ATTRACTANTS OF THE BROWN EAR TICK, *RHIPICEPHALUS APPENDICULATUS,* FOUND IN COW EAR WASHES

BY

BETTY ADHIAMBO NYAGODE


Eger234649

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DECLARATION

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

Signature:..... 25/4/98

Betty Adhiambo Nyagode
Department of Chemistry
Egerton University

This thesis has been submitted for examination with our approval as supervisors

Signature:..... 26/4/98.

Dr. S. O. Were
Department of Chemistry
Egerton University

Signature:..... 27/4/98

Dr. W. Lwande
Behavioural and Chemical Ecology Department
ICIPE.

This work is dedicated to each and every member of my family,

especially my mother, Mary Amolo,

without whose support I would never have come this far.

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ABSTRACT

The brown ear tick, *Rhipicephalus appendiculatus*, is a very important tick species in Africa since it is the vector of *Theileria parva*, the protozoan that causes East Coast Fever (ECF), in cattle. ECF is usually a fatal disease with a mortality rate of 90% and over. In animals that survive, productivity is reduced, weight loss is marked and the hide is damaged.

Tick-borne diseases have conventionally been controlled by acaricides, but these imported chemicals are generally too expensive for third world countries. Alternative methods of tick control are therefore a dire necessity. Modern methods of control seek to utilise semiochemicals or their synthetic analogues in the manipulation of pest populations by modifying arthropod behaviour or disrupting normal physiological processes of the same.

In this study, the chemical cues suspected to be present on the ear pinna, which is the predilection site for the adult *R. appendiculatus* were collected. A comparison between two solvents used for this collection revealed that methanol was more superior to hexane. Hexane was however preferentially used because of its ease of evaporation. When comparing bioassay responses of whole samples tested against females and males, it was clearly evident that the ticks were attracted to some component(s) in the sample. The female ticks

registered higher readings than the male at all dose applications and were subsequently used.

The extracts were separated into basic (Fraction S), neutral (Fraction R), acidic (Fraction Q) and phenolic / enolic (P) fractions. Bioassays that were carried out using each of these fractions revealed attractancy only in Fraction P.

The compounds in this fraction were analysed by gas chromatography and electron impact mass spectrometry. EI-MS spectra were used to tentatively identify compounds by comparison with NIST/NBS mass spectral library of standards. Confirmation of this identification was then achieved by retention time comparison of authentics as well as peak area enhancement by co-injection with commercially available synthetic standards.

The compounds identified from Fraction P were phenol, 2-hydroxybenzaldehyde, 4-methylphenol, 4-hexen-3-one, 4-ethylphenol and 3-ethylphenol. Of these compounds, the authentic samples of the last two were bioassayed against female ticks at doses of 0.0001, 0.001, 0.01, 0.1, 1.0, 10.0 and 100.0 µg per application but failed to show any attractancy. The other three phenolics are compounds that have been documented severally in the past as attractants of this tick. It is no wonder therefore that the inner ear of the cow pinna, a site that contains phenol, 4-methylphenol and 2-hydroxybenzaldehyde should be attractive to the brown ear tick, *R. appendiculatus*. The potential of

using these kairomones for tick control, which resource-poor farmers in third world countries need to reduce costs on imported commercial acaricides, is highlighted.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Ticks are among the most important vectors of life threatening or debilitating human and animal diseases, ranking second only to mosquitoes in this respect [71]. Moreover, ticks transmit a greater variety of infectious agents than any other arthropod group. In many parts of the world, tick-transmitted human diseases, for example, the viral Encephalitis, Lyme disease and Congo-Crimean haemorrhagic fever are endemic and cause significant morbidity and mortality among the population [88].

There are more than 850 species of ticks, all of which are parasitic [32]. Ticks are primarily parasites of wild animals with only about 10% of the species feeding on domestic animals [27]. These relatively few species, however, have prospered on domestic livestock and are responsible for considerable economic loss to farmers in developed countries as well as rural herdsmen in developing countries. The major ticks of importance belong to the genera *Rhipicephalus, Boophilus, Hyalomma* and *Amblyomma*. They are responsible for severe losses caused either by the effect of the tick through: tick worry, blood loss, damage to hides and the injection of toxins or through mortality or debility caused by the diseases transmitted [4,26,41,47,78]. Babesiosis, heartwater and theileriosis are well known tick-borne livestock concerns.

Cattle ticks are destructive blood-sucking parasites which are of considerable importance in tropical and sub-tropical areas of the world and in some regions constitute the most important limiting factor for successful livestock farming [102]. It has been estimated that 80% of the world's 1,266 million cattle are infested with ticks [26]. Although different species of ticks and tick-borne diseases occur in different regions, their impact on animal production is similar in nature and importance. The direct losses due to ticks and tick-borne diseases tend to be less in areas or regions where indigenous animals are kept. The diseases are however of major significance when exotic animals susceptible to them are introduced to tick infested areas [26,41].

East Coast Fever, a form of theileriosis, is a serious disease of bovines caused by the protozoan *Theileria parva*, which is transmitted by the species of ticks, *Rhipicephalus*. This disease is of great importance due to the high rate of mortality it causes in livestock (90% and over), the productivity losses it causes in animals that recover from it and the exclusion of exotic and grade cattle from much of the country [69].

Tick-borne diseases have conventionally been controlled by acaricides, but these imported chemicals are generally too expensive for third world countries [19]. African countries spend about US $ 720 million per year to import acaricides [35]. Acaricides are toxic to both humans and animals, so special care is required in their application. Underuse or misuse of the chemicals leads to resistance to the acaricides. What is worse, reliance on acaricides makes even naturally resistant breeds of cattle completely

susceptible to tick infestation; in case of breakdown of dipping regime for whatever reason, losses in cattle by both tick infestation and disease transmission can be very high. In Africa particularly, where there are economic and social limitations, conditions are not yet appropriate for the execution of successful eradication programs. Alternative control strategies for ticks is therefore a priority [21,23,72].

Relatively simple chemical compounds mediate a number of vital life processes of arthropods by influencing their behaviour in searching for mates, food, shelter, and sites for the deposition of eggs [27,61,67,75,78]. Such information-bearing chemicals are termed semiochemicals, the best known being pheromones, allomones and kairomones. Modern methods of control seek to utilize such chemicals or their synthetic analogues in the manipulation of pest populations by modifying arthropod behaviour or disrupting normal physiological processes of the same [40].

1.2 BIOLOGICAL CLASSIFICATION OF TICKS.

Ticks, which belong to the suborder Ixodida, comprise three families: Ixodidae (hard ticks), Argasidae (soft ticks, so called because they lack a hard dorsal plate or scutum) and Nuttalliellidae whose only representative is *Nuttalliella namaqua* of east and central Africa.

Based on differences in reproductive strategies, ticks can be subdivided into two distinct biological groupings: those that require a blood meal before gametogenesis (Metastriata) and those in which gametogenesis can occur

independently of blood feeding (Prostriata and Argasidae). Species of metastriata and prostriata are subdivisions of the ixodidae.

There are several basic differences between the two larger families of ticks. The argasidae feed moderately but frequently, while the ixodidae, particularly in the nymphal and female adult stages, take one very large feed and increase in size dramatically. The eggs of the argasidae are laid in several small batches, while those of ixodidae are laid in one very large batch after which the female dies.

Rapid feeding by the soft ticks (argasids) and the firm host attachment by the slow feeding hard tick (ixodides) prevents dislodgment and removal by the host. The slow feeding by the ixoxides gives them ample time to ingest large numbers of pathogens from an infected host and to transmit the infection to a new host; it also allows the dispersal of the infected ticks to new areas while attached to the host [41]. Many ixodides have a very high reproductive potential and can live for long periods. They also have a large ability to starve, going without blood-meals for up to 493 days [84]. This is of great survival value and also ensures survival of pathogens in infected ticks for several months, making them efficient vectors of pathogens [46].

The success of both, however, depends on their ability to survive and reproduce within the habitat of the animals they parasitize and from which they draw nutrients in the form of blood and other body fluids [31,41,103].

The family ixodidae (hard ticks) has four subfamilies, one of which is Rhipicephalinae, with eight genera namely *Dermacentor, Cosmiomma, Nosomma, Rhipicephalus, Anomalohimalaya, Rhipicentor, Boophilus* and *Margaropus*. The genus *Rhipicephalus* has seventy species including *R. appendiculatus*, which is of concern to this study.

1.3 RHIPICEPHALUS APPENDICULATUS

1.3.1 DESCRIPTION

Ticks of this species are commonly referred to as "brown ear ticks". This name was given to this species due to the fact that the individuals are brown in colour and the predilection site for the adults is around the ear pinna [10]. This name could just as appropriately be applied to a number of the other species of this genus which also feed on the ears of their hosts and are brown in colour, particularly *R. hurti, R. jeanelli* and *R. pravus*. It should not therefore be assumed that all "brown ear ticks" are *R. appendiculatus*.

The overall length of the adult *R. appendiculatus* is usually less than 5mm long (usually 1.8mm to 4.4mm) by 2mm wide [15]. They are usually brownish or reddish-brown but may be very dark. The legs are always reddish-brown.

The structural differences between the male and the female are highlighted in **Plates 1** and **2**. The most obvious is that in the male the entire dorsal tegument is covered with a thick, rigid scutum unlike in the female. Due to this the male does not change very much in size when it feeds. The body



Plate 1: Photograph of the female brown ear tick,

Rhipicephalus appendiculatus (dorsal aspect)



Plate 2: Photogragh of the male brown ear tick,

Rhipicephalus appendiculatus (dorsal aspect)

proportions are also different in the two sexes. The capitulum in the male is smaller, sometimes half or one-third of the length in the female.

This is a very important tick species in Africa since it is the principal vector of *Theileria parva*, the protozoan which causes theileriosis, commonly known as East Coast Fever (ECF), in cattle [69]. This tick species also infests other domesticated animals for instance rabbits, sheep and goats as well as wild animals causing anaemia, damage to the skin and secondary infections by bacteria through the wounds they inflict on the animals [4].

R. appendiculatus has a three host life cycle. The moulting stages take place in the vegetation while the larva, nymph and adult feed on their respective hosts [89]. Emerged adults survive for periods ranging from 228 to 424 days though dehydration kills them faster [76]. Heavy infestation by this tick causes severe toxaemia; this reduces immunological competence and resistance to other infections and may lead to death.

1.3.2 DISTRIBUTION.

Rhipicephalus appendiculatus will be found ranging from the southern and eastern provinces of South Africa, northward into the Transvaal, through large areas of Zimbabwe, Zambia, Tanzania, Kenya and the southernmost regions of the Sudan (See **Figure 1**) [74]. This important species normally inhabits cool (less than 30 ^{o}C daily maxima), humid (at least 400mm annual

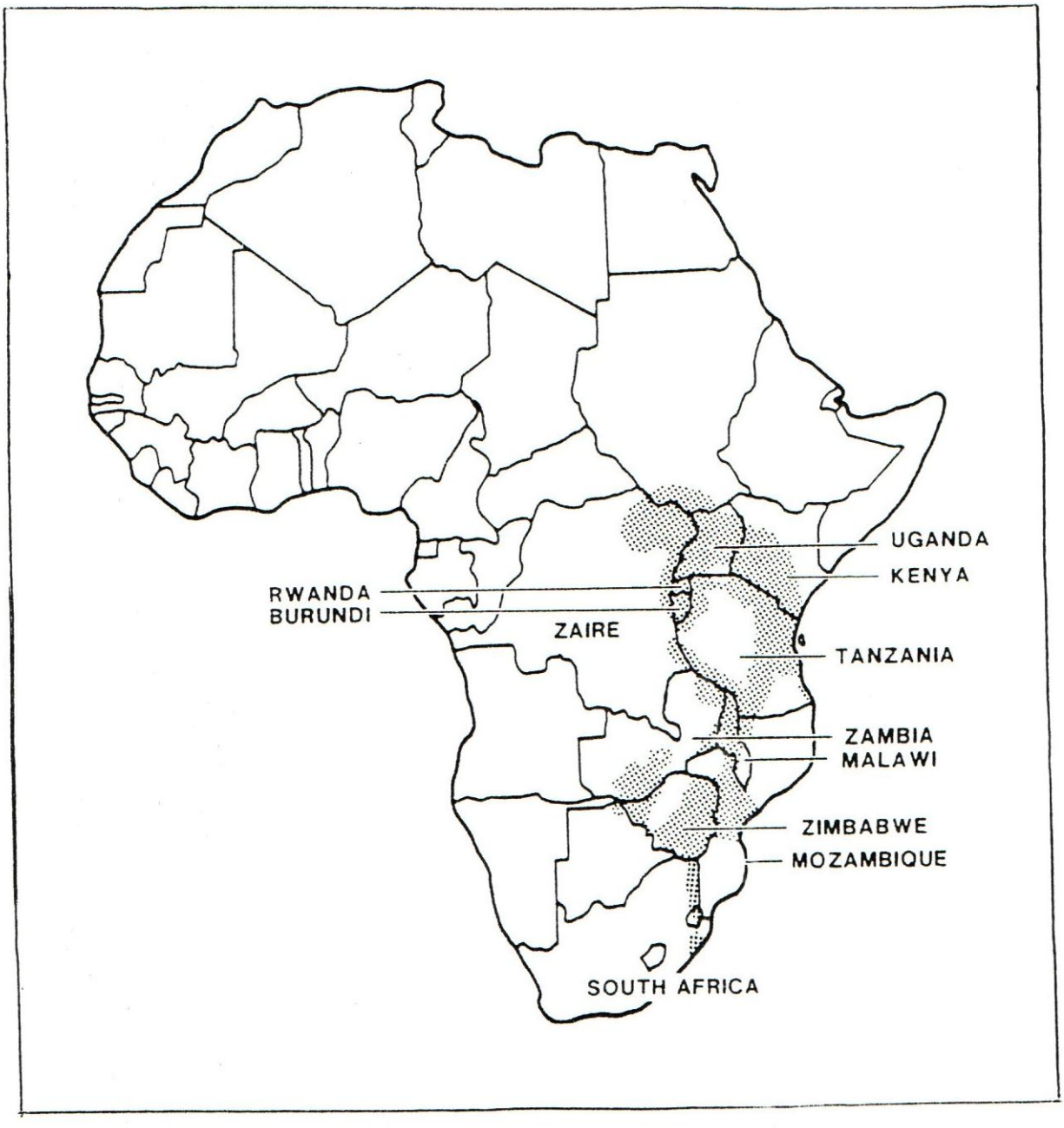


Figure 1: Map of the distribution of *Rhipicephalus appendiculatus* in Africa.

rainfall) biotopes from sea level to about 2300m altitude [25]. This species prefers long rather than short grass [95].

In Kenya, *Rhipicephalus appendiculatus* is restricted to Western, Nyanza, Rift Valley (with exeption of the northern arid districts) and Central provinces as well as in southern areas of Coast Province [95] (See **Figure 2**) .

1.4 EAST COAST FEVER (THEILERIOSIS)

Theilerioses are a group of tick-borne diseases of cattle, water buffalo, sheep, goats and occasionally of wild ruminants, caused by species of protozoa in the genus *Theileria* [46,63,69] which is transmitted only after cyclic development in ticks [15]. In Africa the most important form of theileriosis is caused by *Theileria parva* and is commonly referred to as East Coast Fever (ECF). The disease can be contracted by cattle which graze tick-infested pasture several months after a case of ECF has occurred [63]

In an extensive survey carried out between 1971 and 1974 in 36 districts of Kenya, antibodies to *T. parva* were found in all districts where *R. appendiculatus* were found [39]. It is however interesting to note that ECF does not exist in the North Eastern province of Kenya because the vector *R. appendiculatus* seems not to exist in the area [58].

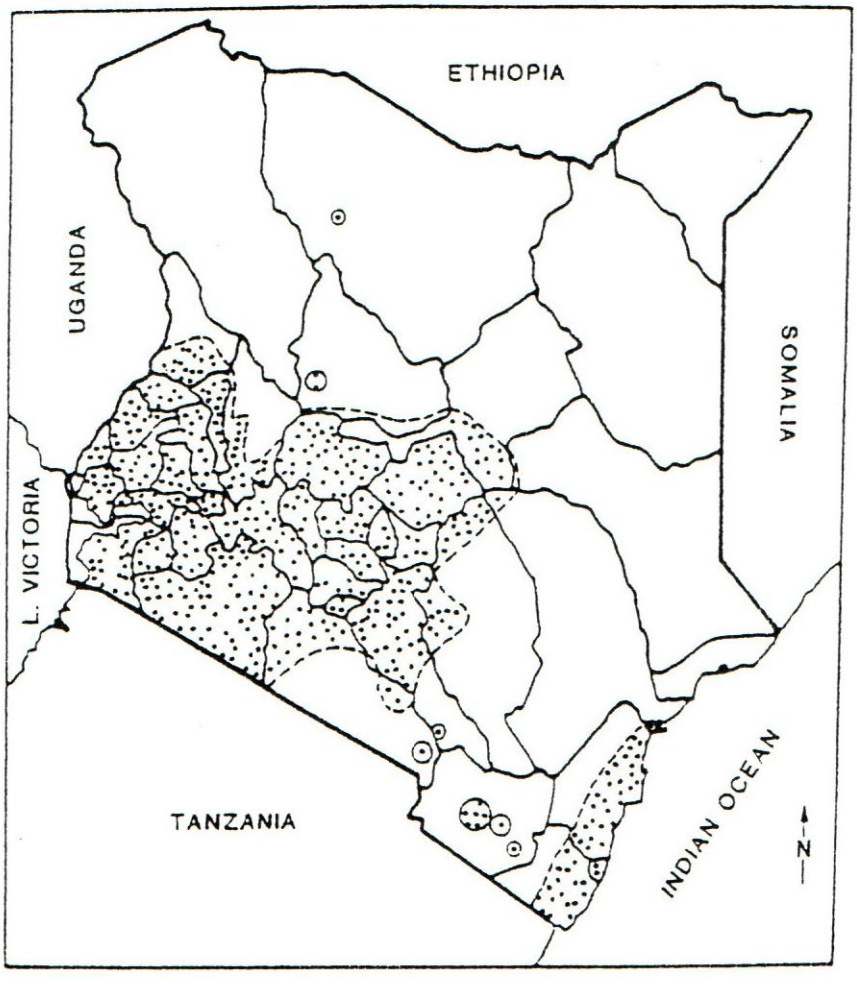


Figure 2: Map of the distribution of *Rhipicephalus appendiculatus* in Kenya.

