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IDENTIFICATION OF SEMIOCHEMICALS FOR THE CONTROL OF THE WEST AFRICAN COCOA MEALYBUG

By

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DECLARATION

I, Tagbor, Phebe do hereby declare that except for references made to other people's work, for which I have acknowledged, this document is the product of my own research carried out at the Department of Chemistry, University of Ghana, Legon, Accra – Ghana and the Cocoa Research Institute of Ghana, New Tafo – Akim, Eastern Region, Ghana, under the supervision of Dr. Enock Dankyi and Dr Akua Konadu Antwi-Agyakwa.

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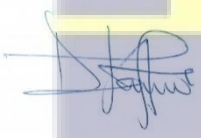

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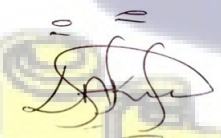

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ABSTRACT

Cocoa swollen shoot virus disease (CSSVD) continues to be a huge challenge undermining the full potential of cocoa production in Ghana, with its main host transmitters being mealybugs (Hemiptera: Pseudococcidae). Control measures using conventional spraying regimes have proven ineffective due to ecological and physiological factors, while the use of systemic pesticides face sustainability limitation including high cost, phytotoxicity, residue effects and consumers demand for organic farming practices.

To address this, Integrated Pest Management, by the use of natural enemies, specifically parasitoids wasps and predatory flies, that have been identified in colonies of cocoa mealybugs are being explored. However, little is known about the role of semiochemicals from these organisms in mediating behaviours such as locating mates, food sourcing, courtship and oviposition.

This study aimed to identify potential semiochemicals influencing the behaviour of natural enemies. Solvent extracts from selected wasp parasitoids and midge predators were tested for their activity in behavioural assays and their chemical compositions were analysed using Gas Chromatography Mass Spectrometry (GC-MS). The results revealed a diverse array of compounds with oxygenated functionalities including alcohols, aldehydes, etc., as well as long-chain hydrocarbons. Three key compounds detected with previously reported pheromonal activity are two methyl-branched alkanes; 2-methylhexacosane and 2-methylnonacosane and a C2 keto compound. Additionally, plant volatiles and mealybugs-derived compounds were found to exhibit kairomonal activity.

The results demonstrate that there is both pheromonal and kairomonal activity in freshly emerged parasitoids and parasites (1-2 days old). These findings highlight the potential of developing semiochemical blends to attract and enhance natural enemy populations in cocoa agroecosystems. This would suppress mealybug pests and thus reduce the spread of CSSVD.

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LIST OF ABBREVIATIONS

m/z	mass-to-charge ratio
VOC(s)	Volatile Organic Compound(s)
CSSV(D)	Cocoa Swollen Shoot Virus (Disease)
HIPV(s)	Herbivore-Induced Plant Volatile(s)
IPM	Integrated Pest Management
CMB	Citrus Mealybug
MB	Mealybug
DDT	dichlorodiphenyltrichloroethane
EO(s)	Essential Oil(s)
HS	headspace (sampling)
GLV(s)	Green Leaf Volatile(s)
SPME	Solid-Phase Microextraction
GC	Gas Chromatography
MS	Mass Spectrometry
CHC(s)	Cuticular Hydrocarbon(s)
EAG	Electroantennography
CoSp	*Coccodiplosis coffeae*
AnSp	*Anagyrus beneficans*
LeSp	*Leptomastix dactylopii*
RI	retention index
ng	nanogram
pg	picogram

CHAPTER ONE

1.0 INTRODUCTION

1.1 SEMIOCHEMICALS

Scientists have long discovered and established that most ecological processes and interactions are chemically-mediated (Barbosa-Cornelio et al., 2019). It is known that volatile organic compounds (VOCs) play a role in how insects interact with plants and other living organisms (insect hosts, predators, and parasites) (Foti et al., 2016). Volatile organic compounds are released by a wide variety of aquatic and terrestrial taxa and are important semiochemicals.

