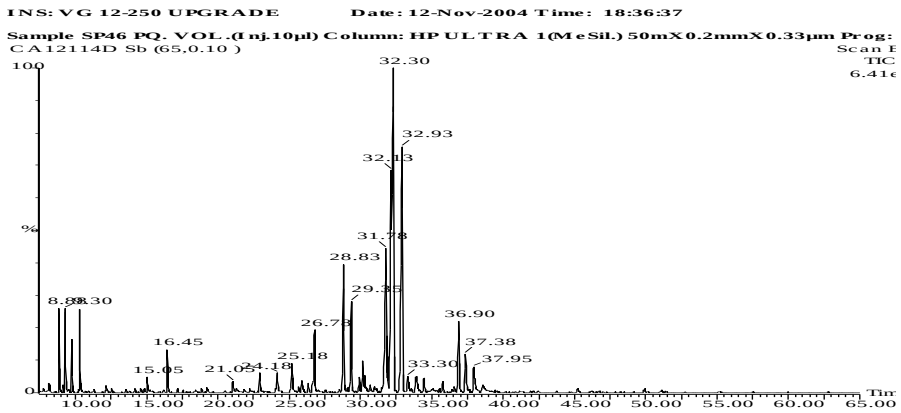
Appendix 3l: Magnified GC profile of floral volatile for cultivar SP52



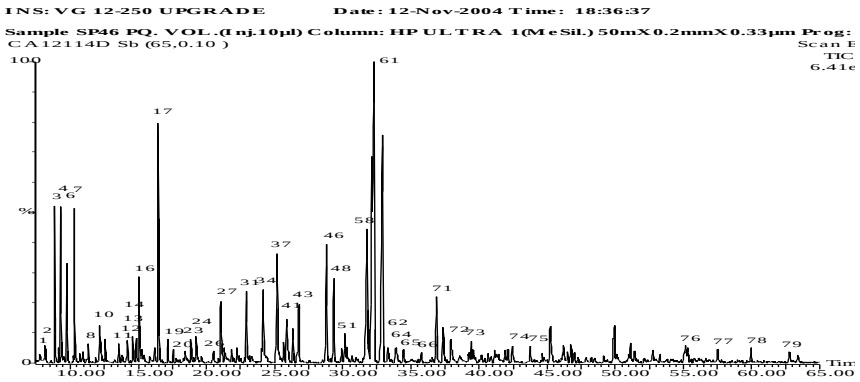
Appendix 4a: GC profile of floral volatile for cultivar ICV 12 trapped in activated charcoal



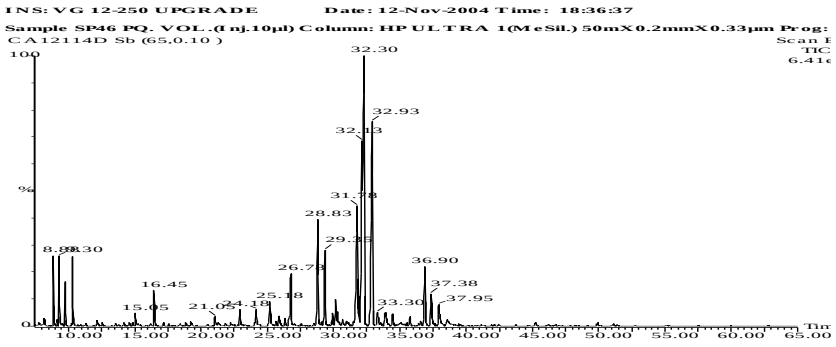
Appendix 4b: Magnified GC profile of floral volatile for cultivar ICV 12 trapped in activated charcoal



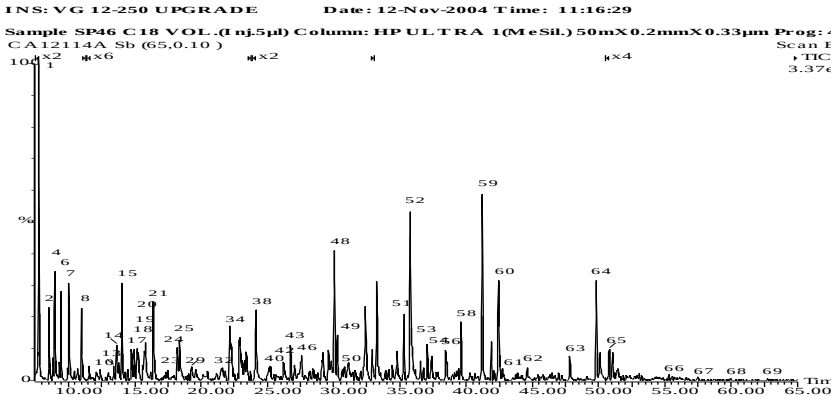
Appendix 4c: GC profile of floral volatile for for cultivar SP 46 trapped in porapak Q



Appendix 4d: Magnified GC Chromatogram of floral volatile for cultivar SP 46 trapped in Porapak Q

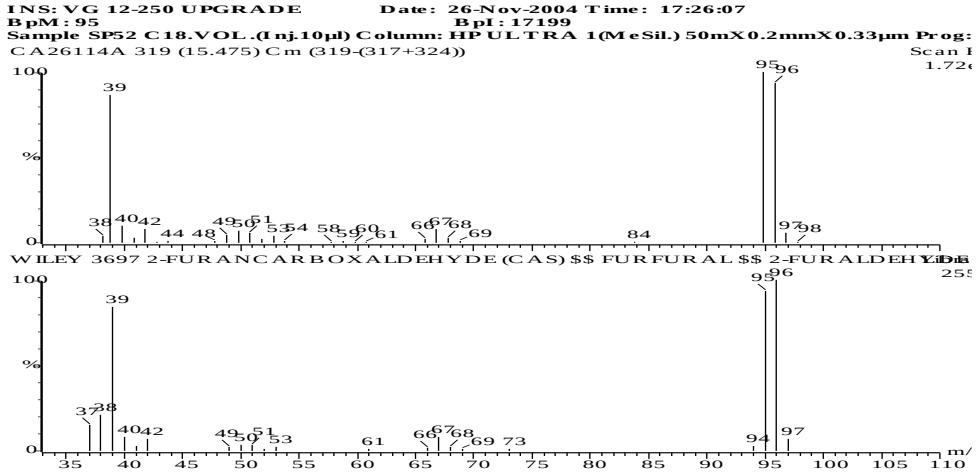


Appendix 4e: GC profile of floral volatile for cultivar SP 46 trapped in C_{18} bonded reverse phase silica