It is known that ECF is not a single disease entity with a uniform etiological agent but a complex of syndromes caused by antigenically similar organisms [46]. In addition to ECF, two other clinical forms of theileriosis have been recognized in cattle. A very virulent form called Corridor Disease is caused by organisms which originate from the wild buffalo and are transmitted by ticks to cattle and a much milder form of the disease, January disease. All were once thought to be different diseases but are now considered to be different expressions of the same disease [65].

ECF is usually a fatal disease of cattle with a mortality of about 90% in new outbreaks of the disease. Animals which recover are commonly known as "salted". After an incubation period of a fortnight or so, the animal becomes dull, listless, loses appetite and runs a high fever. The lymph nodes become enlarged and there may be a discharge from the eyes and nose. Laboured breathing and diarrhoea may also be observed in the animal [103].

1.5 HOST LOCATION IN TICKS

Ticks have a wide range of lifestyles tailored to ensure access to preferred hosts. Among several tick species, host finding is initiated by a positioning phase in which ticks move to a vantage location where they are likely to encounter a host. When a tick finds a suitable position it waits motionlessly, usually with the first pair of legs stretched. As soon as the approaching host is detected, the tick starts questing and (or) appetence behaviour commences. Appetence is the "locomotory hunting for a host or seeking one from a vantage point" [92]. At this stage the tick is prepared to

cling on the passing host. The stimuli emitted by the passing host can be sensed through mechanoreceptors, thermoreceptors and olfactory receptors. Observations have shown that when a warm object is brought next to a questing *R. appendiculatus*, the tick moves towards it immediately and will avidly follow a warm object if it is slowly moved away. This behavioural response is much more impressive if a warm odourous object like a human finger is used [97]

Once the tick is on the host, host specificity and site of feeding are enhanced by factors like odour, temperature and taste stimuli. Ticks have to probe the host's tegument in search of a predilection site with right combination of stimuli that can facilitate subsequent attachment. The stimuli concerned may be non-specific, acting on all types of tick species or may be highly specific for a given species [88,97].

The attachment process starts with the arresting of movement. The tick starts cutting into the host's integument but never enters into the feeding lesion. Feeding progresses and finally detachment occurs [97,99].

1.6 ERADICATION AND CONTROL OF TICKS AND TICK-BORNE DISEASES.

Since the early part of the twentieth century, eradication of vector ticks has been the ambition of governments throughout the world [25,46]. During their lifecycle, ticks spend some time on the host (parasitic stage) and some time away from the host (free-living stage). The parasitic stage, though short-lived for most ticks, has been the easiest stage to control using chemicals [56]. Currently, the best method to control tick-borne diseases is by killing the vector which is the tick [58]. Chemicals that kill ticks are often called acaricides but are sometimes called ixodicides or tickicides [25].

ECF in cattle is presently controlled by intensive application of acaricides to cattle by regular dipping or spraying and may require additional careful hand-dressing because in heavily infested areas dipping alone may not keep the cattle entirely tick-free. Other methods of application may be through ear-tags, pour-ons and implants [88]. In those that show decreased effectiveness, formulations of combinations of acaricides result in synergistic increase in effectiveness.

1.6.1 TYPES OF ACARICIDES

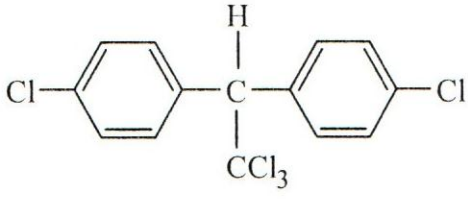
1.6.1.1 Arsenicals

The first widely used acaricides were compounds of arsenic such as arsenic trioxide, As_2O_3. This, however, has gradually been replaced and it's present use is very limited due to it's high toxicity to mammals and especially man [15].

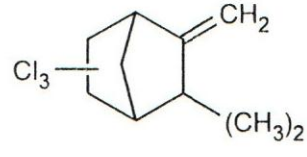
Arsenic acaricides kill ticks by interrupting ATP synthesis and cellular respiration [88].

1.6.1.2 Chlorinated hydrocarbons.

The first of a broad spectrum of compounds known as the chlorinated hydrocarbons was 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (1), popularly known as DDT. This was introduced on a large scale following World War II. Another representative of this group is Toxaphene (2). These are generally very stable chemicals that provide long-lasting residual activity.



(1)



(2)

Chlorinated hydrocarbons are all liposoluble; this characteristic makes them soluble in the lipid-containing membranes of the arthropod's nerve axons. Here they act on the sodium channels (keeping them open), thereby resulting in uncontrollable repetitive action potentials that soon disrupt nerve function and lead to early death.

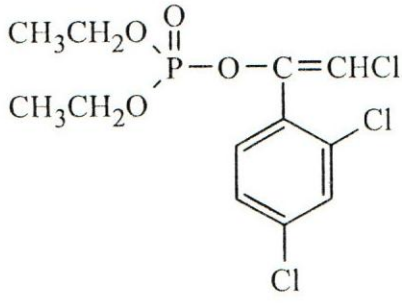
Unfortunately, their stability and absorption in lipids also enable them to accumulate in wildlife, especially fish and birds. It is as a result of this that after a lot of controversy, DDT was banned in the 1970's. Most other chlorinated hydrocarbons have since been withdrawn from general use. Toxaphene is still widely used but was banned in the United States of America in 1983 [88].

1.6.1.3 Organophosphates.

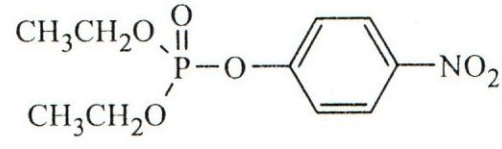
These contain the element phosphorus in some form. The electrophilic nature of the phosphorus atom confers upon it the ability to phosphorylate nucleophiles, including biological nucleophiles.

In general, these are much less stable than chlorinated hydrocarbons and they decompose when exposed to water or ultraviolet light. Examples of these are Chlorfenvinphos (3), Paraoxon (4) and diazinon (5). They are stable in storage vats and are relatively safe when used as directed.

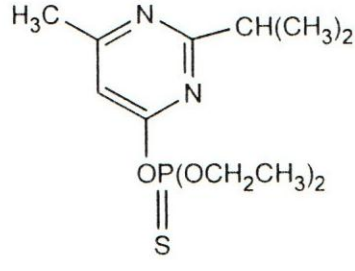
Since these compounds are not absorbed in lipids, they can be detoxified and eliminated from the animal body much more readily than organochlorine compounds. This group of acaricides also gives residual protection and are used as contact pesticides.



(3)



(4)



(5)

Organophosphates have a very specific mode of action and their biological activity is generally destroyed by the simplest chemical or biochemical modification. These interrupt nerve transmission by inhibiting (by phosphorylation) acetylcholinesterase (ACHE), the enzyme responsible for

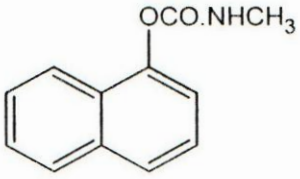
degrading the neurotransmitter acetylcholine (ACH), thereby causing rapid paralysis and death.

Acetylcholine is the chemical transmitter of nerve impulses in cholinergic synapses. Following it's release and diffusion across a synapse, a rapid return to normality is critical to the effective functioning of an organism. This is affected by hydrolysis of the released ACH by an esterase, acetylcholine hydrolase, ACHE. This enzyme, as other carboxyesterases, contains a serine hydroxyl group at it's active site. The organophosphorus insecticides phosphorylate this hydroxyl group and cause the inhibition of this enzyme.

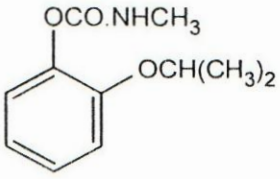
Build-up of ACH in the synapses of these arthropods leads to repetitive firing followed by blockage of nerve transmission. Such severe disruption of the nervous system cannot be tolerated for long and the failure of a critical process leads to the death of the organism.

1.6.1.4 Carbamates.

Examples of these are carbaryl (6) sold under the trade name Sevin, and Propoxur (7) sold under the trade names Baygon and Sendran. The former has the advantage of low mammalian toxicity and short half-life, while the latter has somewhat higher toxicity for mammals but long residual activity. Both compounds have low vapour pressures and function as contact pesticides.



(6)



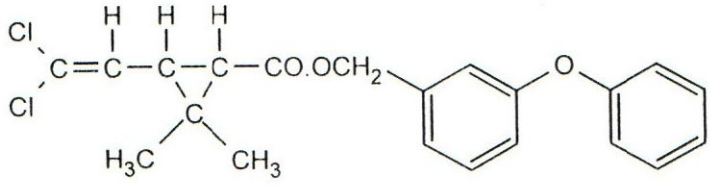
(7)

Carbamates act by antagonising acetylcholinesterase activity in much the same way as organophosphates. A significant difference, however, is that the carbamylated enzyme is much less stable than the phosphorylated enzyme.

Arsenic oxide and chlorinated hydrocarbons can destroy ticks under normal conditions. This however is not the case for organophosphates and carbamates. If 60-75% actually die, the rest merely show disordered biology for instance paralysis, reduced and incomplete oviposition, low oviposition and low hatching percentage [15]. Despite their sometimes not being toxic, organophosphates and carbamates are still considered effective.

1.6.1.5 Pyrethrins.

These toxicants are among the most potent and safest pesticides ever produced. Natural pyrethrins comprise a group of compounds found in chrysanthemum plants (genus *Chrysanthemum*), which are toxic to arthropods. These compounds are exceptionally fast-acting acaricides thereby providing rapid "knock-down" (i.e pests become morbid or die). Synthetic pyrethrins (pyrethroids), are similar to natural ones but are much more stable due to various substitutions. They are relatively non-toxic to mammals. An example is Permethrin (8).



(8)

These compounds are potent neuroactive pesticides. In their action they do not appear to react covalently with a target like the organophosphates and carbamates but are more like the chlorinated hydrocarbon acaricides earlier described.

The major disadvantage of this otherwise effective control method is the ability of arthropods to develop resistance to pesticides [3,104,105]. The existence of tick strains resistant to various acaricides was confirmed about 50 years after arsenic dips first came into use, within 5-10 years for chlorinated hydrocarbons and after about 10 years for organophosphates and carbamates [15]. The number of resistant tick species rose from 2 in 1947 to at least 15 by 1975 [41]. Cross resistance enables these resistant species to survive exposure to related chemicals. Multiple resistance is far more serious as pests display resistance to a variety of classes of pesticides with differing modes of action and different detoxification pathways [106].

Other disadvantages in the chemical approach to livestock tick management include the environmental debt rung up by acaricides, such as

pollution of underground water sources and contamination of meat and milk products.

1.6.2 THE SITUATION IN KENYA.

The use of acaricides in Kenya dates back to the turn of this century [77]. Acaricides are now widely used, especially in areas of high rainfall where the important disease vectors are found. The strategy of tick and tick-borne diseases control in Kenya has focused mainly on controlling *R. appendiculatus* to prevent theileriosis transmission and to introduce improved exotic breeds of cattle and upgraded indigenous populations in ECF-affected areas [5,39,58,77].

The policy of the Kenya Government therefore is to protect susceptible cattle from the effects of ticks and the diseases they transmit by intensive dipping. In ECF-affected areas, tick control measures are enforced by the Cattle Cleansing Ordinance Act, under which the Minister who for time being is responsible for livestock development is empowered to proclaim infected areas and enforce weekly cattle cleansing with government approved acaricides. The table below shows some of the approved and registered acaricides in Kenya in 1988.

TABLE 1: Approved and registered acaricides in Kenya [39]

CHEMICAL NAME	PRODUCT NAME	MANUFACTURER
Dioxathion	Delnav DFF	Coopers
Quintrophos	Bacdip	Bayer
Coumaphos	Asuntol	Bayer
Chlorofenviphos	Supona	Shell
"	Steladone	Ciba-Geigy
"	Superdip	Coopers
Carbaryl	Sevin	May & Baker
Diamide	Triatix	Coopers

Although acaricides are generally available on the market, they are nevertheless too expensive for the majority of the farmers. In an attempt to save money therefore, some farmers fall into the temptation of overdiluting the acaricide or try to reduce the frequency of spraying treatments with the danger of a possible development of resistance to the acaricide concerned.

There is a need for more dips as many herds of cattle converge in one dip. Some of the already few dips do not function because of lack of water as

well as poor management. Some dip attendants are not well trained resulting in improperly conducted dipping procedures.

Socio-economic problems also come into play; some farmers are reluctant to dip heavily pregnant or milking cows for fear of poisoning the milk or killing the foetus. The pastoral tribes migrate with their cattle in search of water and pasture, greatly hampering dipping programs [58]. Livestock are infested by several species of ticks many of which have alternative hosts among domestic animals as well as the wildlife abundant here [25,36,78].

There is need therefore, to re-examine the present setup, and arrive at techniques and strategies that are more compatible with the aspirations and capability of the resources of a poor small scale farmer [72].

1.7 THE SEARCH FOR ALTERNATIVES.

Few agricultural or health problems confronting human societies have proved as intractable as control of ticks and the many diseases they transmit. Ticks are undeniably the most difficult pests and vectors to control [35]. Since their introduction into South Africa in 1890, acaricides have been the most common method of tick control in Africa [38]. Pesticides usually do not eradicate a pest species and their continued use or misuse leads to decreased effectiveness. In addition, the cost of developing new acaricides, in relation to the economic return expected before resistance occurs, has discouraged research leading to new chemicals [24]. Concern for the environment cannot be overemphasised here when one considers the quantities of these chemicals that

are used each year and released to the environment. These phenomena are important factors stimulating interest in alternative methods of tick control, or at least in methods that minimize the amounts of acaricide required to control ticks [6]. Despite the dangers and difficulties that attend the use of pesticides few practical alternatives are available [31].

In this search for alternatives some areas have received more attention than others. Comparatively little has been done concerning utilising host resistance as an alternative for tick control [18,36,87] neither is there much literature on immunisation of cattle against *R. appendiculatus* [25]. Below are enumerated areas in which this concern has been dealt with in some depth.

1.7.1 BIOLOGICAL CONTROL.

The term biological control is generally used to describe the deliberate introduction (or augmentation) of predators, parasites or pathogens to control populations of a pest or vector species.

Predators of ticks include ants, birds, rodents, lizards, shrews, spiders and domestic chickens [32,58]. The known parasitoids are hymenopteran wasps belonging to the genera Ixodiphagus and Hunterellus [51,56]. The entomogenous fungi, *Beauveria bassiana* and *Metarhizium anisopliae* have been shown to induce significant mortalities in adult *R. appendiculatus* and *Amblyomma variegatum* [37].

1.7.2 FLORA TO THE RESCUE ?.

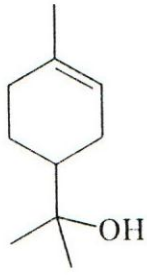
Certain plants have been used since antiquity to keep insect pests away from closets and other storage areas and to keep certain pests off the human body. Plant-produced chemicals repel arthropods, deter feeding and oviposition and have delayed effects on growth and reproduction [33,81]. Laboratory and field observations have been made on the effects of plants on ticks but little chemical analysis has been carried out to identify the active components in these plants or their extracts.

The possible role of anti-tick grasses in maintaining or eliminating field tick populations has been variously examined from as early as 1924 [93]. Molasses grass, *Melinis minutiflora,* has marked tick-repellent properties. Cattle grazing in it carry fewer ticks [93] while all instars of *R. appendiculatus* avoided climbing it's stem during a laboratory study [54].

The use of plant materials as repellents led directly to the extraction and identification of several essential oils that are fairly efficient repellents. An oil extracted from the leaves of a tropical shrub, *Ocimum suave*, was found to repel as well as kill all stages of *R. appendiculatus* [55]. Preliminary experiments undertaken with cattle kept in the field suggest that the oil may have potential in tick control. There are however no published results on the characterization of the components of the oil.

The essential oil of the shrub, *Cleome monophylla*, exhibited repellency higher than or comparable to that of the commercial arthropod repellent,

N,N-diethyltoluamide (DEET), when tested against *R. appendiculatus* and the maize weevil, *Sitophilus zeamais* [61]. 14 compounds were identified in the *C. monophylla* oil by GC, GC-MS and co-injection with authentic samples .The most repellent components against *R. appendiculatus* and *S. zeamais* were 1-∝-terpeneol (9) and 2-dodecanone (10) [62].

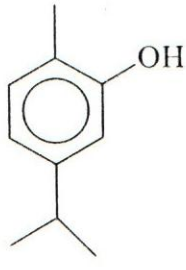


(9)



(10)

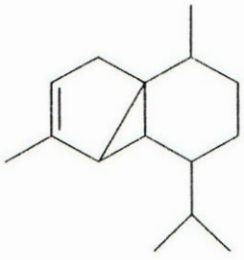
At higher concentrations (10µl per application) however, these two as well as carvacrol (11), 3-undecanone (12) and ∝-(-)-cubebene (13) all repelled better than the commercial arthropod repellent (DEET) (14) [62].