The word Semiochemicals is of Greek origin "semeon" which means, a signal. These chemicals mediate interactions between organisms ((Flint & Doane, 2023). A semiochemical is defined as a message-carrying chemical substance generated from a living thing or an artificial substitute of that substance that elicits a specific behaviour, in organisms of the same or other species (US EPA BRT, 2002). Thus, they are also referred to as "infochemicals" (Boone et al., 2008). These chemical compounds serve the purpose of attractants (luring others to certain food source, a mate, route or to aggregate), deterrents, arrestment (e.g., flight arrestment), repellents, stimulants (e.g. feeding stimulant) among other behaviour-modifying descriptions. They are subdivided into two groups, allelochemical (interspecific/ across species interactions) and pheromones (intraspecific/within species interactions).

Allelochemicals are further categorised by functionality; kairomones (receiver beneficial), synomones (receiver-emitter beneficial), allomones (emitter beneficial), and apneumones (of non-biological origin) (Chauhan & Punia, 2022). Pheromones are usually sub-classified based on the interaction mediated, such as alarm, aggregation or sex pheromone.

The first observation of pheromones was in 1609 when mass bee stinging following a single bee's sting was observed. The stinging bee released a liquid which attracted and directed the attack of other bees towards it (Free & Simpson, 1968) . However, an insect pheromone was isolated and identified for the first time in 1959 by German scientists in the silkworm moth. Subsequently, hundreds, possibly thousands of insect pheromones have been detected by more advanced techniques (Flint & Doane, 2023).

Semiochemicals are produced in specific glands, have specific encoding effect and usually diversify as new species evolve. The pheromonal composition of closely related species tend to be biosynthetically related in spite of variations in makeup. The biosynthesis routes of insect pheromones are variants of

conserved pathways that result in primary and secondary metabolites (Ruther et al., 2019). A vast spectrum of pheromones has been formulated and are utilised by numerous of insects from various orders. High concentrations of pheromones in the air can disrupt mating by making it difficult for the male pests to locate females, thus aiding pest population control (Chauhan & Punia, 2022). Kairomones and synomones are particularly known to increase the efficacy of natural enemies' activities on their host insect pests.

Pheromones, sex pheromones are a common component of several agricultural Integrated Pest Management (IPM) programs (Stenberg, 2017). They fall under the biochemical pesticides which have over the last few years, attracted global attention as a safer pest control strategy as compared to synthetic chemical pesticides (Ndolo et al., 2019). Currently, the most widely used (approximately 75% of all biopesticides) biochemical pesticides consists of *Bacillus thuringiensis* (Bt-) based products, which is witnessing emerging resistance (Olson, 2015). Despite the smaller proportion they consist of in the biochemical pesticide market, pheromones are applied to an estimated one million acres globally annually to mitigate insect pest damage (Biopesticide Industry Aliance, n.d.). In mid-2002, the Environmental Protection Agency (EPA) reportedly registered 36 pheromones, which included more than 200 distinct items (Ware & Whitacre, 2004). As of 2008, the EU had 77 active substances registered as bio-pesticides, while 279 were registered in the United States (Biopesticide Industry Aliance, n.d.)

The use of semiochemicals as biochemical pesticides in pest management can be beneficial in several ways. Firstly, due to their high species and strain specificity, they have little impact on non-target organisms. More so, they have low to no toxicity, do not bio-accumulate and are generally safer to handle. These are helpful in maintaining ecological balance. They can decrease the use of insecticides, serving as non-toxic alternatives to conventional ones. Evidently, semiochemicals incorporate many green chemistry principles (Chauhan & Punia, 2022). They are eco-friendly, presenting low toxicity for the environment and users and degrade quickly. This is in line with the green chemistry principles of designing safer chemicals and design for degradation.

Plants can also release herbivore-induced plant volatiles (HIPVs). These are indirect plant defences that actively lower the population of feeding herbivores by inviting their adversary parasitoids or predators (Arimura et al., 2005).

Thus, semiochemicals are an integral IPM component in controlling insect pests. This work aims at applying this success in biological management of the elusive mealybugs of cocoa.