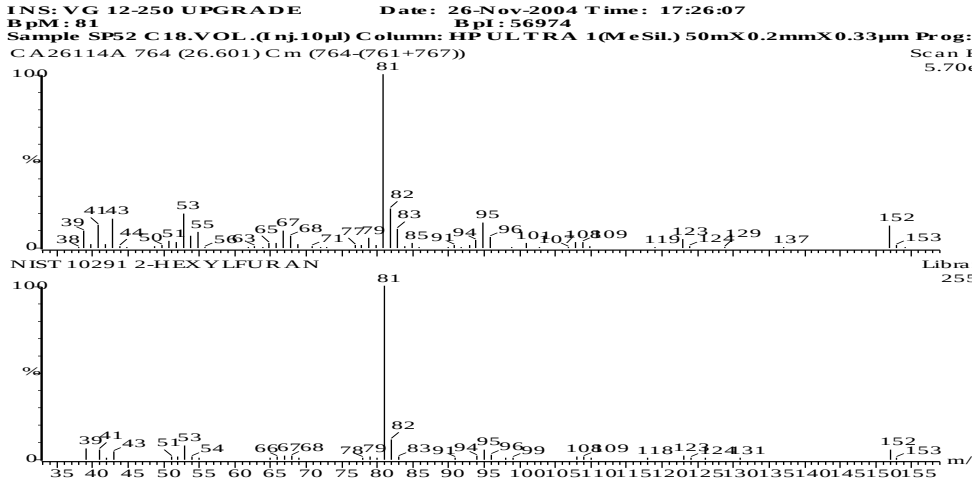


Appendix 4f: Magnified GC Chromatogram of floral volatile for cultivar SP 46 trapped in C_{18} bonded reverse phase silica

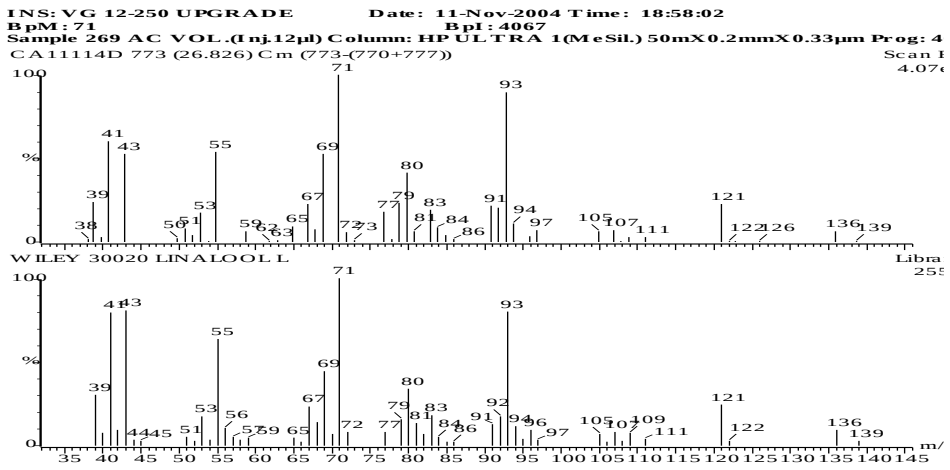
Appendix 5: Mass spectra for selected volatile and essential oil compounds



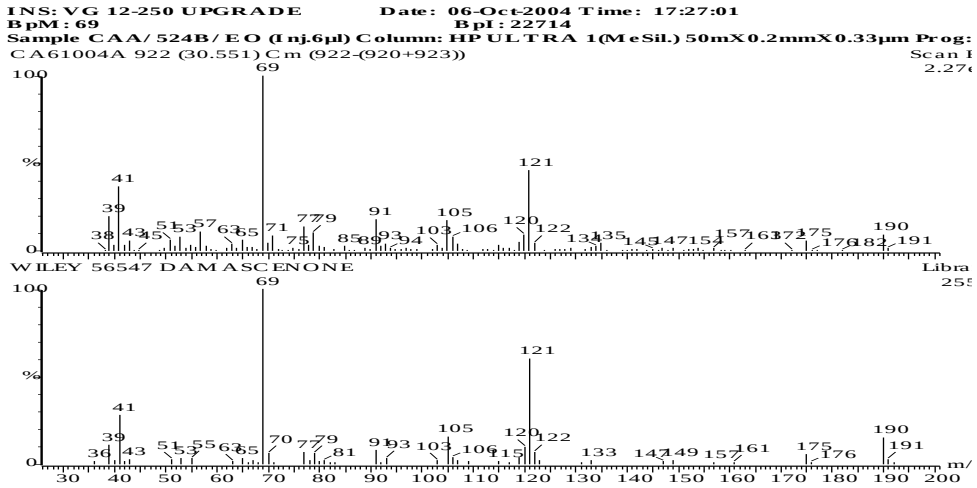
Appendix 5a: Mass spectrum of furfural



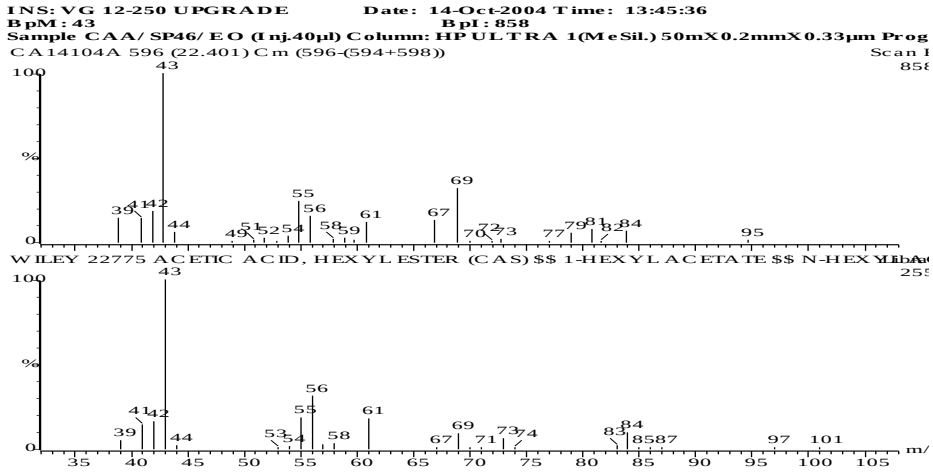
Appendix 5b: Mass spectrum of hexylfuran