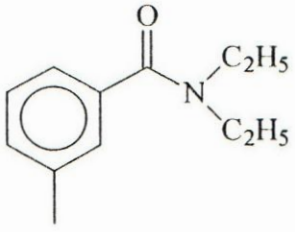
(11)



(12)



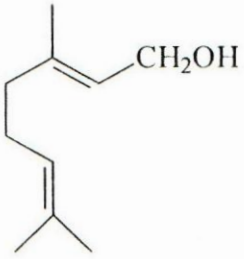
(13)



(14)

Extracts from *Margaritaria discoidea*, an indigenous African plant have shown acaricidal properties against *R. appendiculatus* and *A. variegatum* instars [38]. In adult *R. appendiculatus* feeding on cattle, the 50% oil extract was found to be as effective as the standard concentration (0.05%), of Steladone, a commercial acaricide. The acaricidal components of these extracts however have not been documented.

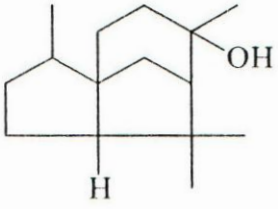
All stages of *R. appendiculatus* and *A. variegatum* ticks were either killed or repelled on exposure to the leaves of *Gynandropsis gynandra* (**L.**) Brig., a shrub abundant in eastern Kenya [48]. Field observations indicated that ticks were not found up to 2 - 5 metres from the plant in where the plant was predominant. The components responsible for this repellency have only been partially investigated. Geraniol (15), linalool (16), (+)-cedrol (17), phytol (18) and n-docosane (19) have all been identified as being components of the essential oil extracted from this shrub. On bioassaying, all components have shown a lower repellency than the commercial arthropod repellent DEET [62].



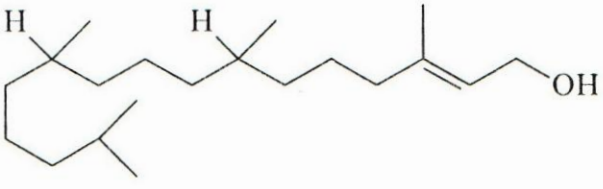
(15)



(16)



(17)



(18)

$CH_3(CH_2)_{20}CH_3$

(19)

Two South American species of *Stylosanthes* produce sticky secretions which immediately immobilize larvae of the cattle tick, *Boophilus microplus*. The larvae are poisoned within 24 hours by an unidentified vapour from the secretions [92].

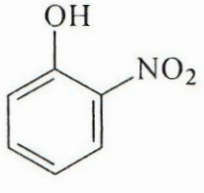
"Kupetaba", ground mixture of dried tobacco leaves and a mineral called "magadi soda" which is mined around L. Magadi in the Rift Valley province of Kenya has proved to be acaricidal against all stages of *R. appendiculatus* [20]. In this instance again, the active components in the mixture remain unidentified.

1.7.3 PHEROMONES

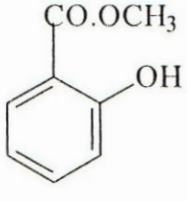
A pheromone is defined as a chemical or mixture of chemicals that is released to the exterior by an organism that causes one or more specific reactions in a receiving organism of the same species [89].

The need for tick control techniques has led to interest in using the ticks' own natural chemicals as a basis for their control. Research has been directed to the use of pheromones as lures, confusants and decoys [88,91].

In 1976 a report was made of *R. appendiculatus* exhibiting aggregation in response to a substance which could be collected from filter papers among which the ticks lived [10]. Later the aggregation-attachment pheromone of *A. variegatum* was found to be composed of a blend of phenols namely O-nitrophenol (20), methylsalicylate (21) and pelargonic (nonanoic) acid (22), all in a precise ratio to one another [83].



(20)



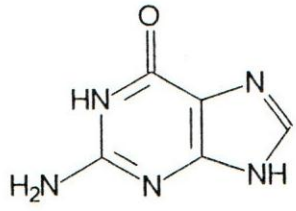
(21)

$CH_3(CH_2)_7COOH$

(22)

They were used together with carbon dioxide to attract and trap unfed adults in the field [68]. The presence of the pheromone on previously infested cattle allowed unfed ticks to discriminate between hosts on which these parasites have fed successfully and those on which they have not [67].

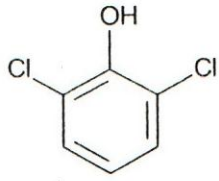
An assembly pheromone was reported in the soft tick, *Argas persicus*, by extraction in saline and not organic solvents [48] and also in the tick *Amblyomma hebraeum* [80]. Guanine (23) has now been identified as an assembly pheromone of ticks [76]. A sex pheromone for *R. appendiculatus* is present in the sterol ester fraction of extracts from the surface of the female [30].



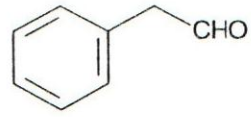
(23)

Pioneering work indicated that ixodid ticks may generally utilize simple phenols as attractants [14,43,52,98,107]. Treatment of *Dermacentor variabilis*-infested dogs with formulations of the sex pheromone 2,6-dichlorophenol (24) significantly reduced mating by the attached ticks [108].

A patented device uses pheromone-acaricide-impregnated plastic tail tag decoys for controlling the bont tick, *A. hebraeum* [64,65]. The tail tags were impregnated with a mixture containing O-nitrophenol, methylsalicylate, 2,6-dichlorophenol and phenylacetaldehyde (25) and one of three different acaricides (cyflumethrin, flumethrin or alphacypermethrin) [66]. Acaricidal effectiveness of synthetic pyrethroids and pheromones was studied against *A. persicus* [101].



(24)



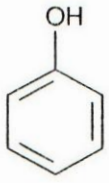
(25)

1.7.4 KAIROMONES

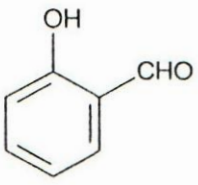
These are interspecific messenger chemicals that benefit the recipient e.g host odours that attract predators or parasites. Stimuli emitted from the host can be sensed through mechanoreceptors, thermoreceptors and olfactory receptors [83]. Host odour substances, as tick attractants may be used in conjunction with acaricides in the control of ticks [60,99].

The scent of the host (dog) fur has been shown to attract the tick *Ixodes persulcatus* [59]. Substances produced by or associated with the leg glands of the white-tailed deer, *Odocoileus virginiasnus* may influence *Ixodes scapularis* climbing behaviour [12].

More recently a study carried out showed that *R. appendiculatus* was attracted to hexane washes of the host ear [85] while ear-trapped attractants of the same tick have been identified as simple phenolic compounds most of which are also pheromones: phenol (26), salicyladehyde (27), 4-cresol (28), 2,6-dichlorophenol and 2,6-dibromophenol (29) [70].



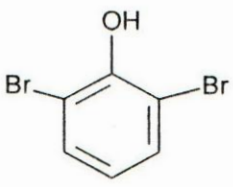
(26)



(27)



(28)



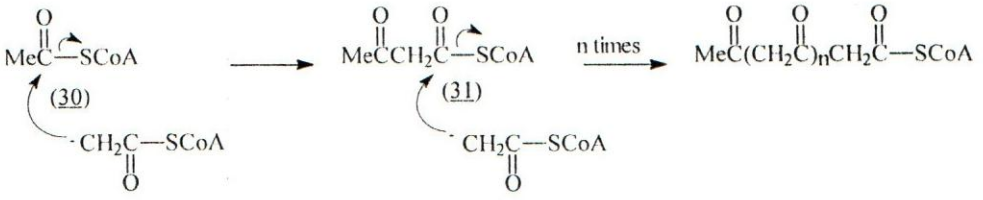
(29)

1.8 PHENOLIC COMPOUNDS.

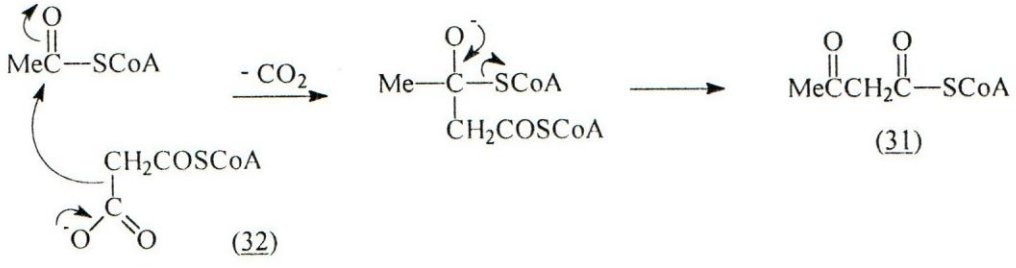
Simple phenolic compounds may be produced aand modified by a variety of sequences, according to the organism involved and the function the metabolite fulfils. Many of the reactions are part of a denerative sequence that is employed by a particular organism [17].

1.8.1 THE BIOSYNTHESIS OF PHENOLIC COMPOUNDS.

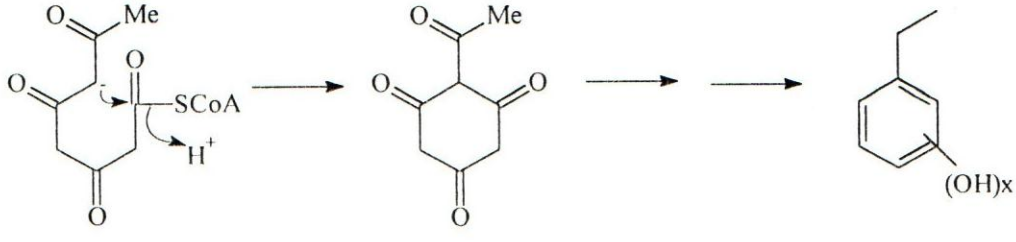
In the living organism, chemical compounds are synthesised and degraded by means of a series of chemical reactions each mediated by an enzyme. A large number of natural products are derived from acetyl co-enzyme A (30), one of the basic bulding blocks for secondary metabolism, which itself is formed from acetate [49]. An inherently simple mode of biosynthesis would be a linear polymerisation of acetyl (CH_3CO-) units, which chemically quite acceptable. Hence a series of Claisen condensations, via acetoacetyl-CoA (31) could conceivably produce a linear polyketide as shown below [2,49].



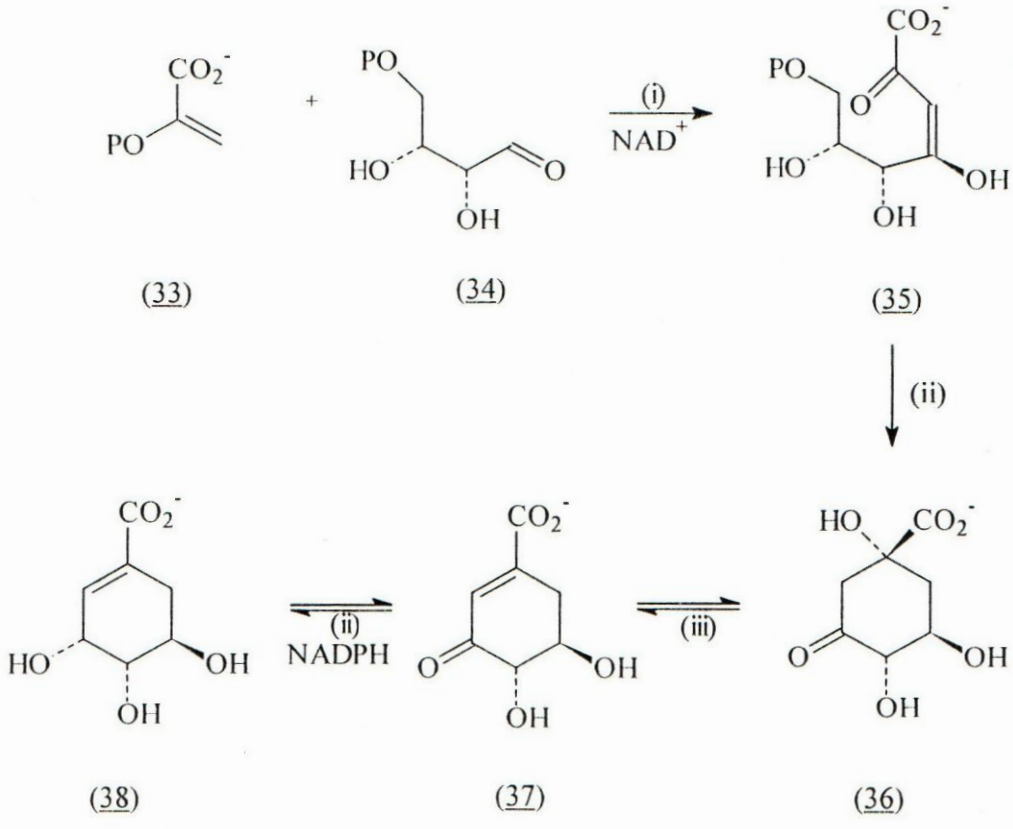
Alternatively malonyl-SCoA (32), may be the source of C_2 units which are added to acetyl-SCoA, as a starter unit:



At some stage, coiling of the polyketide chain leads to intramolecular aldol or Claisen condensations to produce phenols [1,49].



Phenolic compounds have also been demonstrated to originate via the Shikimate pathway [49]. See **Figure 3.**

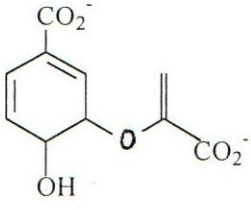


(i) DAHP synthase
(ii) 3-Dehydroquinate synthase
(iii) 3-Dehydroquinase
(iv) Shikimate dehydrogenase

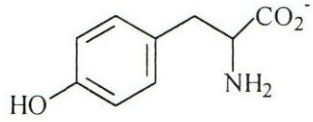
Figure 3: The Shikimate Pathway.

The first reaction in the Shikimate Pathway involves condensation of phosphoenolpyruvate (PEP) (33) with erythrose-4-phosphate (34), giving 3-deoxy-arabino-heptulosonate-7-phosphate (DAHP) (35). The latter is then converted to 3-dehydroquinate (36) in a complex sequence of reactions requiring an oxidation, an α-elimination, a reduction and an intramolecular aldol condensation. The 3-dehydroquinate is finally dehydrated to 3-dehydroshikimate (37) which is eventually reduced to shikimate (38).

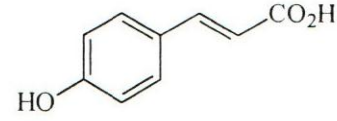
The shikimate is then converted sequentially to the chorismate (39) from which eventually tyrosine (40) is made.



(39)



(40)



(41)

The stereospecific, anti, elimination of ammonia from tyrosine is facilitated by an amino acid ammonia lyase to yield the cinnamic acids e. g. p-coumaric acid (41). These then become the precursor of several so called shikimate metabolites. (See **Figure 4**).

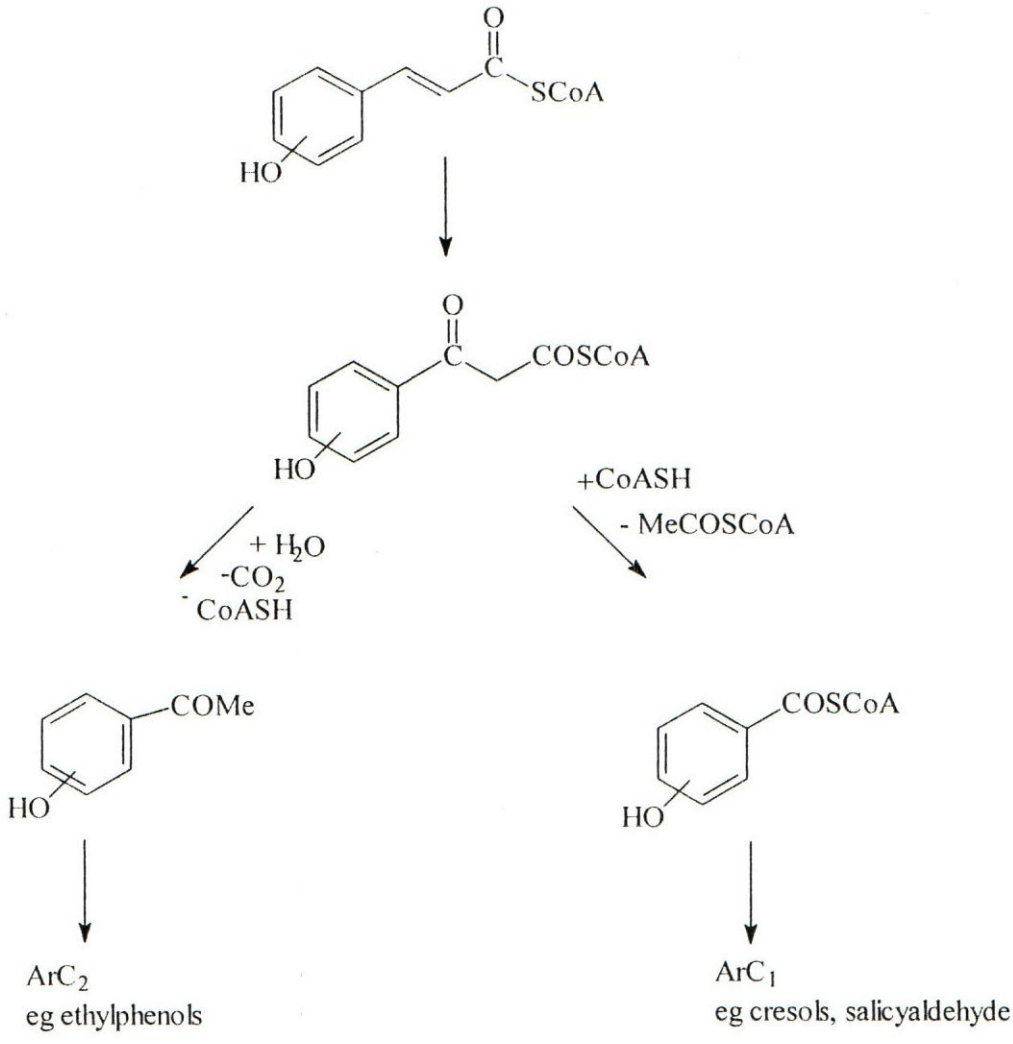
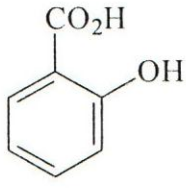
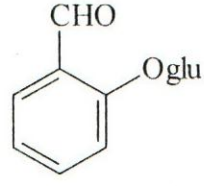


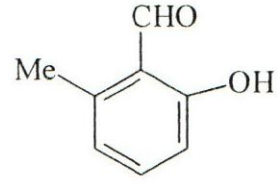
Figure 4: Proposed biosynthetic pathway to some Shikimate metabolites

It is interesting to note that salicylic acid (42) and helicin (43) derive from chorismic acid in contrast to 6-methylsalicylic acid (44) which is synthesized from acetate and malonate [29,50].





glu = glucose



(42) (43) (44)

One may merely speculate as to why nature has chosen to synthesise these rather similar compounds via widely differing biosynthetic pathways.