1.2. THE COCOA MEALYBUGS

The soft-bodied, polyphagous, sap-sucking insect pests known as mealybugs (Hemiptera: Pseudococcidae) have a worldwide diversity of around 2000 species, and are classified as scale insects (Hemiptera: Coccoidea) (Subramanian et al., 2021). They are referred to as "mealybugs" because they secrete mealy wax all over their bodies (Subramanian et al., 2021). Mealybugs are phloem feeders (Subramanian et al., 2021). They use their piercing-sucking mouth parts feed to extract plant fluids inside the plants' vascular tissue. Their distinctive leaf-curling damage may be caused by calcium extraction connected to salivary pectin esterases' degradation of pectin (Subramanian et al., 2021). Stunting, leaf yellowing, leaf drop, an increased chance of pathogenic infection, and occasionally death are the outcomes of direct plant injury. The emission of honeydew, a transparent, sticky liquid that acts as a growing medium for black sooty mould fungi and can hinder plant photosynthesis, is the source of indirect damage to plants. Ants also use honeydew as a food supplement (Willmott, 2012). There are more than 160 mealybug species that are designated pests of crops (Subramanian et al., 2021). They are considered global invasive pests because of their rapid spread, broader distribution their polyphagous nature.

Several mealybug pests also serve as carriers for multiple virus infections (Subramanian et al., 2021). This is the case of the West African Cocoa mealybug, *Formicococcus njalensis* (Laing) (Figure 1) and the Citrus mealybug, *Planococcus citri* Risso, implicated as Cocoa Swollen Shoot Virus Disease (CSSVD) vectors in Ghana. The CSSVD was initially identified in Ghana's Eastern Region in 1936 (Andres et al., 2017). *Formicococcus njalensis* (Laing) and *P. citri* (Risso) accounted for around 90 percent of the mealybug population on cocoa. The mealybugs gain the virus by feeding on any part of infected cocoa trees. The now vectoring mealybugs feed in interconnecting branches of nearby cocoa trees as they move, get blown by wind or conveyed by attendant ants (*Crematogaster* spp. and *Camponotus* spp.), causing an outbreak far from the origin of infection (Ameyaw et al., 2016; Domfeh et al., 2016). Red vein banding, leaf chlorosis, stem swellings, decreased growth and vigour, branch dieback, and plant mortality are typical signs of CSSVD. If viruliferous mealybugs do not encounter another infected plant within 48 hours of contracting the virus, they lose ability to spread CSSV to nearby cocoa (Domfeh et al., 2016).

Figure 1: Colonies of Female Formicococcus njalensis mealybugs on cocoa pods

1.3. MEALYBUGS AND THEIR CONTROL

Mealybug populations are challenging to control because of biological and behavioural traits. Above the cuticle, they have a thicker, waxy waterproof layer that keeps most insecticides from penetrating (Ameyaw et al., 2016; Willmott, 2012). In addition, mealybugs tend to group together and settle in tree bark cracks and fissures, and under fruit sepals, and leaf sheaths (Ameyaw et al., 2016; Andres et al., 2017; Subramanian et al., 2021). These prevent insecticides from fully contacting the mealybug pest (Avila et al., 2023). Ants protect and tend mealybugs, disrupting biological control by predators and parasitoids (Ameyaw et al., 2016; Bostanian et al., 2012; Willmott, 2012) as well as chemical control (spraying of conventional pesticides), by sheltering them in mud tents (Ameyaw et al., 2016) or moving them away from poisoned location to prevent their feeding. The ants in exchange, get honey dew from the mealybugs (Ameyaw et al., 2014).

The efficacy of insecticides on mealybugs has been found to be varying, especially for *Formicococcus njalensis*. Thus, no practically feasible method for farmers is available (Andres et al., 2017). Systemic insecticides are commonly used to control hemipteran insect pests' populations (Willmott, 2012). They are absorbed by the plant and then translocated in the vascular system. Xylem and phloem feeding insect pests which include mealybugs would therefore be poisoned by them. They can be applied to soil or the growing medium in granular form or as foliar sprays or drenches (Cloyd et al., 2011) or much recently onto seeds. They are good for protecting new growth that would be omitted after applying contact

insecticides. This trait ensures long-lasting protection making them suitable for use against cryptic pests (Willmott, 2012).