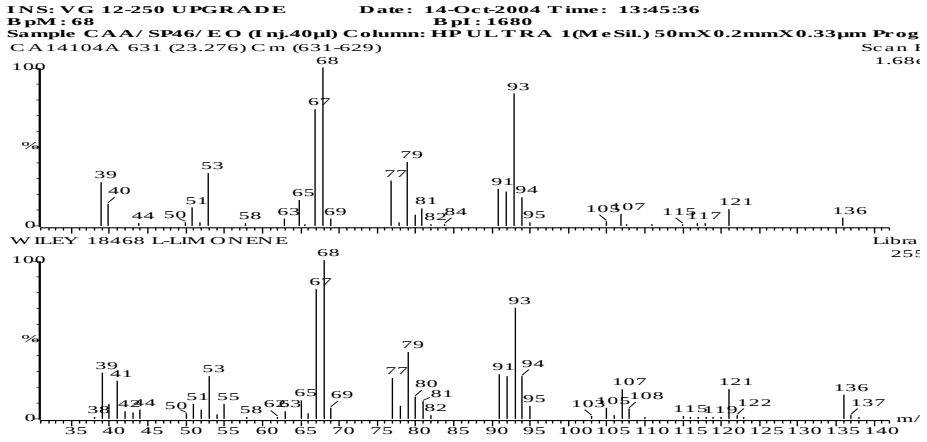
Appendix 5c: Mass spectrum of linalool



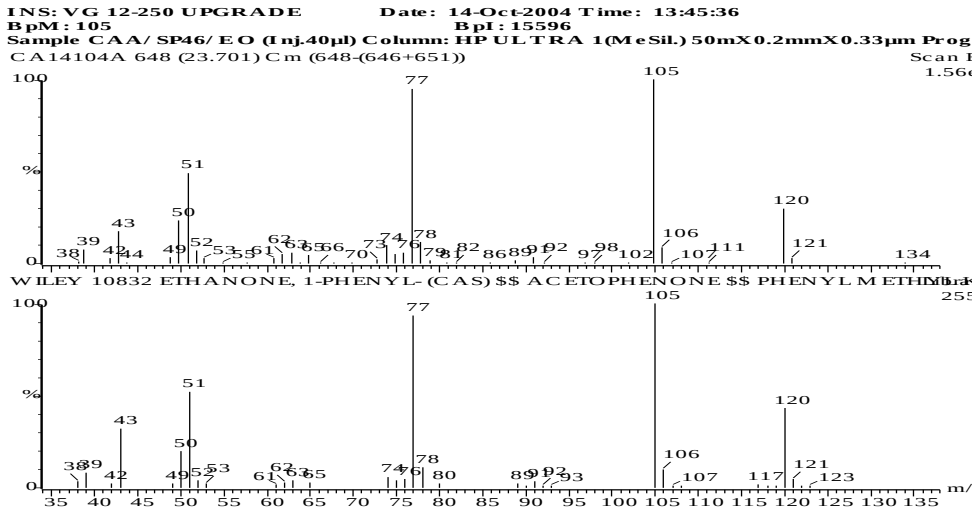
Appendix 5d: Mass spectrum of *β*-damascenone



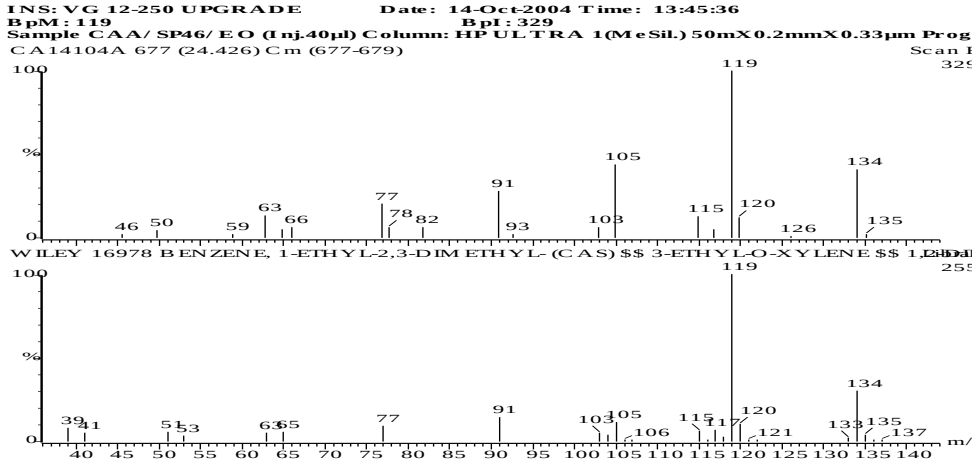
Appendix 5e: Mass spectrum of hexyl acetate