1.9 OBJECTIVE OF THE PROJECT.

Literature survey points towards the fact that there is a kairomanal cue that attracts the brown ear tick, *Rhipicephalus appendiculatus*, to the ear of the host. Little work however has been done to ascertain the chemical identity of any compound that may attract the tick to this site. It was therefore the aim of this project to:

i) Collect body chemicals from the ear of the tick host, the cow.

ii) Carry out bioassays against the tick, thereby establishing the existence of tick attractants emitted by the host.

iii) To separate the various components of the body chemicals in order to analyse and identify the kairomones.

CHAPTER TWO

RESULTS AND DISCUSSION

2.1 COLLECTION OF TICK ATTRACTANTS FROM THE COW EAR.

It is a documented fact that host odours are made of assorted air-borne compounds of different volatility prone to extraction by organic solvents. The solvent of washing is then expected to create a partial or complete absence of active compounds from the predilection site on a temporary basis [85]. Prior to the commencement of sample collection a suitable solvent was sought. Two solvents with opposite polarity, hexane and methanol, were investigated in order to determine an efficient solvent for the collection of samples throughout the study.

Bioassay responses of female *Rhipicephalus appendiculatus* to cow ear washes using hexane and methanol are summarised in **Table 2.** At the doses tested, hexane and methanol extracts of the ear evoked significant attraction to female *R. appendiculatus* as compared to their respective controls. This is indicated by the positive Percentage Attractancy, (PA), values recorded in the bioassays.

Table 2: Bioassay responses of female *R. appendiculatus* to hexane and methanol extracts of cow ear washes.

Amount of sample applied(µl)	Fraction used	Number of ticks		PA (%)
		N_t (%)	N_c (%)	
12.5	H	12 (40.0)	11 (36.7)	4.35
12.5	M	11 (36.7)	9 (30.0)	10.0
25.0	H	13 (43.3)	7 (23.3)	30.0
25.0	M	19 (63.3)	8 (26.7)	40.0
50.0	H	10 (33.3)	12 (40.0)	-8.3
50.0	M	12 (40.0)	9 (30.0)	14.3

Where N_t = Number of ticks that prefered the treated arm
N_c = Number of ticks that prefered the control arm
PA = Percentage attractancy
H = Hexane extract
M = Methanol extract
N = 30, the total number of ticks used for each assay

Figure 5 compares the results obtained by assaying the different solvents. At a glance the figure seems to imply that hexane generally gave lower PA values at any given dosage of application. However, statistical analysis points out that the PA values are not significantly different at the 95% level.

The bar graph also clearly depicts that no negative attractancy values are recorded for any methanol application while the hexane extract recorded one negative PA value at 50 µl. On comparing the two solvents therefore, methanol is seen to be the more superior solvent in this study. This is in keeping with results previously obtained in which hexane, acetone and methanol were compared but hexane was preferentially used because of it's ease of evaporation [85].

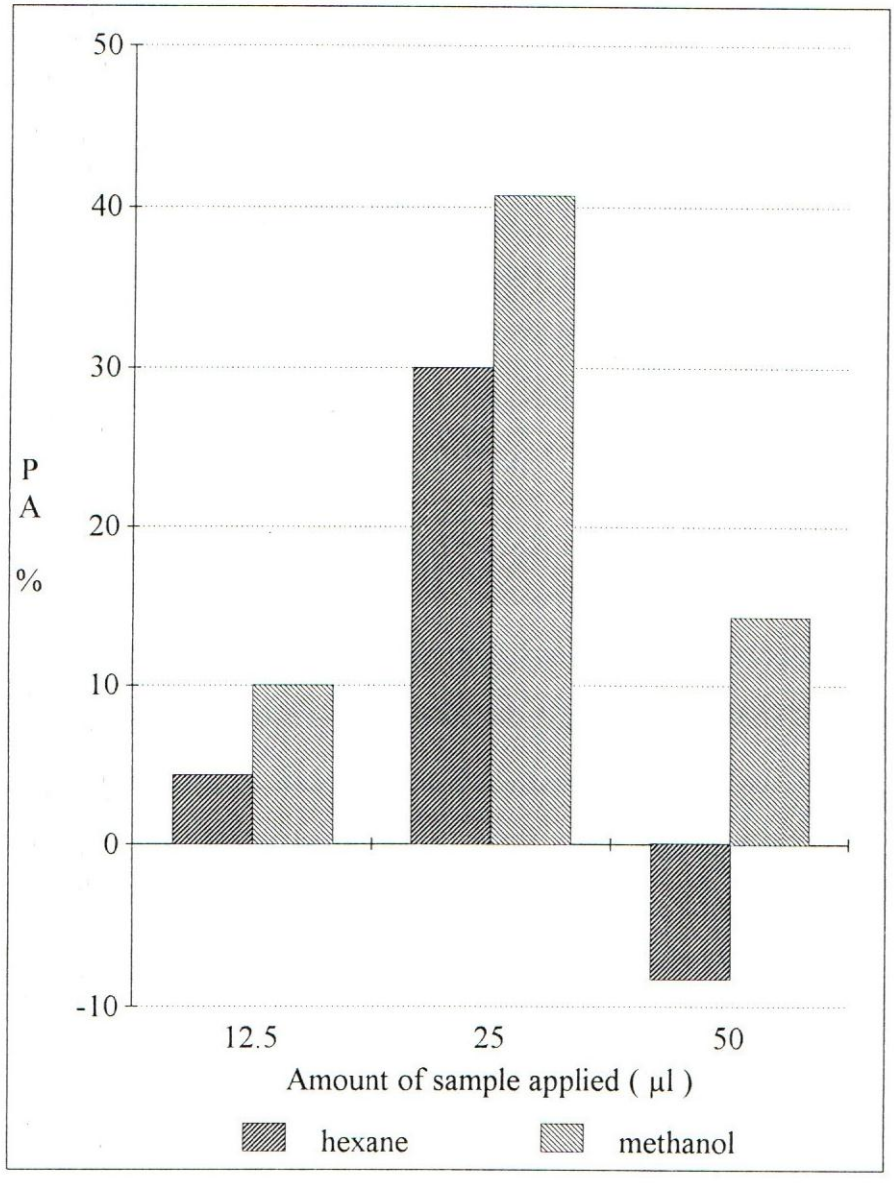


Figure 5: Comparison between Hexane and Methanol

2.2 COMPARISON OF RESPONSES BETWEEN FEMALE AND MALE *R. APPENDICULATUS*

Studies by Walker involving the collection of ticks from the field indicated that there was a higher proportion of male than female *R. appendiculatus* [96]. This excess of males over females fits in with the known tendency of males to remain longer on the host than the female, which detach themselves immediately upon repletion. Naturally therefore one is left to wonder whether the two sexes respond in the same way to the attractant cues already established to be present. The female and male ticks were hence subjected to assays against crude extract. The results of these assays are compiled in **Table 3**.

Both female and male ticks preferred the treated to the control arms of the T-tube at all dose applications.This is a clear indication that both sexes of ticks were attracted by the crude sample applications. On comparing bioassay responses between male and female ticks, it was interesting to note that in both cases the intermediate doses, (25.0 µl), gave highest percentage attractancy.

Figure 6 graphically presents the comparison between the percentage attractancy (PA) values obtained while assaying the female and males. This figure clearly depicts that at all the three levels of application it is evident that the PA readings of the female ticks is higher than those of the male. This points to the fact that female ticks are more receptive to this sample.

Table 3: Bioassay responses of female and male *R. appendiculatus* to whole extracts of cow ear washes.

Amount of sample applied(µl)	Tick used	Number of ticks		PA (%)
		N_t (%)	N_c (%)	
12.5	F	11 (36.7)	7 (23.3)	22.2
12.5	M	15 (50.0)	10 (33.3)	20.0
25.0	F	14 (46.7)	5 (16.7)	47.4
25.0	M	12 (40.0)	7 (23.3)	26.3
50.0	F	18 (60.0)	8 (26.7)	38.5
50.0	M	13 (43.3)	8 (26.7)	23.8

Where N_t = Number of ticks that prefered the treated arm
N_c = Number of ticks that prefered the control arm
PA = Percentage attractancy
F = Female ticks
M = Male ticks
N = 30, the total number of ticks used for each assay

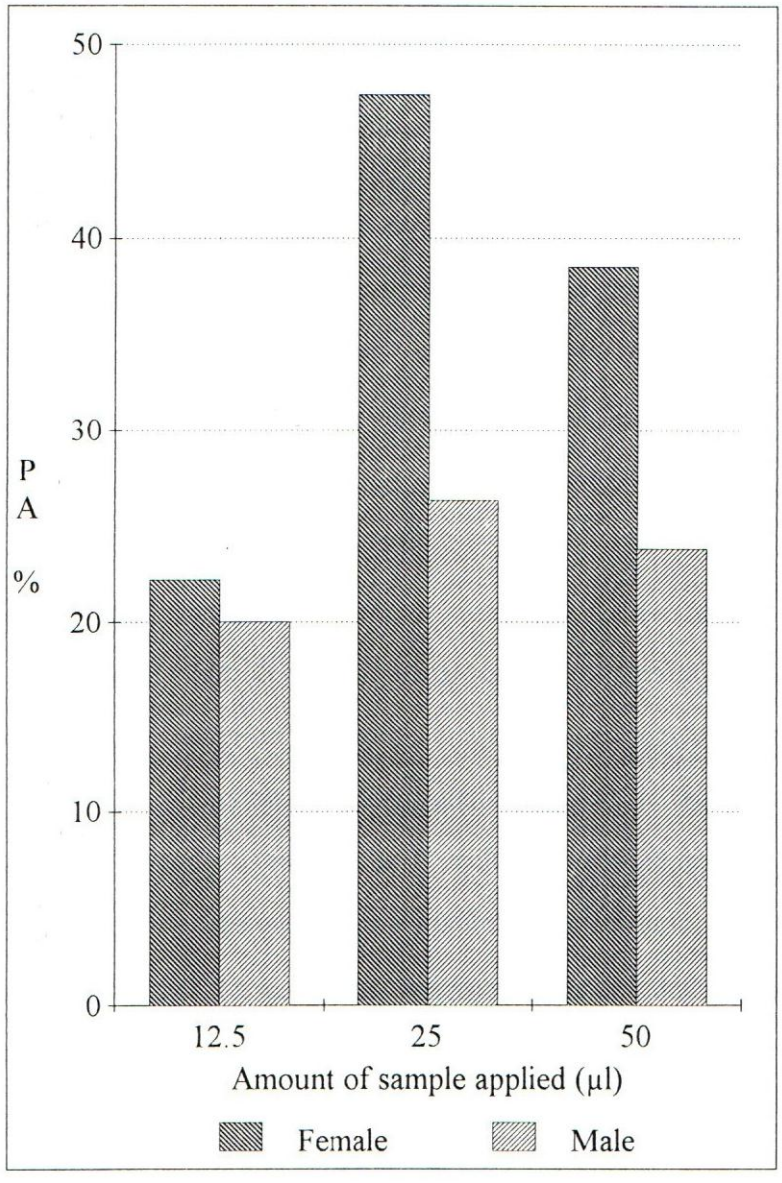


Figure 6: Comparison between responses of female and male *Rhipicephalus appendiculatus* to whole sample.

On the strength of this evidence therefore, only female ticks were used for subsequent bioassays.

2.3 BIOASSAY RESPONSES OF THE VARIOUS SEPARATED FRACTIONS.

Separation of the various components of the cow ear washes involved manipulation of the physical properties of the compounds. (See **Figure 7**). Ethylacetate as a solvent was chosen since all the wash was soluble in it, it is immiscible with water, evaporates relatively easily and was readily available.

2.3.1 Fraction P

The initial addition of an aqueous solution of sodium hydroxide to the crude sample converted all organic acids into their water soluble sodium salts. These were separated from the organic ethylacetate layer during partitioning. The aqueous mineral acid, hydrochloric acid, readily converted these salts back into free organic acids which are more soluble in an organic solvent than in water. Most phenols are weak acids and will not dissolve in aqueous bicarbonate solutions like carboxylic acids. The organic Fraction P, was therefore expected to contain phenolic or enolic compounds. This fraction showed marked attraction for the ticks (**Table 4**).

The response was least for the lowest concentration at 12.5µl. The response increased noticeably at the next application (25.0 µl) and was higher still at the 50µl dose. The 35.7% attractancy recorded for the 50 µl

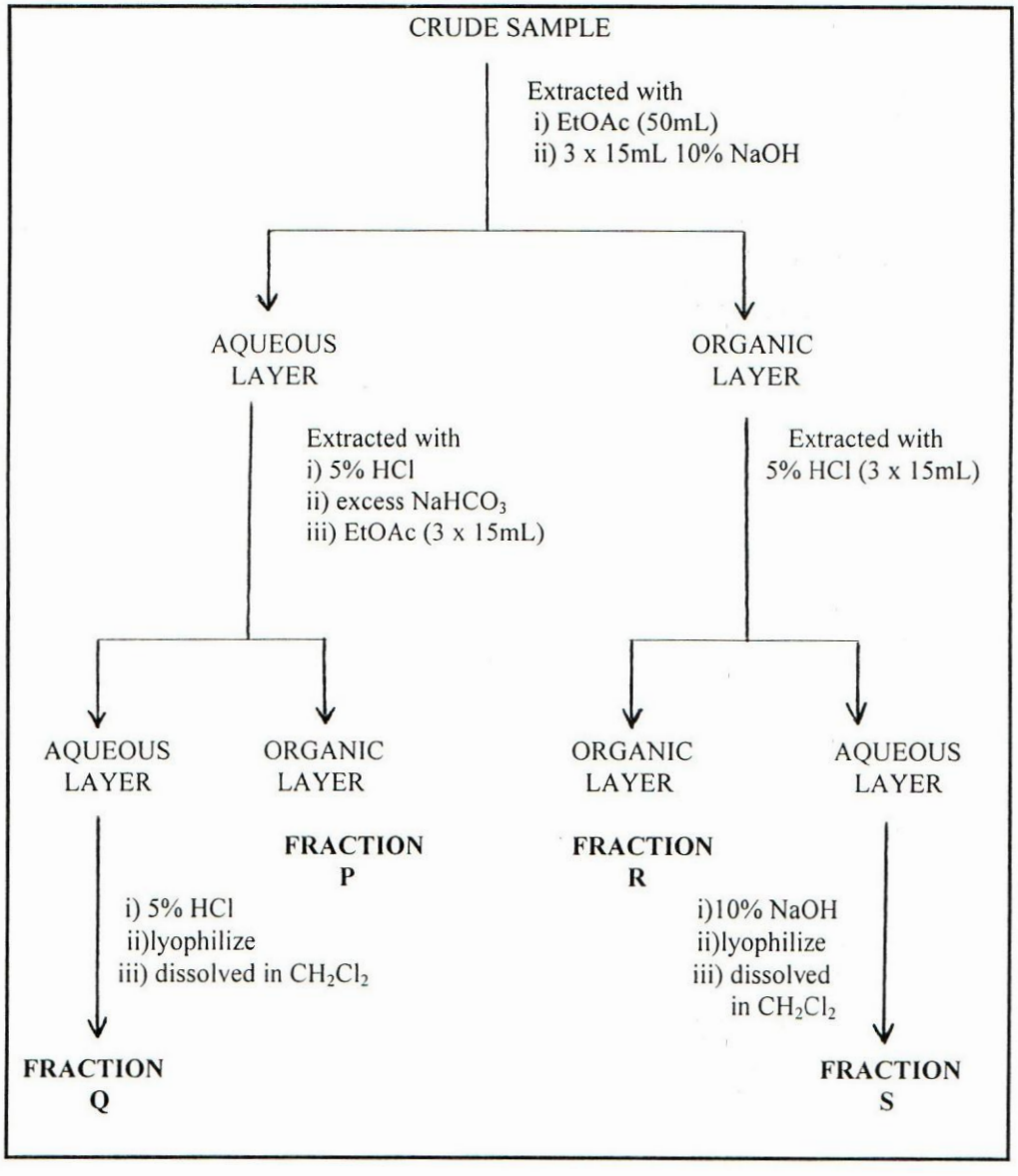


Figure 7: Flow chart for the separation of components of the crude sample.