Willmott (2012) studied the efficacy of some systemic insecticides on Citrus mealybugs (CMB) greenhouse set-ups in Kansas, USA. Results indicated minimal CMB mortality. Azadirachtin, spirotetramat, and neonicotinoid insecticides (imidacloprid, dinotefuran, and thiamethoxam) at the recommended and double the recommended dosages were unable to effectively control CMB under greenhouse conditions. Similarly, in Ghana, insecticides introduced from the 1940s (nicotine sulphate and dichlorodiphenyltrichloroethane (DDT), endrin, dieldrin, heptachlor and Lindane) aimed at controlling cocoa insect pests including mealybugs (*F. njalensis* (Laing), *P. citri* (Risso) and *Ferrisia virgata* had little success. The use of systemic, mainly organophosphate insecticides such as Monocrotophos SC was a more convenient method but suffered a setback when it caused tainting of beans (Adu-Acheampong et al., 2015).

During 1948-55, some 14 parasites and 7 predator species of *F. njalensis* were released in West Africa (Herren et al., 1983). This biological control program for *F. njalensis* was said to have failed. Failure was attributed to ant interference and also to the fact that *F. njalensis* is a native species on which most of the introduced natural enemies did not develop (Herren et al., 1983). Ackonor, (2001) recovered some natural enemies of *F. njalensis* and *P. citri* from farm-obtained colonies. Their Eastern Region study sought for populations of mealybugs and their natural enemies in farms run by researchers (CRIG) and farms run by farmers. Depending on the mirid season, either propoxur or lindane was sprayed on the CRIG fields while the peasant farmers sprayed unspecified dosages of any available insecticide. They found that the insect populations in both farming systems did not differ significantly. Thus, the mealybugs and their natural enemies survived in spite of toxic insecticide applications. This however is insufficient information to conclude that natural enemies of mealybugs are not negatively impacted by the insecticides used, as the paper appears to imply. There could be other factors accounting for their survival.

A review by Avila et al., (2023) located 14 scholarly publications describing the insecticidal effects of essential oils (EOs) on mealybugs. The findings showed that 13 of the 24 EO genera examined were efficacious against mealybugs. Although indirect contact and fumigation were the most commonly employed techniques, all were successful. A more uniform distribution of EOs is made possible via fumigation. Typical contact insecticide treatments offer poor effectiveness, whereas essential oils can penetrate those insects' waxy covering because of their lipophilicity.

In order to attract males for mating, females of many mealybug species generate pheromones from scent glands in the abdominal region, a phenomenon which has been leveraged for the development of pheromones for effective control and monitoring. The natural enemies of mealybug are also drawn to herbivore-induced plant volatiles (HIPVs), and this occurrence could be applied for conservative biological control (Subramanian et al., 2021).

1.4 THE PESTICIDE PROBLEM AND INTEGRATED PEST MANAGEMENT (IPM); THE CASE FOR BIOLOGICAL CONTROL

Chemical control remains the dominant strategy for insect pest control although it is well appreciated that for the most part, chemical insecticides only provide short-term solutions for pest issues (DeBach & Rosen, 1991). There has been increasing concern about chemical pesticidal toxicity as a major environmental problem as well as toxicity in humans and many other non-target organisms (Aminu et al., 2020; Antwi & Reddy, 2015; Asogwa et al., n.d.; Gunasekara et al., 2007; Miyamoto & Katagi, 2011; Rico-Martínez et al., 2022). There has been reported resurgences and upsets of natural balance of organisms described correlated with the use of chemical pesticides (Bostanian et al., 2012; DeBach & Rosen, 1991).

However, there is an array of biological, chemical, mechanical, cultural and autocidal strategies for effective pest control in modern agro-ecosystems called Integrated Pest Management (IPM) (Flint & Doane, 2023). Integrated pest management (IPM) is based on the understanding that there is no one-size-fits-all method of controlling pests and that the optimum crop protection can be achieved by combining different strategies and practices, grounded in sound ecological principles (Flint & Doane, 2023).