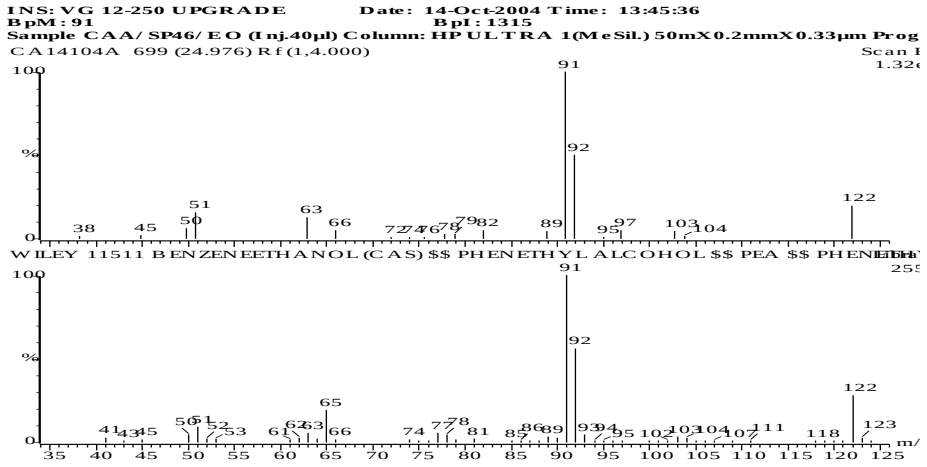
Appendix 5f: Mass spectrum of limonene



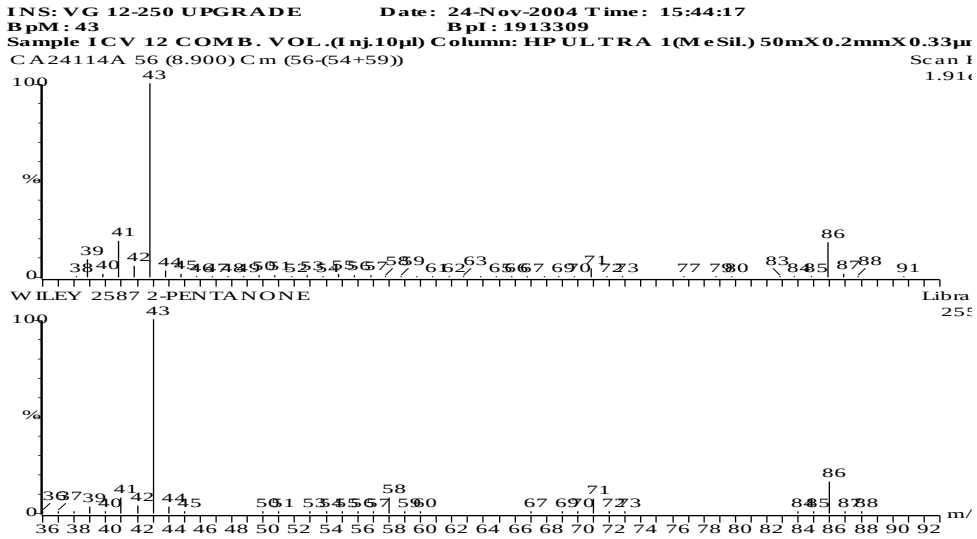
Appendix 5g: Mass spectrum of acetophenone



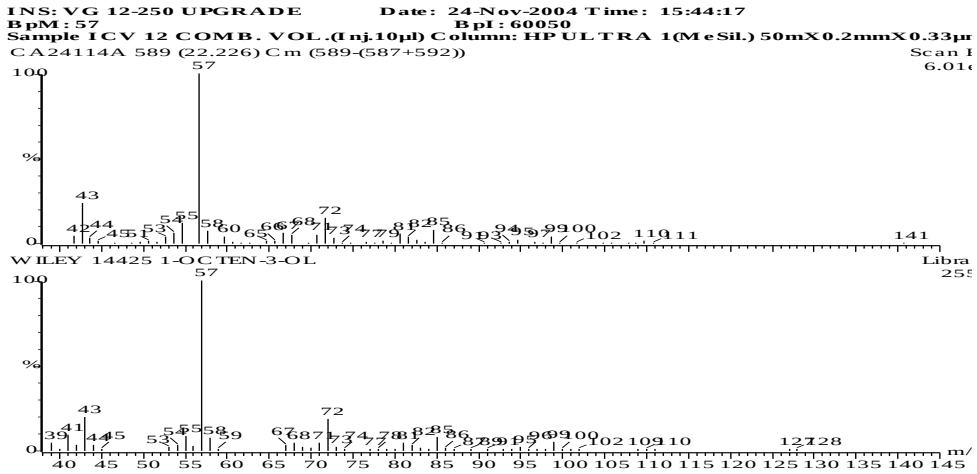
Appendix 5h: Mass spectrum of O -xylene



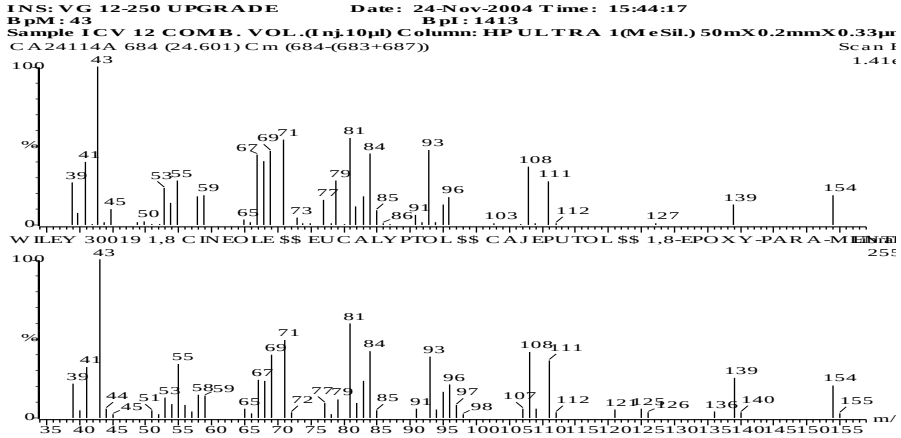
Appendix 5i: Mass spectrum of benzeneethanol