Table 4: Female *Rhipicephalus appendiculatus* bioassay responses to various fraction treatments.

Amount of sample applied(µl)	Fraction used	Number of ticks		PA (%)
		N_t (%)	N_c (%)	
12.5	P	12 (40.0)	10 (33.3)	9.1
12.5	Q	12 (40.0)	14 (46.7)	-7.69
12.5	R	10 (33.3)	15 (50.0)	-20.0
12.5	S	6 (20.0)	12 (40.0)	-33.3
25.0	P	13 (43.3)	8 (26.7)	23.8
25.0	Q	6 (20.0)	19 (63.3)	-52.0
25.0	R	2 (6.7)	18 (60.0)	-80.0
25.0	S	11 (36.7)	12 (40.0)	-4.3
50.0	P	18 (60.0)	8 (26.7)	35.7
50.0	Q	9 (30.0)	15 (50.0)	-23.1
50.0	R	0 (0)	19 (63.3)	-100.0
50.0	S	13 (43.3)	14 (46.7)	-0.04

Where N_t = Number of ticks that prefered the treated arm
N_c = Number of ticks that prefered the control arm
PA = Percentage attractancy
P = Fraction P
Q = Fraction Q
R = Fraction R
S = Fraction S
N = 30, the total number of ticks used for each assay

was quite comparable to the 38.5% recorded at the bioassay of adult females against the crude sample. This may point towards the fact that most or even all of the samples attractive to these ticks were present in this fraction. It was concluded then that this sample be subjected to further analysis by Gas Chromatography - Mass Spectrometry (GC-MS). (See p53)

2.3.2 Fraction Q

Into this fraction were extracted any carboxylic acids which were released from their bicarbonate salts by acidification, with hydrochloric acid, of the aqueous solution from which Fraction P was separated.

For the first application (12.5µl) of this fraction, the number of ticks choosing either arm of the T-tube are almost equal (**Table 4**). This is however not the case at the next application when a little more than half move to the control, while for the last dose a little less than half move to the control. There was no significant difference, at the 95% probability level ($p < 0.05$), between the number of ticks that prefered the treated and the control arm of the T-tube in each case. Further to this fact, there were no positive PA readings. It was therefore evident that ticks are not attracted to this sample and no further analysis was undertaken of this fraction.

2.3.3 Fraction R

This fraction ought to have consisted of any compounds that were neither basic nor acidic. It was considered to be the fraction consisting of the neutral components of the ear washes. Percent female tick bioassay responses to disks treated with various amounts of sample from Fraction R gave surprisingly strong negative attraction responses in a dose dependent fashion (**Table 4**).

An increase in the amount of sample applied was reflected in the increase of negative attractancy. As the aim of this study was to identify the components of ear washes that are attractive to these ticks, it was concluded that since there is no attractancy resulting from this fraction, there was no reason for analysing it further. It would however be interesting to know what compound(s), if any, cause such a strong negative response in these ticks, and the reason as to why this effect is masked in the crude sample.

2.3.4 Fraction S

This is the fraction that contained any basic compounds. Analysis of the movement of ticks at all three levels of application showed the percentage attractancy levels as being comparatively less negative than those of Fraction R (**Table 4**).

The 12.5 µl application recorded a higher negative value than that of Fraction R. The -4.3% and -0.04% readings at 25µl and 50µl respectively seems to imply that there is not much preferance for either the control or the

treated arm of the T-tube.There were no positive attractancy readings recorded. On the strength of these observations therefore, it was concluded that there was no sufficient evidence to warrant further analysis of this fraction.

Figure 8 compares the percentage attractancies due to the four fractions. From this graph, one observes that the only fraction with consistent positive readings is Fraction P. This means that it is the only fraction that was attractive to the ticks at all dose applications. It is therefore evident that the interest of this study lies in Fraction P.

The GC analysis of this Fraction P revealed several peaks as is evident in **Figure 9.** Subsequently, the GC-MS analysis led to the identification of some of the compounds responsible for these peaks.

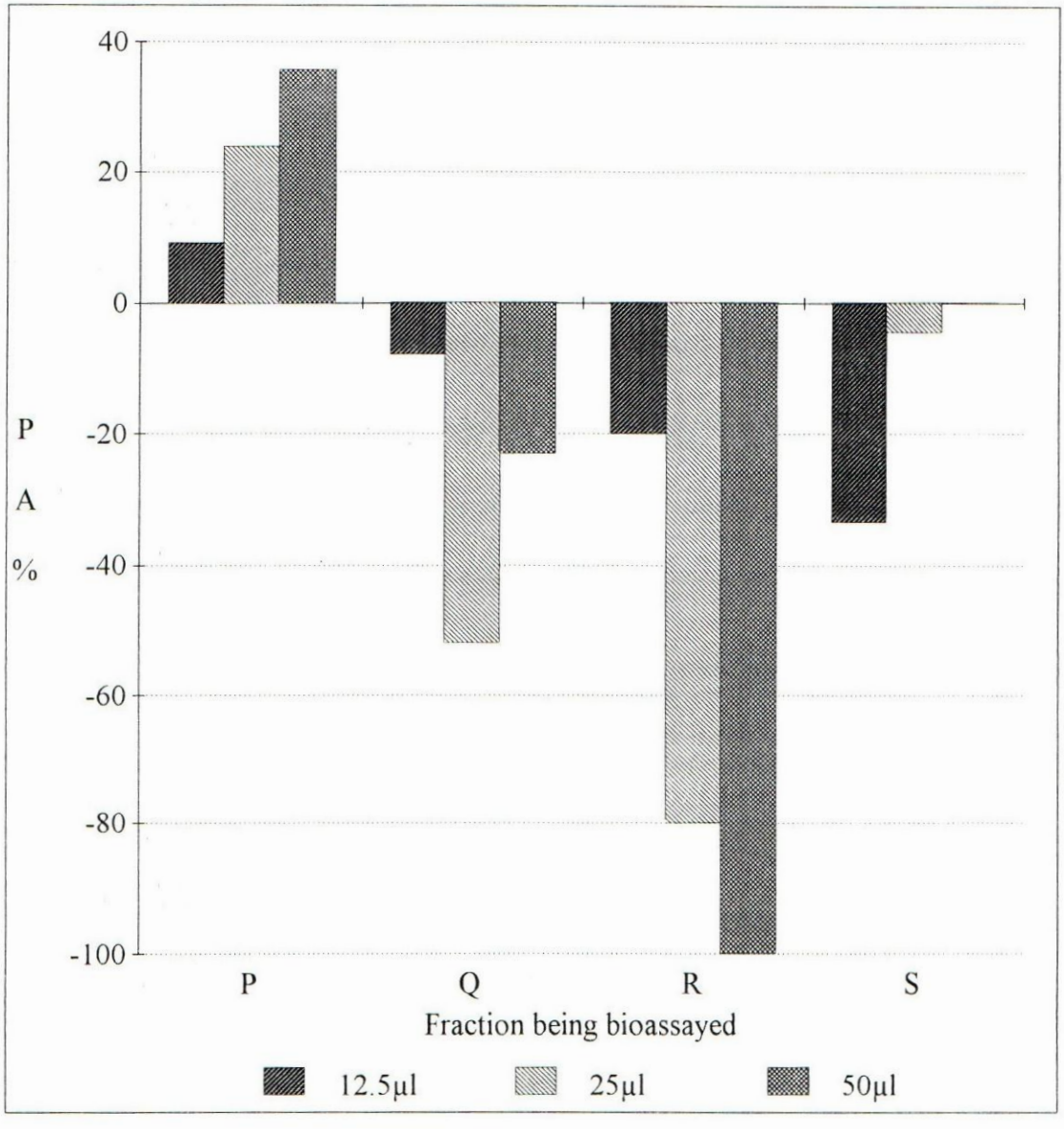


Figure 8: Comparison of the Percentage attractancies for the different fractions

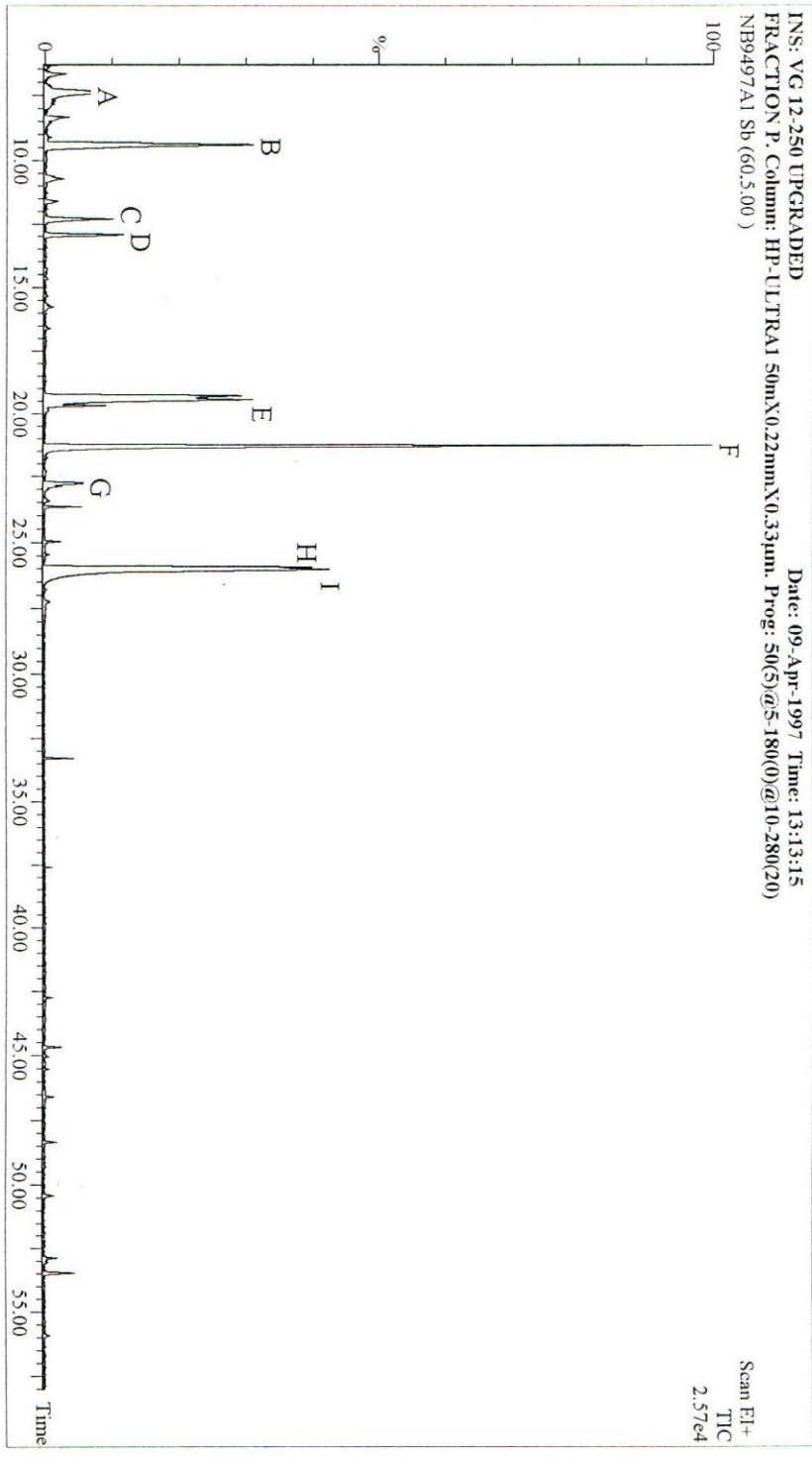


Figure 9: Gas Chromatogram of Fraction P of cow ear washes

2.4 CHARACTERIZATION OF VARIOUS COMPONENTS OF FRACTION P.

Only peaks with areas greater than or equal to 1.6% of the total area covered by sample (and excluding the area due to solvent) were considered for analysis.

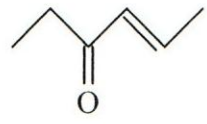
2.4.1 COMPOUNDS A, B AND C

Compound A, with a retention time of 7.375 minutes, comprised 3.82% of the total sample composition while compounds B and C eluted at 9.400 and 12.300 minutes respectively and made up 4.08 and 3.17 % of the sample. All the three samples gave scant spectra which matched those of several compounds in the library. All the possible authentic compounds however had different retention times from those of these three compounds. These compounds were therefore not characterised.

2.4.2 COMPOUND D

The compound D with retention time 12.925 minutes comprised about 4.03% of the total sample composition. It's electron impact mass spectrum revealed an apparent molecular ion at m/z 98 (See **Appendix 1a**). The base peak, m/z 69, indicated a loss of an ion of m/z 29 which may correspond to an ethyl group. The other significant peaks appeared at m/z 41, 40, 39 and 38. The peak at m/z 41 may have arisen due to the loss of carbon monoxide (CO) from the base peak.

A library search gave 4-hexen-3-one (45) as the closest fit to the spectrum of the compound eluting at retention time 12.925 minutes (See **Appendix 1b**).



(45)

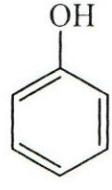
Indeed as is typical of ketones, the major fragmentation of 4-hexen-3-one occurs at the C-C bonds adjacent to the oxygen atom to give the base peak (m/z 69) as a result of the loss of the larger alkyl group. The fragment at m/z 41 may have lost subsequent hydrogen atoms to give rise to the other peaks at m/z 40, 39 and 38.

2.4.3 COMPOUND E

A compound E, whose electron impact mass spectrum (**Appendix 2a**) showed a likely molecular weight of 94 atomic mass units was eluted at 19.451 minutes. This compound comprises 3.63% of the total sample and has a base peak at m/z 94. The spectrum of this compound revealed prominent peaks at m/z 66 (M - 28), m/z 65 (M - 29), m/z 40 and m/z 39.

The peak at m/z 66 may have come about as a result of the loss of carbon monoxide (CO) from the parent molecular ion, while that at m/z 65 may have arisen due to the loss of the elements of CHO. The elimination of a neutral acetylene molecule (28 amu) each from the m/z 65 and m/z 66 respectively could have borne the prominent peaks at m/z 39 and m/z 40.

A library search for the best fit of Compound E's spectrum gave phenol (26) See **Appendix 2b.**



(26)

Phenol, like most aromatic compounds, has an intense molecular ion peak, M^+, in this case at m/z 94. Phenols usually do not fragment by loss of either the -OH hydrogen or the -OH group itself thus the M - 1 (m/z 93) and M - 17 (m/z 77) are very weak.

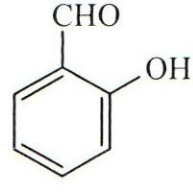
The most important fragmentation of phenols is associated with the loss of 28 atomic mass units which is accompanied by an M - 29 species of only slightly reduced abundance due to the loss of the elements of CHO. Phenol itself has no CO group, clearly therefore, a rearrangement of hydrogen away from the -OH group must first take place [9].

2.4.4 COMPOUND F

Compound F was eluted at 21.301 minutes. This is the most abundant component of Fraction P, comprising 28.47% of the total. It's electron impact mass spectrum revealed a possible molecular ion at m/z 122. See **Appendix 3a.** Other major peaks were those at m/z 121(M - 1), m/z 104 (M - 18), m/z 93 (M - 29), m/z 76 (M - 46), m/z (M - 57) and m/z 39.

The molecular ion may have fragmented into two resulting in a peak at m/z 29 and an ion at m/z 93. The m/z 76 peak may have arisen due to first, the elimination of a water molecule to give the m/z 104 peak followed by the elimination of a carbon monoxide molecule.

The library search gave 2-hydroxybenzaldehyde (27) (**Appendix 3 b)** as the most probable structure for compound F:



(27)

The presence of both the M-1 and m/z 29 ions in a spectrum are very typical of aldehydes. Further to this, a typical aromatic aldehyde would be expected to have large molecular and M-1 ion peaks in their spectra. This is

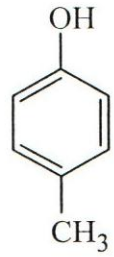
the case in this compound. Co-injection with an authentic sample confirmed that compound F indeed is 2-hydroxybenzaldehyde.

The M - 1 ion may have eliminated CO to give the hydroxyphenyl ion (m/z 93) which in turn lost yet another CO to give m/z 66 and subsequently m/z 65 by loss of 1 amu. The loss of a neutral acetylene each from the ions at m/z 66 and m/z 65 may have next been responsible for the peaks at m/z 39 ($C_3H_3^+$) and m/z 40 ($C_3H_4^+$).

2.4.5 COMPOUND G

Compound G which comprised 1.66% of the total composition of the sample had a retention time of 22.751 minutes. Electron Impact - Mass Spectrometry (**Appendix 4a)** revealed an apparent molecular ion peak at m/z 108. Other prominent peaks were at m/z 107 (M-1), m/z 79 (M - 28), m/z 77 (M - 31) and m/z 39. The base peak of this compound is that which occurs at m/z 107.

A library search revealed 4-methylphenol (28) (**Appendix 4b**) as the most probable fit to the spectrum of compound G. Confirmation of this compound was achieved through co-injection with an authentic sample which resulted in the enhancement of compound G's peak area.



(28)

As is typical of any cresol, 4-methylphenol has a M - 1 (m/z 107) peak larger than the molecular ion. This is as a result of a facile C - H cleavage which gives rise to a hydroxytropylium ion that is highly stabilized by resonance involving the - OH group as well as the ring carbons [86]. The M - 1 peak (m/z 107) is the base peak in this compound.

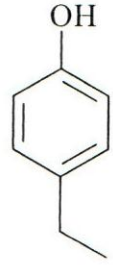
Fragmentation of the hyroxytropylium by loss of a carbon monoxide (CO) molecule may have given rise to the peak at m/z 79. Subsequent rearrangement of the latter ultimately may then have given rise to the phenyl ion at m/z 77 [11].