Of all the available strategies, biological control using natural enemies is the most effective and promising solution to the solo recourse to synthetic pesticides (Huffaker & Dahlsten, 1999; Reddy et al., 2020; Rico-Martínez et al., 2022). The modern history of biological control can be dated from the successful management of the cottony cushion scale (*Icerya purchasi*) with introduced natural enemies on citrus in California in 1888 (DeBach & Rosen, 1991). Around 1946-1947, the widespread use of DDT, parathion and related compounds caused the virtual extinction of vedalia beetle (*Rodolia cardinalis*), the natural enemy of the citrus scale insect pests and an unbelievable population spike of several species of the pest. In order to re-attain control as rapidly as possible, growers paid $1.00 each for the vedalia beetle (Grafton-Cardwell, 1999). Ever since 1888, hundreds of biological control projects have been successfully carried

out in numerous locations. Areas included Texas in 1957, California (1940) and South Africa (1913), later Bedford, 1976, Israel in the 1990s, and many other documented occurrences across the globe in a wide array of crops including cocoa (DeBach & Rosen, 1991). DeBach & Rosen, (1991) argue that ill-effects on non-target organisms and quick recovery of target pests will be automatically resolved if pesticide use is drastically reduced by conserving and utilising natural enemies and other non-chemical techniques of control.

When biological control is viewed from an ecological standpoint, it is the process by which parasites, predators or pathogens keep another organism's average population density lower than it would be in their absence (DeBach, 1964). Its efficacy has already been demonstrated in several species (Huffaker & Dahlsten, 1999).

Semiochemicals can improve the presence and effect of biological control agents in pest management. Embedded lures are being used to improve biological control techniques by attracting and keeping of the required natural enemies (Ayelo et al., 2021). Large volumes of various herbivore-induced plant volatiles (HIPVs) are released by plants in response to herbivore attacks, attracting arthropod predators and parasitoids of the herbivore pest (Xiu et al., 2019). Letourneau & Altieri (1999) identify one habitat management technique to increase parasitism of crop pests as applying diatomaceous earth or fake eggs laced with kairomones to simulate high pest densities.

Typical examples of this pest control strategy include but are not limited to the following; the use of hydrocarbons (such as tricosane) found in extracts of moth scales (*Heliothis zea*) increased the efficiency of their location by parasitoids, thus increasing their parasitism in the field (Ayelo et al., 2021); The invasive papaya mealybug, *Paracoccus marginatus* were successfully suppressed by natural enemies particularly, *Acerophagus papayae* (Mani et al., 2012). Several accounts of plant volatiles increasing parasitism are also given, such as *Hippodamia variegata*, a key biological control agent for aphids in cotton-planting in China (Jiang et al., 2023); *Encarsia formosa* for Whitefly (*Bemisia tabaci*) pest of the Chinese broccoli (Li et al., 2014), among others.

1.4.1 Problem statement and justification

It is generally reported that the numbers of natural enemies in the cocoa ecosystem is low (Ackonor, 2002) with several explanations such as frequent use of chemical pesticides, availability of alternate

hosts, absence of shade (Ackonor, 2001; Ambele et al., 2023; Daghela Bisseleua et al., 2013). Thus, parasitism rates are generally low.

Some studies have investigated the biology of some natural enemies of the West African Cocoa Mealybugs. They include parasitic wasps' species from *Leptomastix* and *Anagyrus* genera, *Aenasius abengouroui*, the gall midge *Coccodiplosis coffeae*, beetle species of *Hyperaspis* and *Scymnus* among other uncommon or unidentified species. These insects occur all year round and so far have provided parasitism levels of not more than 5.2% (Donald, 1956; Entwistle, 1972).

Both the developmental and adult stages of these natural enemies feed on mealybug species. Parasitic flies and wasps and predator beetles depend on chemical cues to locate herbivores as host and oviposition sites. In the cocoa ecosystem however, this chemical ecology has not yet been explored. Unearthing the chemical ecology and chemical cues mediating interactions and activities of the natural enemies of the West African cocoa mealybugs would provide useful puzzle pieces to recruit and increase their populations, subsequently improving their effect on the mealybugs.

1.4.2 Aims and Objectives

This study seeks to determine Volatile Organic Compounds (VOCs) involved in the ecology of mealybugs and their natural enemies, *Anagyrus* spp., *Leptomastix* spp., and *Coccodiplosis coffeae*.

The specific objectives are;

i. To determine the role of volatiles and extracts of pest and host plant in natural enemy attraction.
ii. To analyse and compare VOCs to identify potential compounds that serve as cues for natural enemies.
iii. To identify bioactive compounds that mediate attraction in natural enemies of mealybug.