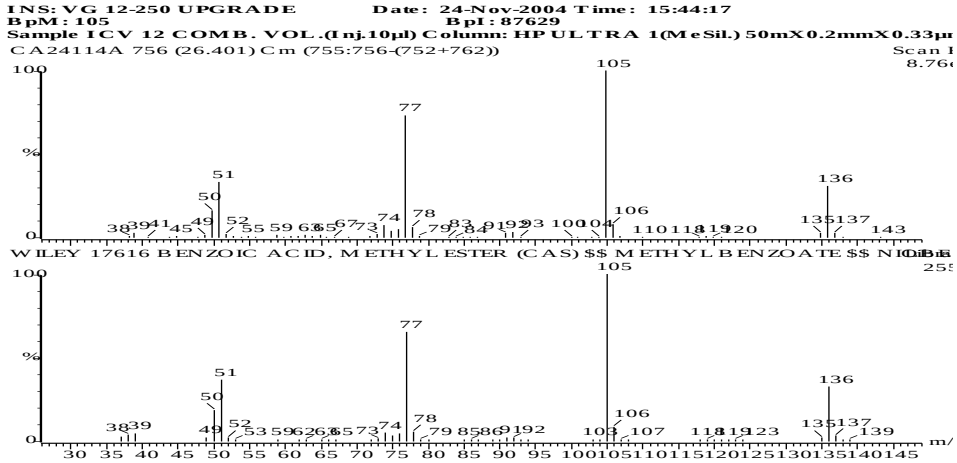
Appendix 5j: Mass spectrum of 2-pentanone



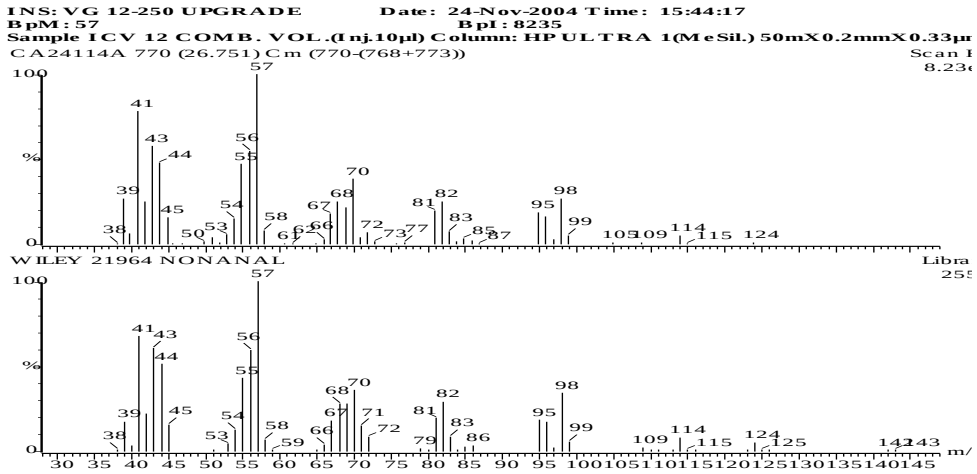
Apppendix 5k: Mass spectrum of 1-octen-3-ol



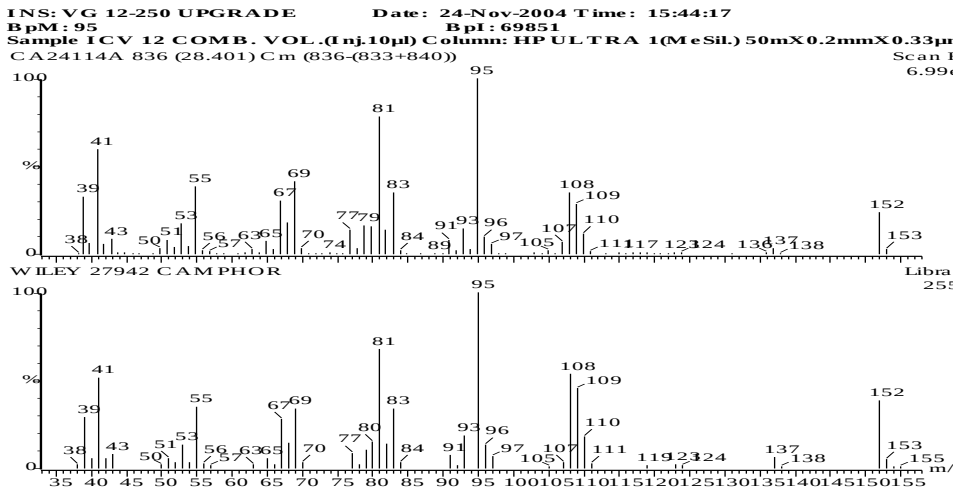
Appendix 5l: Mass spectrum of eucalyptol (1, 8-cineole)



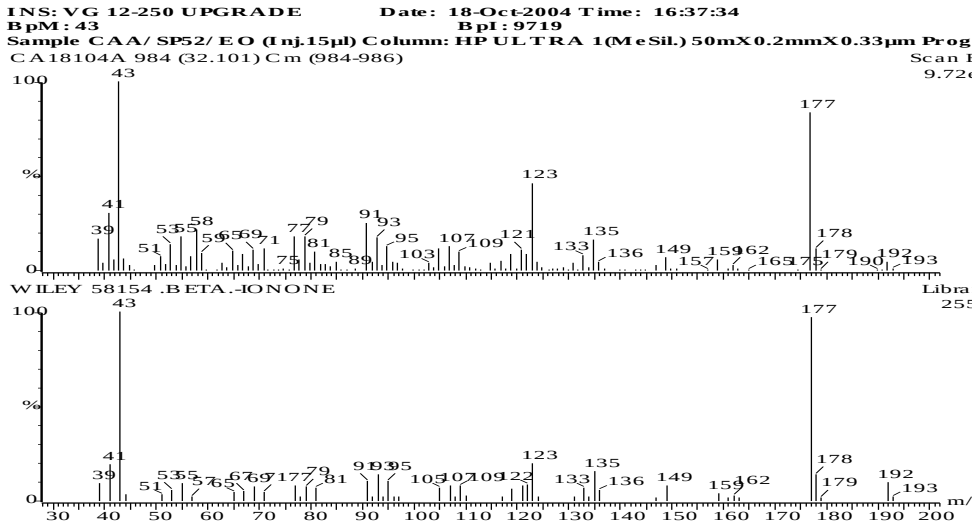
Appendix 5m: Mass spectrum of methylbenzoate