2.4.6 COMPOUNDS H AND I

A compound H, with a retention time of 25.926 minutes, comprised 9.57% of the total sample composition. It's electron impact mass spectrum, see **Appendix 5a,** revealed an apparent molecular ion at m/z 122 and prominent peaks at m/z 107 (M-15), m/z 77 (M-45) and m/z 39. The base peak for this compound was at m/z 107.

The peak at m/z 107 may have resulted from the loss of 15 amu in the form of a methyl (-CH_3) group. The presence of a peak at m/z 77 pointed

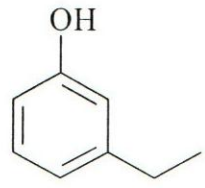
towards the fact that this compound is aromatic. This was further strengthened when a library search gave a possible fit for compound E as being 4-ethylphenol (46), see **Appendix 5b**. Confirmation of this was done when co-injection with an authentic 4-ethylphenol sample resulted in peak area enhancement of the region coinciding with compound H.



(46)

Compound I, which comprised about 12.94% of the total sample composition, had a retention time of 26.026 minutes. This is the second most abundant compound in the Fraction P sample. The electron impact mass spectrum, see **Appendix 6a,** displayed data that was very similar to those of compound I. An apparent molecular peak occurred at m/z 122 and the base peak was that at m/z 107 (M - 15). Other prominent peaks were at m/z 77 (M - 45) and m/z 39.

A library search of this compound uncovered 3-ethylphenol, (**Appendix 6b**), a structural isomer of 4-ethylphenol, as compound I. Co-injection with an authentic sample of 3-ethylphenol confirmed the identity of compound I which was therefore assigned structure 47.



(47)

The spectra of different ethylbenzenes are not identical because the hydroxytopylium ion, m/z 107, formed from the various isomers have differing amounts of excess energy and will fragment to different degrees. The peaks thus formed are therefore similar but of different abundance [11].

A typical ethylphenol spectrum should depict the fact that the methyl group is lost more readily than the α- hydrogen [11]. This fact is quite in evidence in both these spectra since the M - 1 peak, m/z 121, is much less intense than the M - 15 peak (m/z 107).

The peak at m/z 79 may have arisen due to the hydroxytropylium ion eliminating a carbon monoxide molecule. Further hydrogen rearrangement may then have produced the peak at m/z 78 and the phenyl ion at m/z 77.

The method of extraction used to yield Fraction P was designed to isolate enolic as well as phenolic compounds into this fraction. Those compounds identified are therefore in keeping with this expectation. The table shown below summarises the compounds identified from Fraction P.

Table 5: Compounds identified from Fraction P.

No.	Compound	RT (mins)	Method of identification	Relative %
A	Unknown	7.375	-	3.82
B	Unknown	9.400	-	4.08
C	Unknown	12.300	-	3.17
D	4-hexen-3-one	12.925	L	4.03
E	Phenol	19.451	L,C	3.63
F	2-hydroxybenzaldehyde	21.301	L,C	28.47
G	4-methylphenol	22.751	L,C	1.66
H	4-ethylphenol	25.926	L,C	9.57
I	3-ethylphenol	26.026	L,C	12.94

L = Confirmed by library search
C = Confirmed by co-injection with authentic sample.

The fact that this fraction is attractive to the tick, *Rhipicephalus appendiculatus,* therefore does not come as a surprise in the light of the identity of the phenols present in this fraction since for a long time now it has been known that simple phenols are used as attractants by ixodid ticks [8,9,107]. Besides, the volatile sex attractant 2,6-Dichlorophenol, which is released by partially fed females of a great variety of metastriate species, and a mixture of 4-methylphenol and phenol, which replaces 2,6-Dichlorophenol in some species of *Rhipicephalus* [107], there are other simple phenols

which have been identified as tick pheromones or as a components of a tick pheromone complex.

The aggregation-attachment pheromone produced by the fed males of the tropical bont tick, *Amblyomma variegatum* consists of 2-nitrophenol, methylsalicylate and pelargonic acid. Of the individual components, only the first induced a significant, although not complete aggregation response. The other two contribute to complete pheromone activity [83]. Salicyaldehyde is also a component of ixodid tick sex pheromone [8,9,107]. Feeding females attract sexually mature males by producing a sex pheromone reportedly composed of 2,6-Dichlorophenol, phenol, p-cresol and salicyaldehyde [8,9,14,28].

The results obtained from the bioassay of both ethylphenols, (See **Table 6),** did not seem to reveal a new attractant for the brown ear tick. This conclusion is arrived at since the percentage attractancy values worked out are either negative or have insignificantly small positive values as is portrayed in **Figure 10**.

Table 6: Female *Rhipicephalus appendiculatus* responses to various doses of 4-ethylphenol and 3-ethylphenol.

Amount of sample applied(µl)	Compound used	Number of ticks		PA (%)
		N_t (%)	N_c (%)	
0.0001	1	11 (36.67)	13 (43.33)	-8.33
0.0001	2	9 (30.0)	8 (26.67)	5.88
0.001	1	14 (46.67)	12 (40.0)	7.69
0.001	2	13 (43.33)	14 (46.67)	-3.70
0.01	1	12 (40.0)	12 (40.0)	0.0
0.01	2	11 (36.67)	12 (40.0)	-4.35
0.1	1	10 (33.33)	12 (40.0)	-9.09
0.1	2	14 (46.67)	10 (33.33)	16.67
1.0	1	10 (33.33)	9 (30.0)	5.26
1.0	2	9 (30.0)	11 (36.67)	-10.0
10.0	1	10 (33.33)	11 (36.67)	-4.76
10.0	2	10 (33.33)	12 (40.0)	-9.09
100.0	1	12 (40.0)	12 (40.0)	0.0
100.0	2	14 (46.67)	11 (36.67)	12.0

Where N_t = Number of ticks that prefered the treated arm
N_c = Number of ticks that prefered the control arm
PA = Percentage attractancy
1 = 4-ethylphenol
2 = 3-ethylphenol

N = 30, the total number of ticks used for each assay

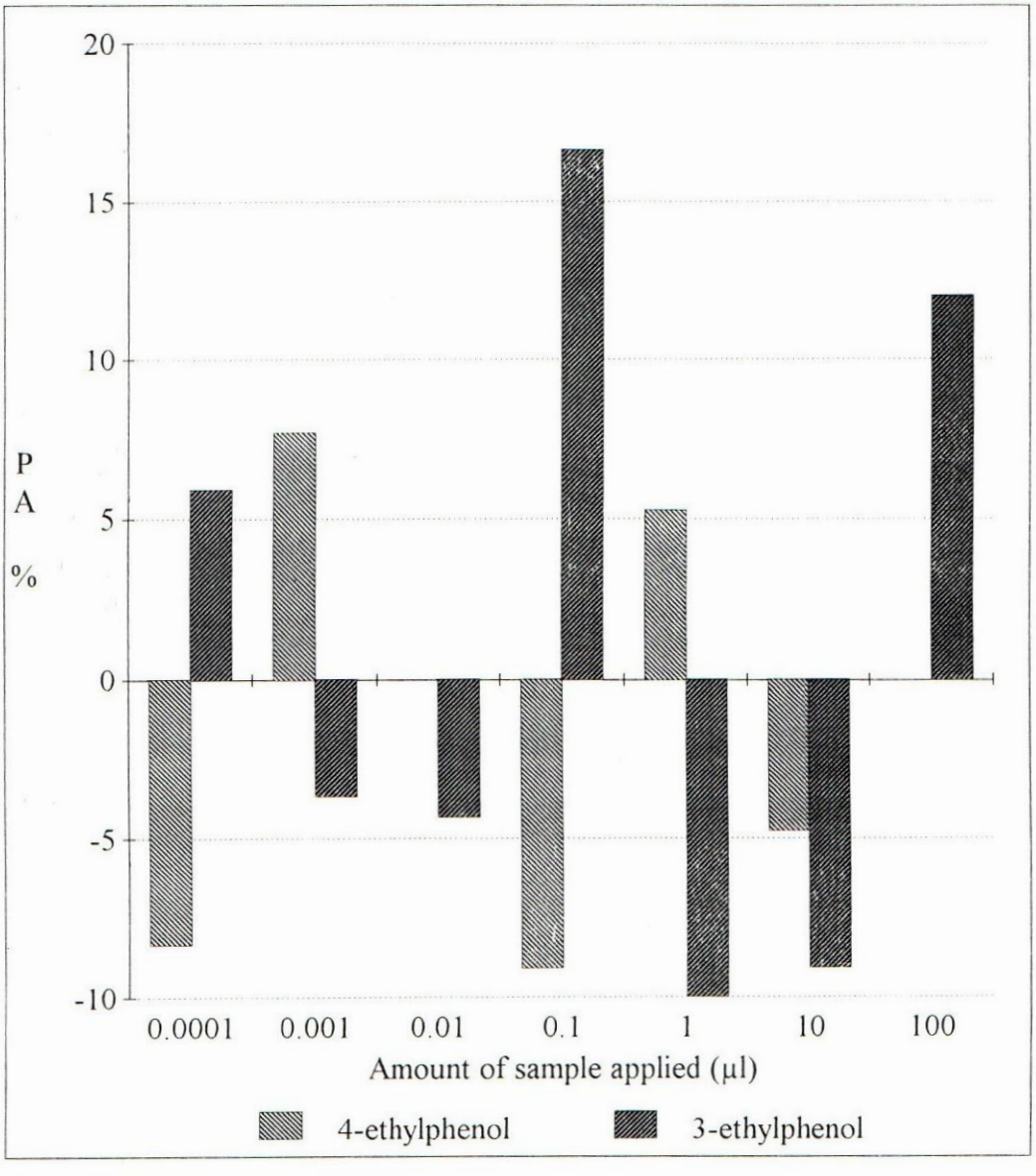


Figure 10: Female *Rhipicephalus appendiculatus* responses to doses of 4-ethylphenol and 3-ethylphenol.

The compounds identified from Fraction P are very comparable to those identified as being the phenolic components of oxen skin secretions or sebum as seen in the table below:

Table 7: Phenolic components of sebum and their proportions relative to the major phenolic component, 4-methylphenol [100].

4 - methylphenol	100.0
3 - methylphenol	6.4
Phenol	5.0
4 - ethylphenol	4.4
3 - ethylphenol	3.5
3 & 4 - propylphenol	2.3

It is interesting to note that it is these very compounds that are found in the most attractive fraction of buffalo urine as tsetse attractants [34]. In the two species of tsetse, *Glossina morsitans morsitans* Westwood and *Glossina pallidipes* Austen, responses to 3-alkylphenols increased as the alkyl chain increased from one carbon atom to three, while the opposite trend was observed with 4-alkylphenols. These results indicated that 4-cresol and 3-propylphenol are the most stimulating of the two respective groups of phenols [82].

In view of all this as concerns tick attractants, it is no wonder therefore that a fraction containing phenol, 4-methylphenol and salicyaldehyde should be attractive to ticks. These compounds have been reported as attractants of *R. appendiculatus* on several occasions.

Earlier work revealed that phenol alone is not highly attractive but attractivity of phenol and p-cresol mixtures, of p-cresol alone and of 2,6-dichlorophenol could be (and have been) confirmed [107]. This information is echoed by the fact that trapped volatiles from the host are significantly more attractive to the ticks than the washes but their blend is more attractive still [85]. The additive effect of the blend suggests a dual kairomonal set of components comprising short-range or contact signals and volatile components which mediate feeding site selection in *Rhipicephalus appendiculatus*.

One would therefore be inclined to think that these compounds are imbibed by the arthropods during blood meals and later used as semiochemicals. Were this the case then, it would only be fair to expect a trace of the ubiquitous 2,6-dichlorophenol in sebum sample. This compound however is not in evidence. One should expect this since the presence of halogenated compounds is not very usual in land animals but has been recorded in marine animals [94]. There is no evidence as yet that

2,6-dichlorophenol is produced by *R. appendiculatus* [98] but behavioural studies done show that phenol and p-cresol are the sex pheromone of this tick [107].

Due to unavailability of time however, a comprehensive bioassay into the level of attraction and what dosage each of the identified compounds is most active has not been done. This bit of work needs to be completed so that precise attraction ratios for these attractants can be established for purposes of tick population control in integrated pest management.

2.5 COMMENTS AND CONCLUSION

Ticks are haematophagous parasites and to secure a blood meal, they have to locate their hosts. Tick positioning in the natural environment depends on the tick species concerned and predisposes certain potential hosts more than others, hence partly forming the begining of host specificity. Once the tick is on the host, host specificity and site of feeding are enhanced by factors like odour, temperature and taste stimuli.

Chemical compounds in sweats and other skin secretions, detectable by olfactory and/or tactile receptors are believed to facilitate the selection of feeding sites [87]. The preffered feeding sites can be positively said to contain behaviourally active compounds as is demonstrated by the relatively high rate of success in locating their predilection sites [83] as well as the attraction of ticks to ear washes.

Further to this, experiments show that solvent washed ears attracted less ticks than unwashed ears. Attraction of *R. appendiculatus* to washed ears dropped significantly ($p < 0.001$) from an average of 47.8% arrivals at untreated ear sites to an average of 35.2% reaching washed ears. This reasoning should therefore rule out the possibility that the reason a tick moves to the cow ear is that once on the host, ticks tend to avoid dessication by moving to less exposed areas or parts of the host.

Results from this study support suggestions put forward way back in 1975 [104] that simple phenols are indeed attractants of ixodid ticks. As

such, these compounds may then be used as lures in integrated pest management (IPM), a strategy that uses several compartible control strategies simultaneously to reduce pest populations and maintain their abundance below the levels at which disease transmission or economic losses are likely to occur [85].

My suggestion then is that a formulation of these kairomones together with a relatively affordable but effective acaricide should be dispensed as a pour-on applied in small concentrated volumes down the middle of the animals' backs. From this point of initial application, the active ingredients can rapidly diffuse to provide an effective concentration over the entire bodysurface. The advantages inherent in this are four-fold:

i) It uses up less amounts of acaricide and is therefore cheaper than dipping.

ii) There is no need for fixed installations such as dips and spray races.

iii) It can be used in times of water shortage.

iv) There is no need for animals to trek long distances to the few available and functioning dips.

Implementation of such measures would definitely go a long way in the up-hill task of controlling the brown ear tick, *Rhipicephalus appendiculatus*, as well as in helping poor third world farmers maintain quality herds of cattle at a price that is comfortable to their pockets.

CHAPTER THREE

EXPERIMENTAL

3.1 GENERAL EXPERIMENTAL PROCEDURES

3.1.1 TICKS

The adult female *R. appendiculatus* ticks used for the bioassays were generously supplied by the Insectary Department of the International Centre of Insect Physiology and Ecology (ICIPE), Duduville, Nairobi, Kenya. The colony was originally established with a strain obtained from Kenya Agricultural Research Institute (KARI), Muguga, Kenya since 1952. This ICIPE colony has since been maintained without introduction of outside stock and were reared as described by Bailey [5].

When not in use, the ticks were kept under humid conditions (85% relative humidity) in saturated pottasium chloride solution [99] (See **Table 8**). The choice of this salt was guided by it's availability and cost. The solution was prepared by dissolving enough solid pottasium chloride (Serva, Feinbiochemica GmbH & Co., Research Grade) to saturate at boiling and allowing this to partially cool at which time a small amount of solid was added. After the mixture cooled, considerably more solid was added and this allowed to stand for several days to ensure saturation before use.

Table 8: Relative humidity values over saturated solutions at various temperatures

Temperature °C	10°	15°	20°	25°	30°	35°	40°	45°
NH_4Cl	79.0	79.5	78.0	77.5	77.5	-	74.0	-
$(NH_4)_2SO_4$	80.5	81.0	80.5	80.0	80.0	79.5	79.0	79.0
$LiCl.H_2O$[a]	13.5	13.0	12.5	12.0	11.5	11.5	11.0	11.0
$MgCl_2.6H_2O$	34.0	34.0	33.0	32.5	32.5	32.5	32.0	31.5
$Mg(NO_3)_2.6H_2O$	58.0	56.0	55.0	53.0	52.0	50.5	49.0	47.5
P_2O_5	00.0	00.0	00.0	00.0	00.0	-	00.0	-
KCl	88.0	86.5	85.0	85.0	84.5	83.0	82.0	81.0
KNO_3[b]	96.0	95.5	93.5	92.5	91.0	89.5	88.0	86.5
K_2SO_4	98.5	99.0	98.0	97.5	96.5	96.0	96.5	96.0
K Tartrate[c]	-	75.0	75.0	75.0	74.0	-	74.0	-
NaCl	76.5	76.0	76.0	75.5	75.5	75.5	75.0	75.0
$Na_2Cr_2O_7.H_2O$[d]	60.0	56.5	54.5	53.0	52.5	51.0	50.0	48.5

[a] $LiCl.XH_20$ = $LiCl.H_20$ at 12.5 °C

[b] Large temperature coefficient

[c] Pottasium tartrate. Excellent

[d] Very good; small temperature coefficient

In preparation for bioassays, the ticks were removed from their storage compartments, sexed and put into clean vials separately then slowly dehydrated over 24 hours using Phosphorus pentoxide (Aldrich Chemical Co. Ltd. Gillingham, Dorset-England).

3.1.2 COWS

A young freisian and later a sahiwal and a mixed breed steers were used for the duration of this research. They were kept in a large animal accomodation unit at ICIPE, Duduville Nairobi. The animals were fed on hay, cattle pellets and occasionally on fresh grass. Water was also supplied constantly.

3.1.3 SOLVENTS

All solvents used were of analytical grade from Merck (Germany). Dichloromethane and n-hexane were double distilled in a Vigreux column before use.

3.1.4 EXTRACTION OF BODY CHEMICALS.

Extraction of body chemicals was done using cottonwool which was first cleaned as follows: Cottonwool was first cut into sizeable pieces using a pair of scissors. The pieces were then refluxed in a soxhlet apparatus for up to 48 hours in dichloromethane. Taking care not to touch these clean pieces, the excess solvent was wrung out and the cotton wool kept overnight in an oven to dry at $100 \pm 2^{\circ}C$.

The clean cottonwool, held by forceps to avoid contamination, were then soaked in n-hexane and used to swab the inner parts of the cow ear pinna. The pieces of cottonwool were then soaked in more of the solvent to allow the sample collected to dissolve into solution. The solution collected was then filtered and concentrated using a rotary evaporator, labelled and kept in the refridgerator. Methanol extracts were also collected using the same procedure.

3.1.5 GAS CHROMATOGRAPHY

Capillary column gas chromatography was performed on a Hewlett Packard 5890 Series II gas chromatograph fitted with a flame ionisation detector (FID). The capillary column employed was an HP-ULTRA 1 crosslinked methylsilicone gum with a length of 50m, an internal diameter of 0.2 mm and a film thickness of 0.33 μm (50m x 0.2mm x 0.33μm).

Temperature changes were programmed starting at 50^{o} for 5 minutes then 5^{o}C per minute rise in temperature till 180^{o} after which the rate was increased to 10^{o}C per minute to a final temperature of 280^{o}C where the temperature was maintained for twenty minutes. The carrier gas used was nitrogen. The injector temperature was 250^{o}C while that of the detector was 240^{o}C. Peaks were analysed using a Hewlett-Packard 3393A integrator.

3.1.6 GAS CHROMATOGRAPHY - MASS SPECTROMETRY (GC-MS)

Gas Chromatography-Mass Spectrometry (GC-MS) was performed on an upgraded VG12-250 quadrupole mass spectrometer interfaced with a Hewlett Packard model 5790A seties gas chromatograph. The GC column used was an HP-ULTRA 1 crosslinked methylsilicone gum with helium as the carrier gas. The programme at which the sample was run was similar to that detailed above but the injection port temperature was 270°C. The chromatogram obtained is seen in **Figure 8**. (See pp 48)

The spectrometer was operated in the electron impact mode at 70 ev and data is given as mass to charge ratios (m/z), with relative ion intensities in parentheses.

3.1.7 AUTHENTIC SAMPLES.

The following authentic samples were used.

1. p-Cresol (99+%) Gold label, Aldrich Chemical Co. Ltd. Gillingham, Dorset-England.
2. m-Ethylphenol ICN Phamaceuticals Inc. Life Sciences Group. Plainsview, New York.
3. p-Ethylphenol 97% Aldrich Chemical Co. Ltd. Gillingham, Dorset-England.
4. Phenol (zur analyse) ACS Merck, Darmstadt, Germany.
5. Salicyaldehyde 98%, Aldrich Chemical Co. Ltd. Gillingham, Dorset-England.

3.1.8 PREPARATION OF STANDARDS FOR BIOASSAYS

10.31 mg of 4-ethylphenol were weighed on an analytical balance (Mettler AT261 Delta range) and dissolved in 10 ml of dichloromethane. This made up the stock solution with a concentration of 10 μg / μl. Serial dilutions of this were then done to enable the dispensing of a range of concentrations from 0.0001 μg / disc to 100 μg / disc.

10.00 mg of 3-ethylphenol was treated in a similar manner. This concentration range was chosen because it is comparable to that used in the bioassay of other phenolic compounds against *R. appendiculatus* [107].

3.1.9 BIOASSAY TECHNIQUE.

The T-tube bioassay technique [4,70,85,107] was used at each stage to establish whether or not attractancy existed. The T-tube is a clear glass apparatus with a stem 2.5cm high, a crossbar 6cm long and an internal diameter of 0.8cm. (**Figure 11**).

A 12.5μl solution of sample was dispensed on a small piece of filter paper (Whatman No. 1) and the solvent left to evaporate. This treated paper was then made into a plug and inserted into one arm of the T-tube. A similar piece of paper with the same volume of solvent but no sample, was then plugged into a control arm of the tube.

This tube was then placed over a single tick which was allowed to climb up the stem of the apparatus. On reaching the junction, the tick made a choice between either of the arms of the T-tube. This first choice was then

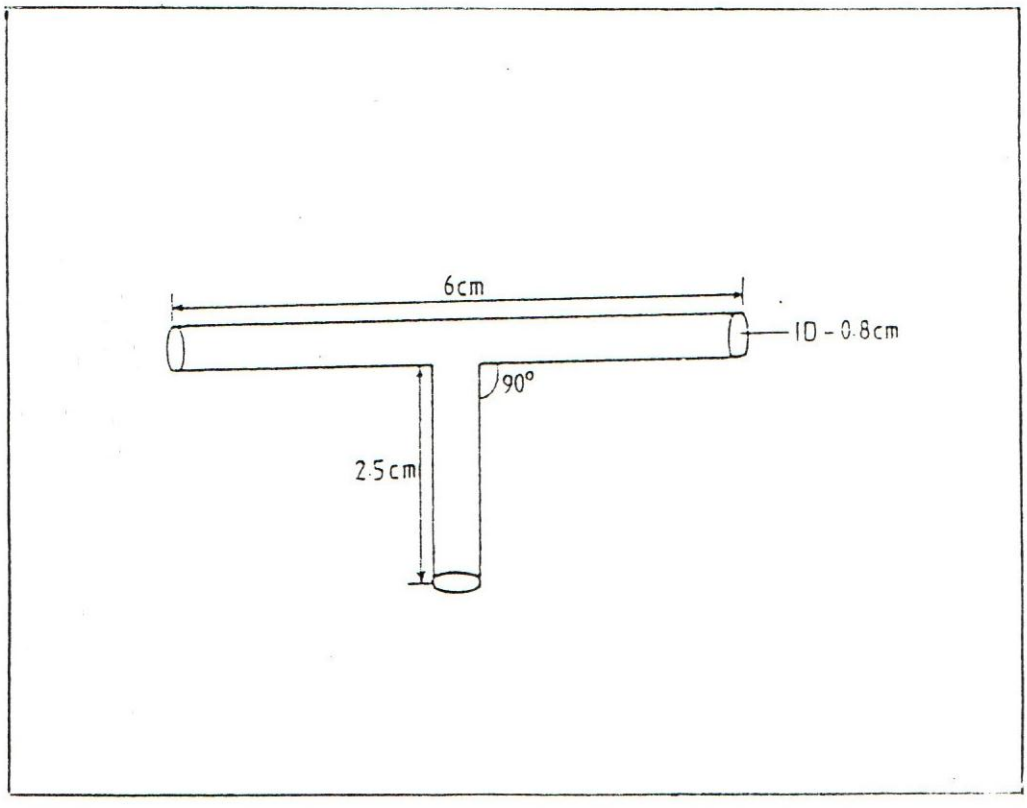


Figure 10: Diagram of the T-tube used for bioassays.

recorded and the assay repeated with a total of thirty ticks for each dose of sample. Each assay was let to run for twenty minutes each time. Higher doses of sample were applied using 25µl and 50µl respectively. These results are summarised in **Tables 2, 3, 4** and **6.**

3.2 ANALYSIS OF DATA.

3.2.1 BIOASSAYS

The first step in analysis of bioassay data was to establish whether the ticks were chosing to move to the various arms of the T-tube randomly or in a pattern. Using the following notation,

a) N_t as the number of ticks which chose the treated arm and

b) N_c as the number of ticks which chose the control arm,

It was assumed then that if,

a) $N_t > N_c$, then attraction was implied

b) $N_t < N_c$, then no attraction was implied

c) $N_t = N_c$, then random movement of ticks was implied.

The Percentage Attractancy, PA, was calculated using the formula,

$$PA = (N_t - N_c) / (N_t + N_c) * 100$$

Treatment differences in bioassays were subjected to statistical analysis relevant to the type of experiment and results obtained. The data was transformed using both square root and logarithmic transformations so as to normalize the data and minimize correlation between the mean and

variance of the data. For these, Statistical Analysis System (SAS), version 6.04 was used [79].

3.2.2 SPECTRA AND CHROMATOGRAMS.

The various components in the mixture were tentatively identified by GC-MS through computerized matching of the sample spectra with a stored mass spectral library. This was achieved using a personal computer based masslynx software with National Institute of Standards and Technology / National Bureau of Standards (NIST/NBS) mass spectra library.

Confirmation of the above identification was then achieved by retention time comparison of authentics as well as peak area enhancement by co-injection with authentic samples. The latter was not always possible however since not all authentic samples required for tests could be obtained.

3.3 SEPARATION OF COMPONENTS.

The crude samples obtained after each collection were pooled and evaporated to about 10ml using the rotary evaporator and then blown to dryness using nitrogen gas. The sample remaining was dissolved in 50ml of ethylacetate and partitioned with 10% sodium hydroxide solution (3 x 15ml) resulting in an aqueous and an organic fraction.

The aqueous layer was acidified with 5% hydrochloric acid then an excess of sodium hydrogen carbonate added to it. This was extracted with ethylacetate, (3 x 15ml), to give a phenolic/enolic organic fraction (P). The

remaining aqueous layer was then acidified with 5% hydrochloric acid to give the acidic Fraction Q.

The organic fraction originally set aside was then extracted with 5% hydrochloric acid (3 x 15ml). The remaining organic layer yielded the neutral Fraction R. The acidic extracts were then made alkali with 10% sodium hydroxide solution to release the basic compounds in Fraction S.

Both Fractions R and P were evaporated to dryness and the residues taken up in dichloromethane for bioassay and further analysis. The aqueous fractions S and Q were subjected to freeze-drying, taken up in dichloromethane and bioassayed. The results obtained during these bioassays are compiled in **Table 4,** (See page 43), while the GC-MS results for Fraction P are shown in **Appendices 1 - 6** and summarised below.

The mass spectrum of compound D (See **Appendix 1**) revealed the following data:

EIMS m/z (relative intensity): 98 (6, M^+); 69 (100, M - C_2H_5); 57 (1, CH_3CH_2O); 41 (70, M - CH_3CH_2CO); 40 (4, C_3H_4); 39 (37, C_3H_3); 38 (3, C_3H_2)

The mass spectrum of compound E (See **Appendix 2**) revealed the following data:

EIMS m/z (relative intensity): 95 (4, M + 1); 94 (100 M); 66 (35, M -CO); 65 (27, M - CHO); 55 (2, M - $C_3H_3^+$); 40 (19 $C_3H_4^+$); 39 (39, $C_3H_3^+$)

The mass spectrum of compound F (See **Appendix 3**) revealed the following data:

EIMS m/z (relative intensity): 123 (7, M+1); 122 (100 M); 121 (99, $(M - 1)^+$); 104 (18, M - H_2O); 94 (6, M - CO); 93 (26, M - CHO); 76 (33, M - H_2O - CO); 66 (16, C_5H_6); 65 (49 C_5H_5); 51 (7, $C_4H_3^+$)

The mass spectrum of compound G (See **Appendix 4**) revealed the following data:

EIMS m/z (relative intensity): 108 (75, M); 107 (100, M - 1); 90 (4, M - H_2O); 79 (14, M - CO); 78 (1, M - CO); 77 (22, C_6H_5); 53 (2, C_4H_5); 51 (4, $C_4H_3^+$); 39 (13, $C_3H_3^+$)

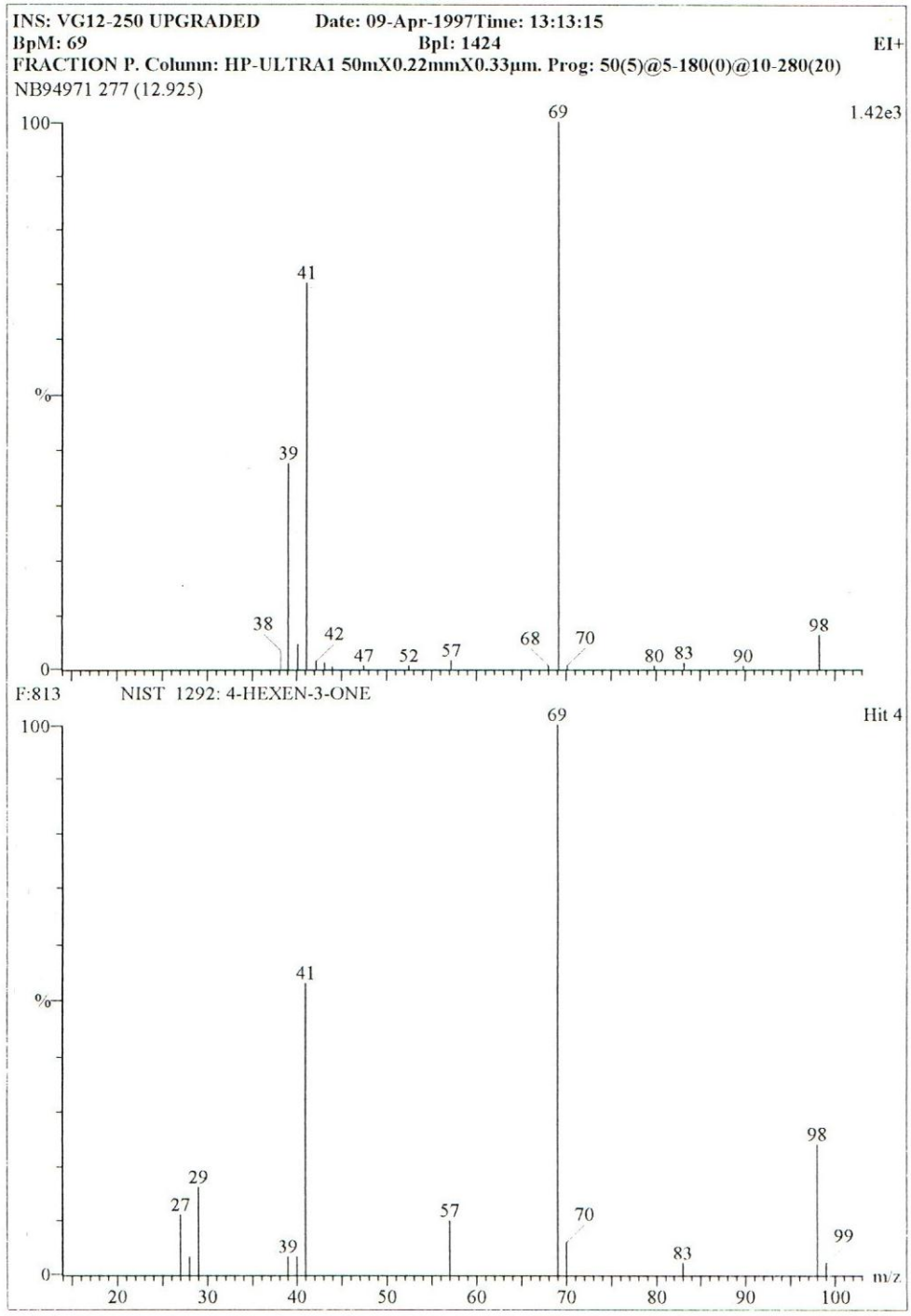
The mass spectrum of compound H (See **Appendix 5**) revealed the following data:

EIMS m/z (relative intensity): 122 (21, M); 107 (100, M - CH_3); 91 (4, M - C_2H_7); 79 (2, C_6H_7); 78 (3, C_6H_6); 77 (19, C_6H_5); 53 (3, C_4H_5); 51 (4, $C_4H_3^+$); 39 (13, $C_3H_3^+$)

APPENDIX 1

(a)

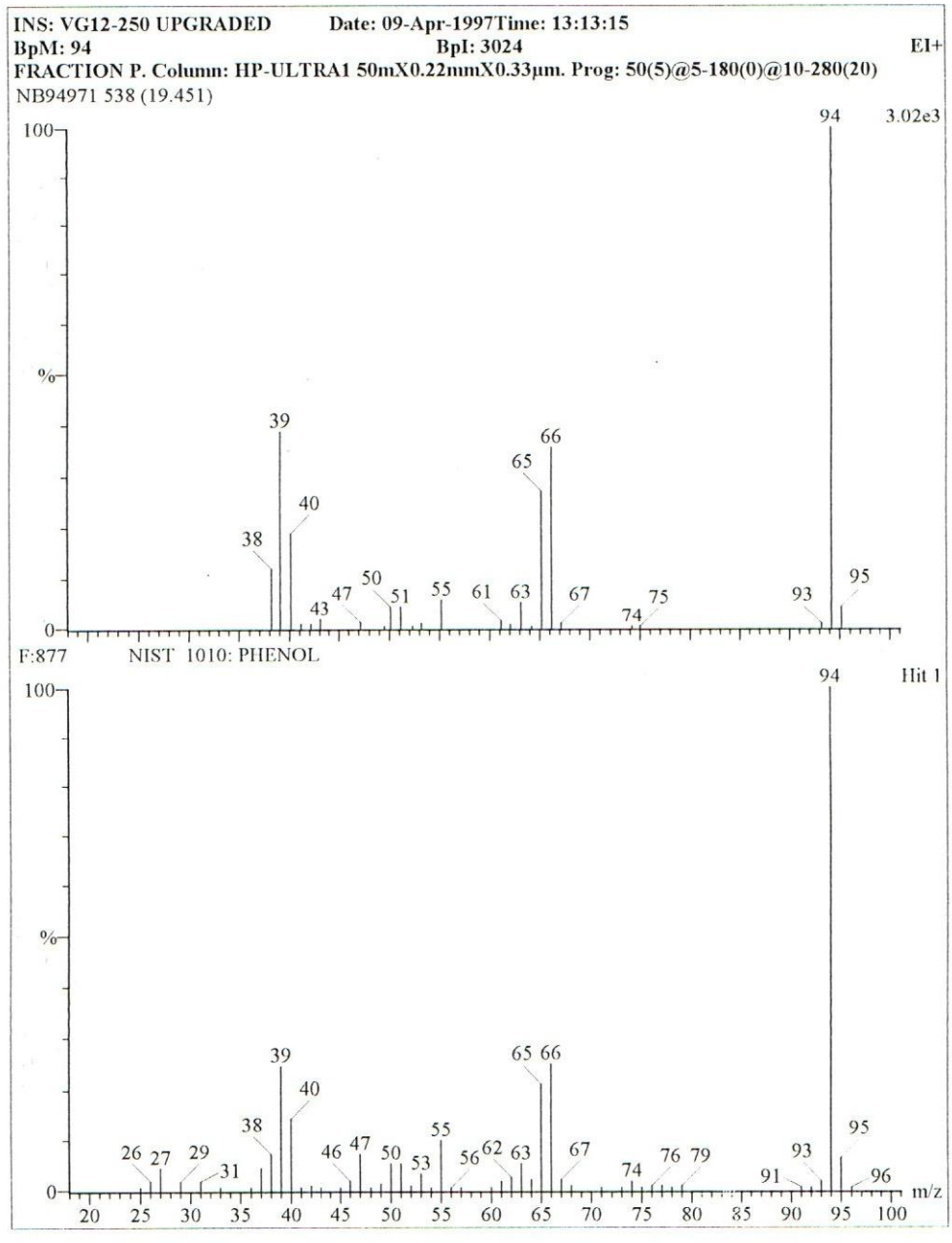
(b)