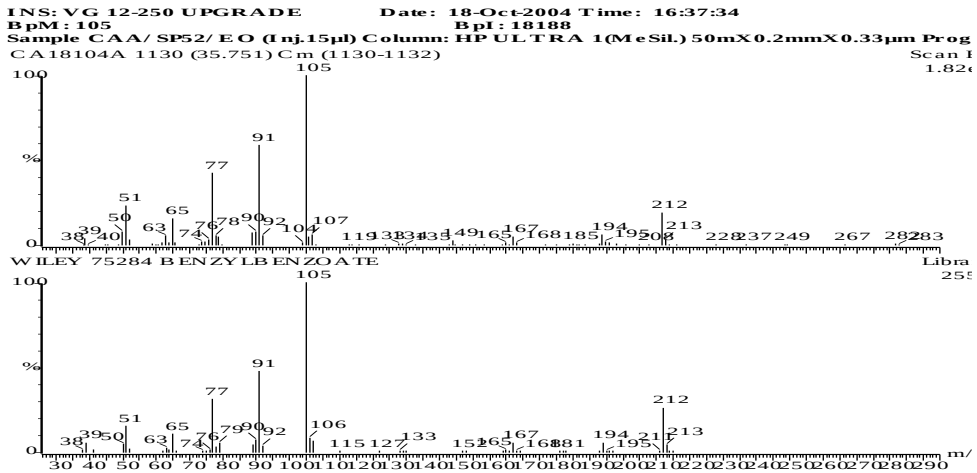
Appendix 5n: Mass spectrum of nonanal



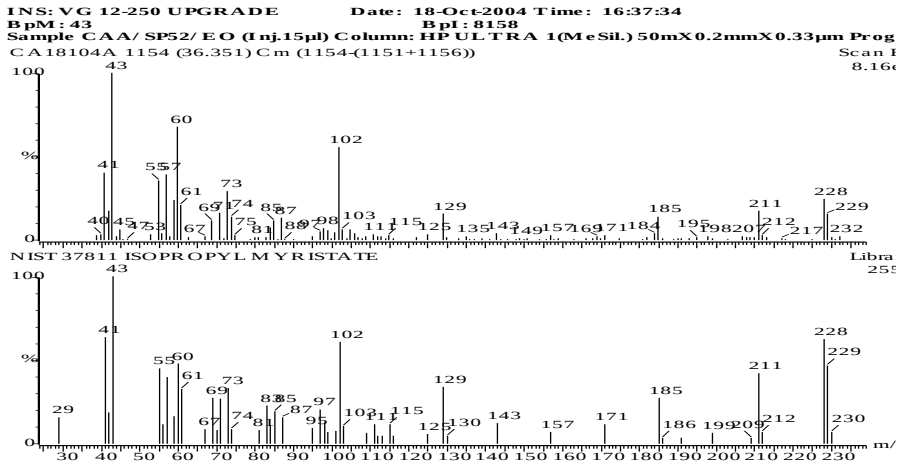
Appendix 5p: Mass spectrum of camphor



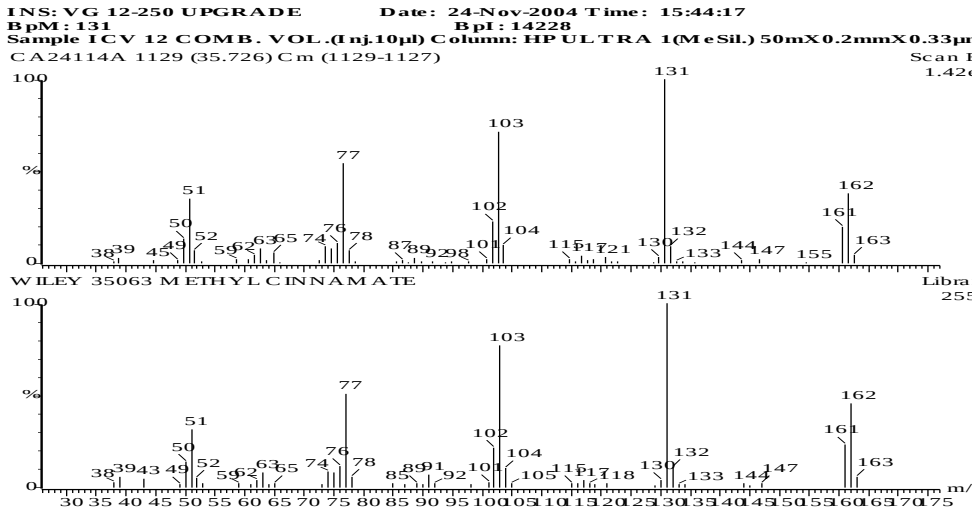
Appendix 5q: Mass Spectrum of *β*-ionone



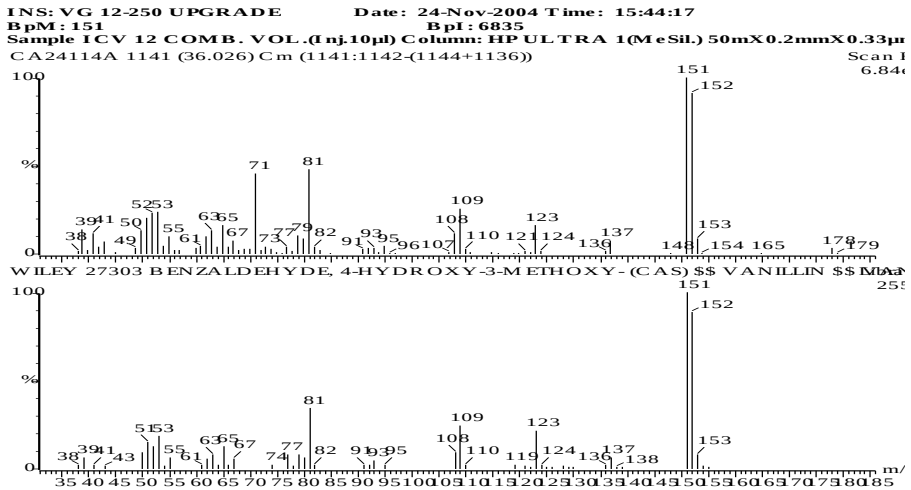
Appendix 5r: Mass spectrum of benzyl benzoate



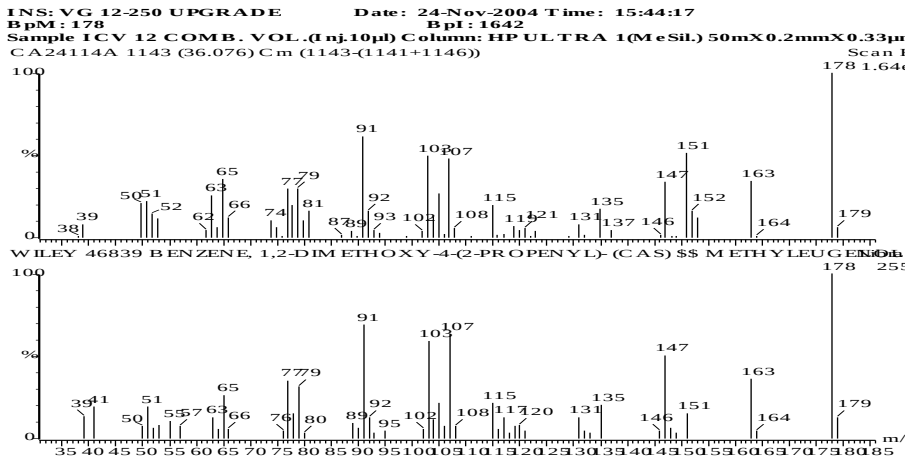
Appendix 5s: Mass Spectrum of isopropyl myristate



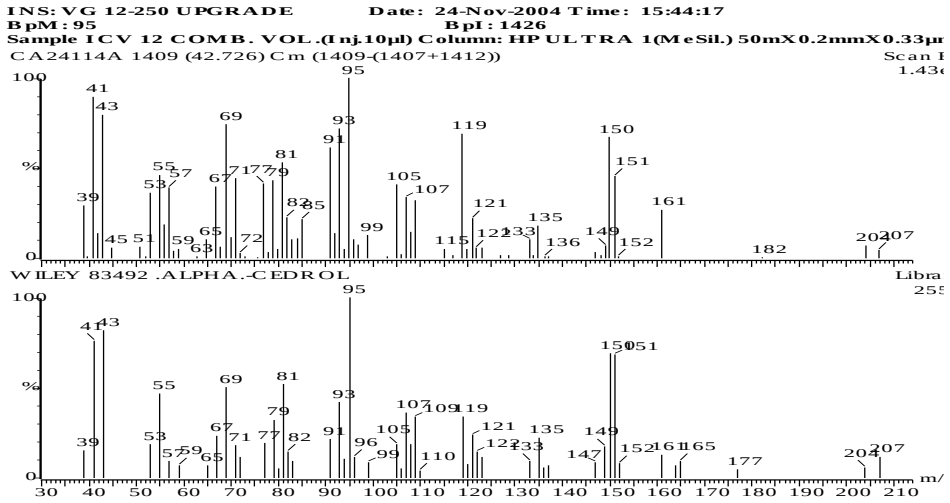
Appendix 5t: Mass spectrum of methyl cinnamate



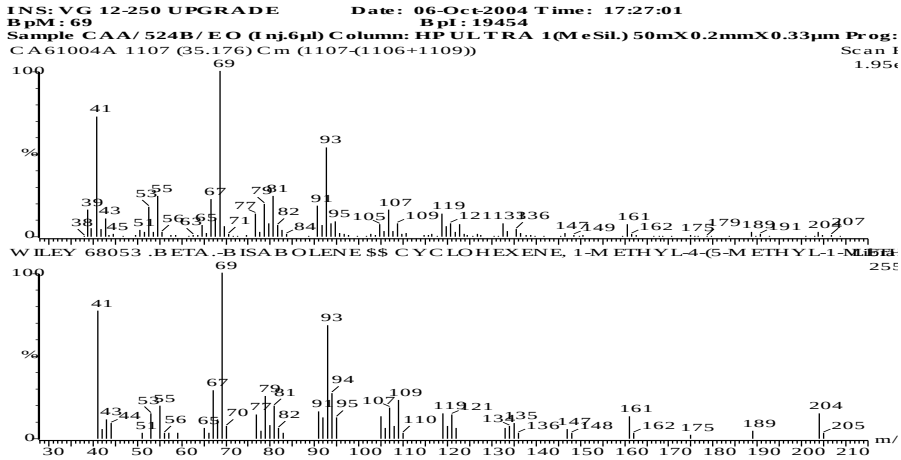
Appendix 5u: Mass spectrum of benzaldehyde



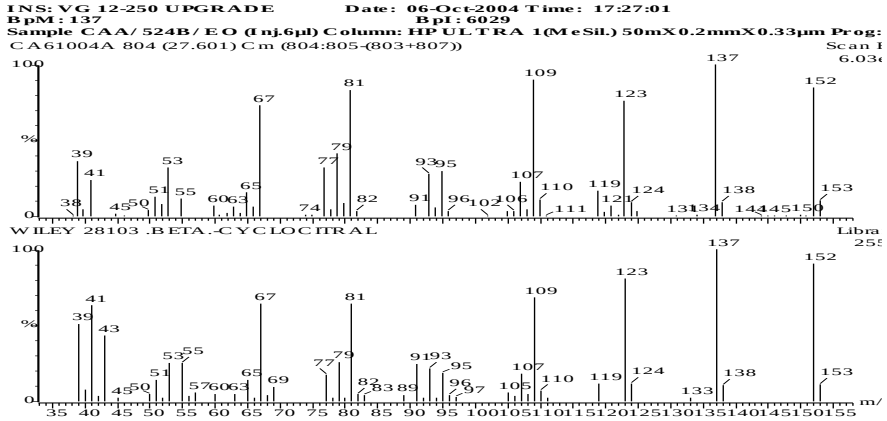
Appendix 5v: Mass spectrum of methyleugenol