Mass spectra of (a) compound A from Fraction P

(b) authentic 4-hexen-3-one

APPENDIX 2



(a)

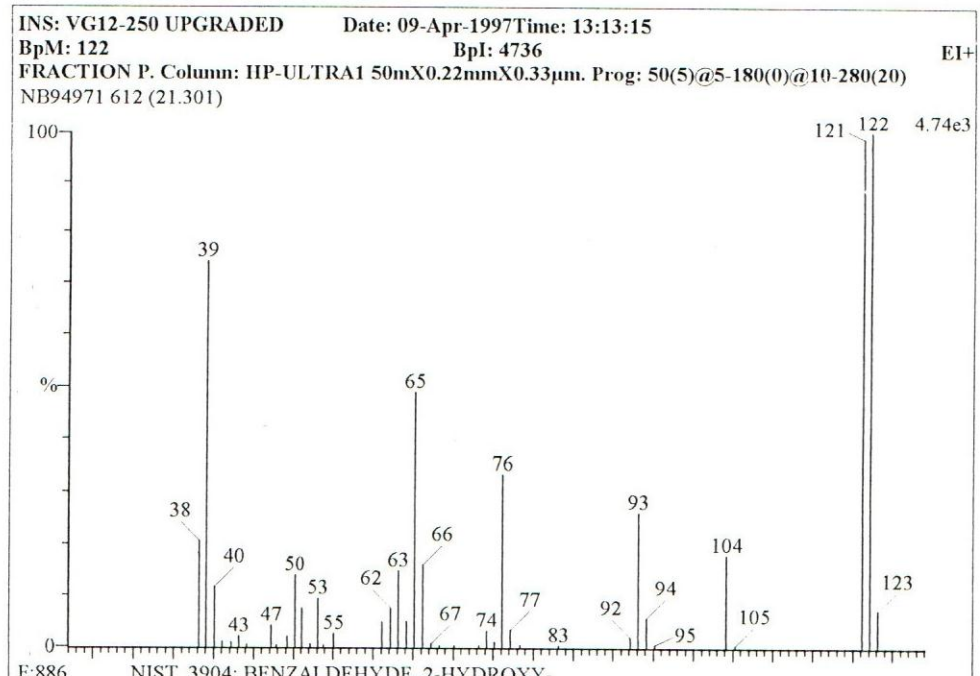
(b)

Mass spectra of (a) compound B from Fraction P

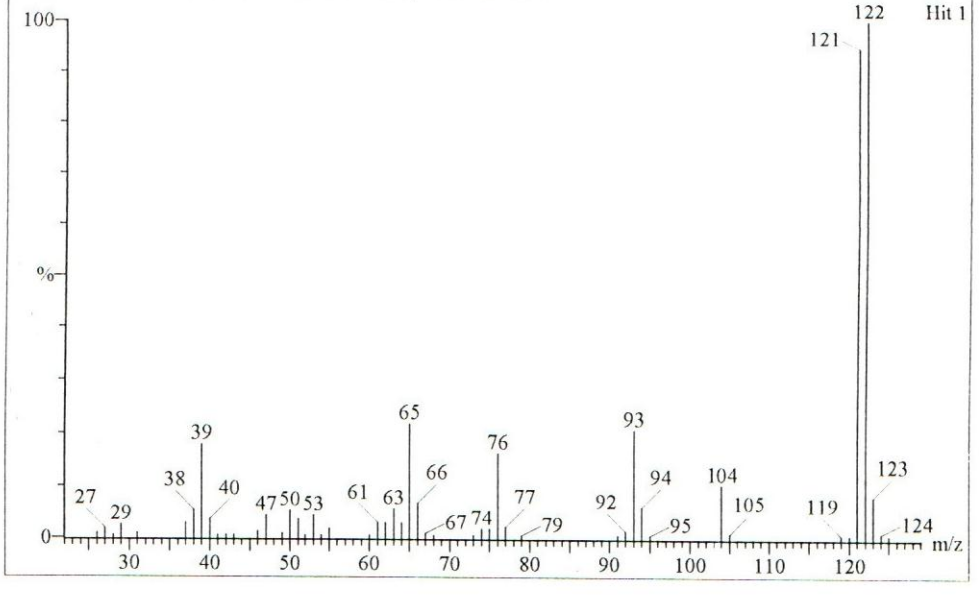
(b) authentic phenol

APPENDIX 3

(a)



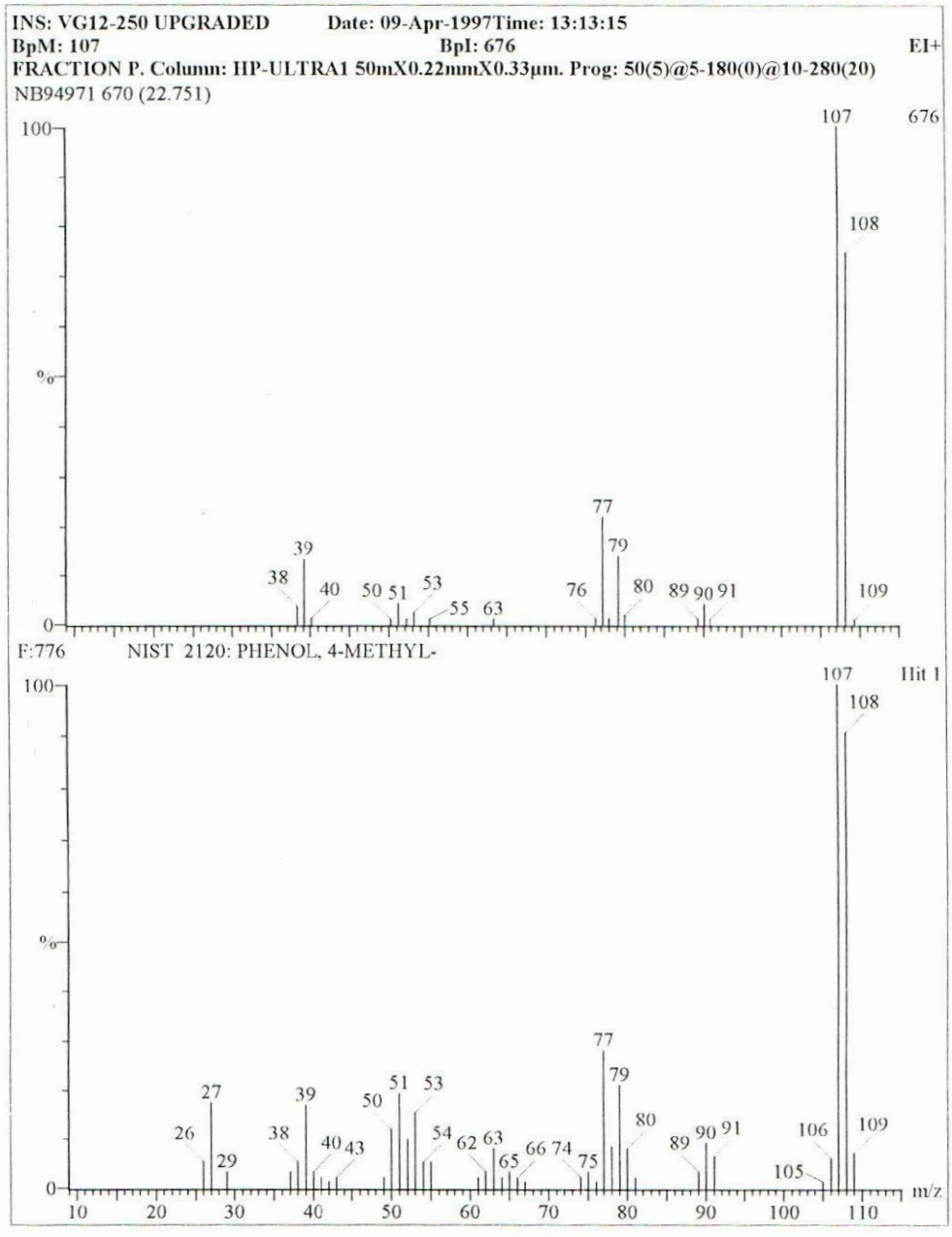
(b)



Mass spectra of (a) compound C from Fraction P

(b) authentic 2-hydroxybenzaldehyde

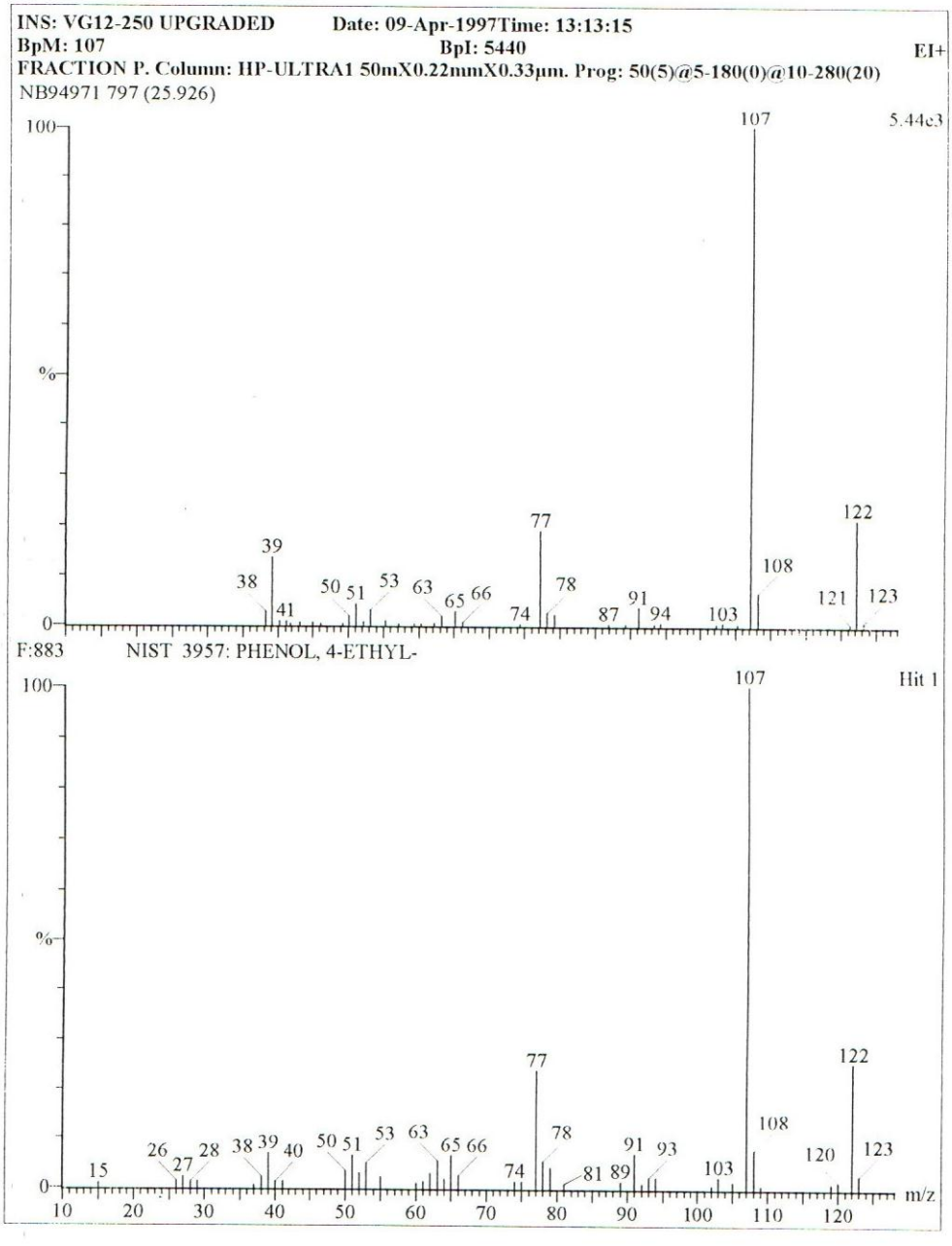
APPENDIX 4



Mass spectra of (a) compound D from Fraction P

(b) authentic 4-methylphenol

APPENDIX 5



(a)

(b)

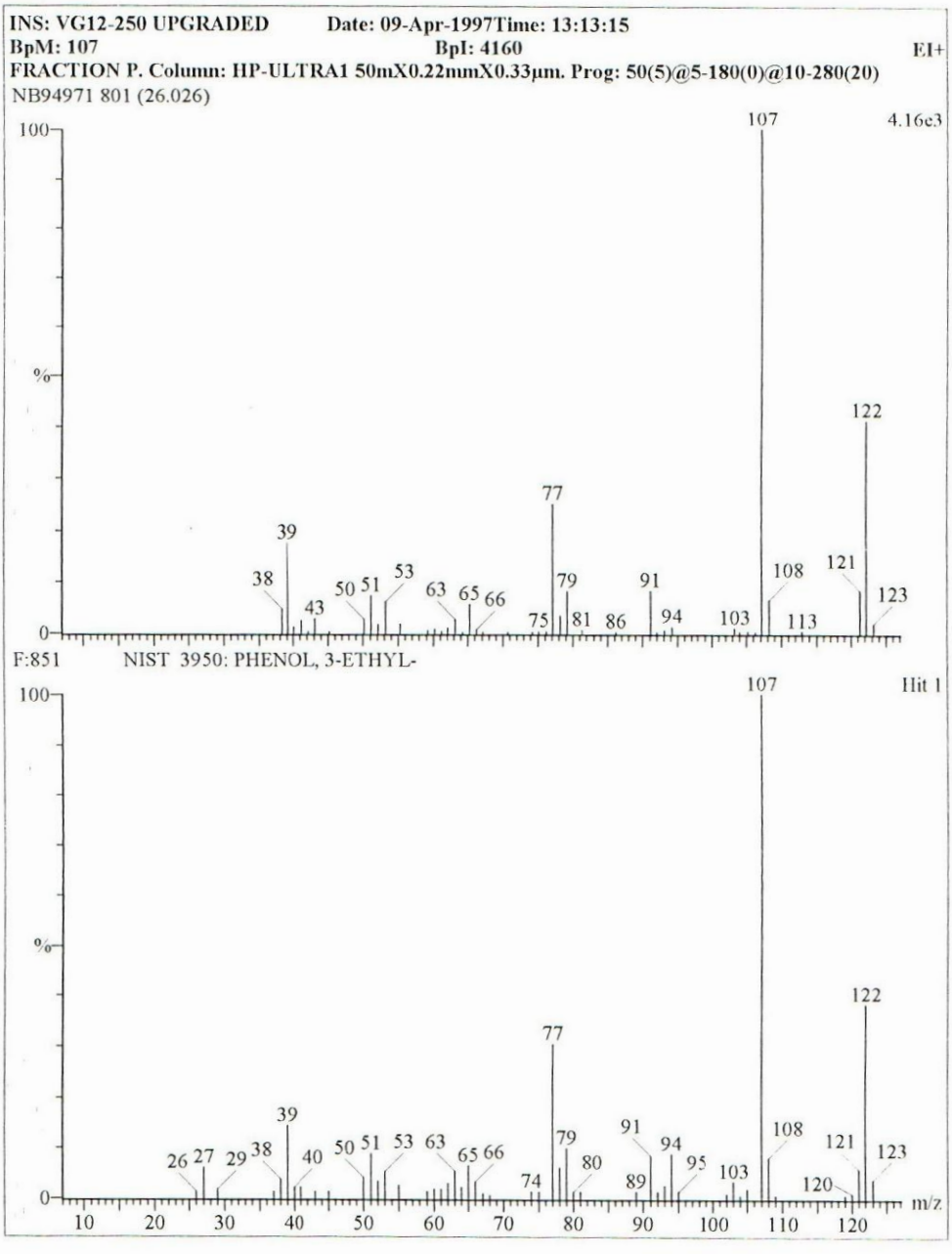
Mass spectra of (a) compound E from Fraction P

(b) authentic 4-ethylphenol

APPENDIX 6

(a)

(b)



Mass spectra of (a) compound F from Fraction P

(b) authentic 3-ethylphenol

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