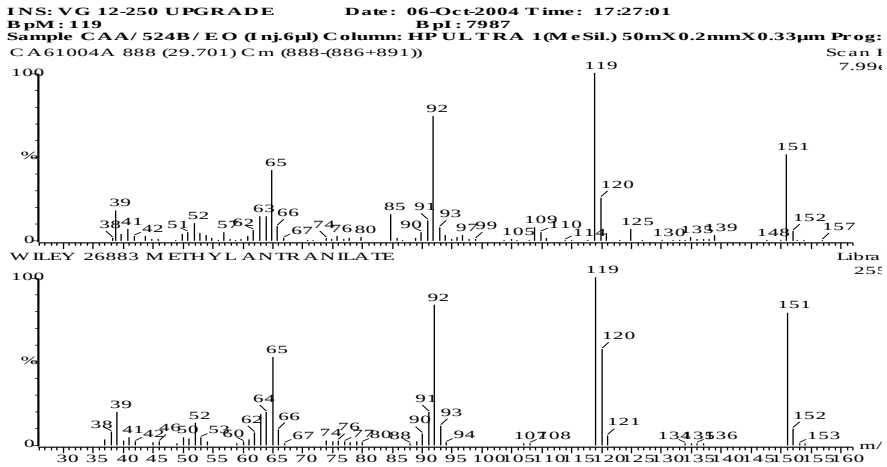
Appendix 5w: Mass spectrum of *α*-cedrol



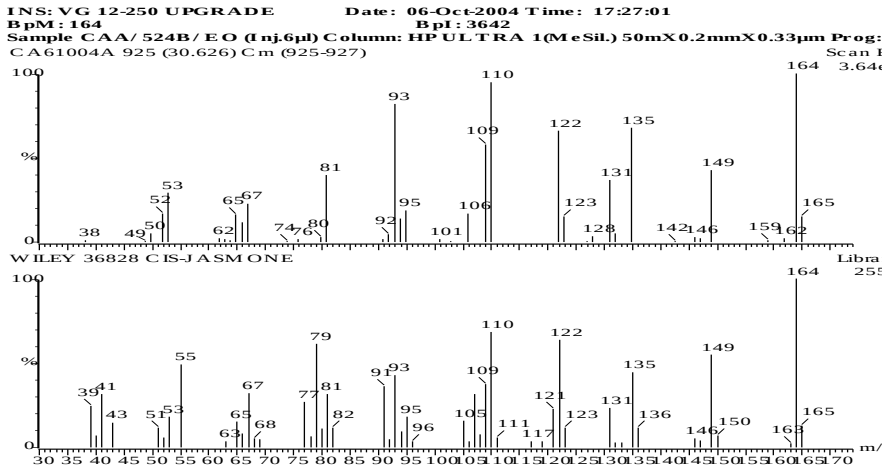
Appendix 5x: Mass spectrum of *β*-bisabolene



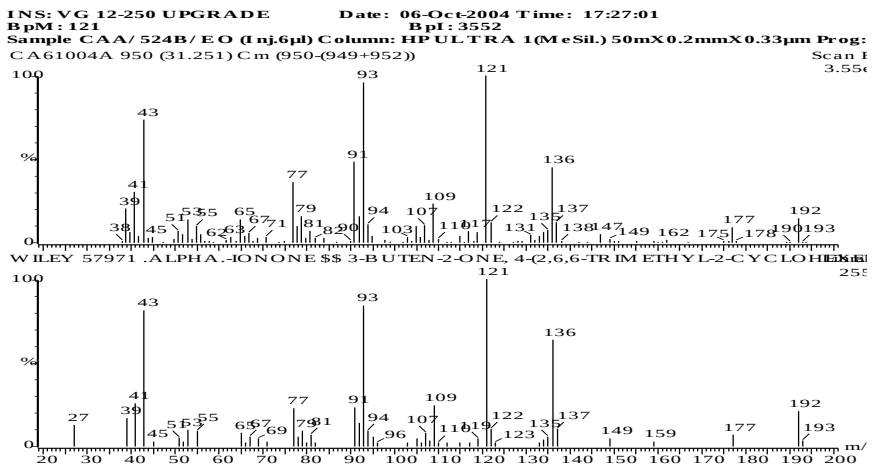
Appendix 5y: Mass spectrum of *β*-cyclocitral



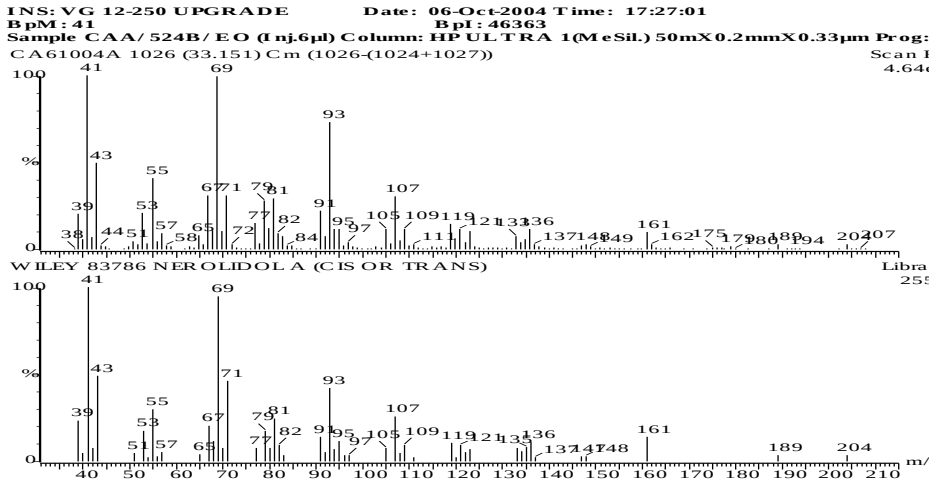
Appendix 5z: Mass spectrum of methyl anthranilate



Appendix 5ii: Masss of *cis*-jasmone



Appendix 5iii: Mass spectrum of *α*-ionone



Appendix 5iv: Mass spectrum of nerolidol