Semiochemicals identified would be systematically exploited for the development and implementation of IPM strategies for mealybug control in the cocoa agro-ecosystem.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Chemical communication in insects

Integrated Pest Management (IPM) is now widely practiced in many agricultural pest control systems. Among established strategies, biological control by the promotion, conservation and augmentation of natural enemies are said to be the most agriculturally sustainable (Letourneau & Altieri, 1999) and self-sustaining (Huffaker & Dahlsten, 1999). One mechanisms being explored to enhance the activity of these natural enemies to successfully suppress pests usually is identification of semiochemicals at play in the insects' interactions (Francke & Schulz, 2010). Successes have been reported in environmental manipulation by the use of semiochemicals. Semiochemicals are included in IPM programs in various ways including but not limited to monitoring, disruption, mass trapping, attract-and-kill, and push-pull tactics (El-Ghany, 2019).

Semiochemicals are a type of natural products, secondary metabolites, usually volatile organic compounds, that perform specific functions. They are chemical signals that can transmit data between organisms of different species (allelochemicals) or between members of the same species (pheromones) (Chauhan & Punia, 2022). El-Shafie et al., (2017) highlights the complexities involved in the studies to identify and develop semiochemical formulations for IPM from the aggregating semiochemical compound of red palm weevil (RPW) *Rhynchophorus ferrugineus*. An interesting study by Frago et al. (2017) demonstrated that when there is a chemical attenuation (by microbial activities) in certain wasp parasitoids attraction, the level of their attacks drops. These few examples indicate the extensive impact of semiochemicals and its implications for species population control. Modern techniques involving chemical analytical and ecological study are employed from laboratory identification to their field applications in these studies (Chauhan & Punia, 2022 and Flint & Doane, 2023).

2.1.1 Pheromone

The use of semiochemicals, including pheromones, is still a developing area of science. However, many insect Integrated Pest Management techniques frequently use pheromones (Flint & Doane, 2023). These compounds are secondary metabolites produced in specialized glands or tissues of the insect through conserved biosynthetic pathways, dependent on the taxon of origin, and are used in intraspecific communications (Stökl et al., 2014; Ruther et al., 2019).

Several reports for virtually all insect taxonomic families have been made, on a wide variety of compounds, through meticulous chemical and electrophysiological studies (Guo et al., 2022).

Peculiarities exist in biological production and propagation; example, some parasitoids upon emergence do not have pheromone in their gland, but titres rise within a few days. Some scale insects cease production of pheromones after some activity, e.g., mating. They may be laid on a solid substrate or released as aerial plumes (Kost, 2008). Discontinuous trails may function as odour beacons and are thought to help three-dimensional orientation of comrades (Kost, 2008). (Keeling et al., 2004) described twelve functional categories of response to a pheromone:

1. Alarm; alerts species or colony members of imminent danger
2. Attraction; helps to attract a suitable mate
3. Recruitment; attracting species mates to a food source or new nest site
4. Grooming; indicating the presence of an "exciting" odour, also a response of worker bees to their queen, or a parent to its egg or offspring.
5. Trophallaxis; oral and anal liquid exchange for sharing of nutrients and other resources
6. Solid food particle exchange
7. Group effect: facilitating or inhibiting a specific activity to complete a larger complex task
8. Recognition of nest mates, members of specific castes, discriminating injured from dead individuals
9. Caste determination by stimulation or inhibition
10. Management of rival reproductives for example inhibiting ovary development in workers to ensure the queens survival in bees.
11. Nest markers, home range signals, and territorial signals
12. Sexual communication, includes synchronization of sexual activity, sex and species recognition, and evaluation during sexual competition.

Insect pheromones may be a single compound or a blend of several compounds occurring in ratios that are species-specific (Schulz and Francke, 2010). For instance, the sex pheromone of female apple leaf miner, *Lyonetia prunifoliella,* in Korea are three, namely, 10,14-dimethyl-1octadecene (M25), 5,9-dimethyloctadecane and 5,9-dimethylheptadecane. In North America, the main compound, 10,14-dimethyl-1octadecene is a singular component pheromone of the same moths. A 100:5 mixture of (Z)-6-henicosen-11-one and (Z)-6-henicosen-9-one (A184) is used by *Orgyia thyellina* moth while *Orgyia leucostigma* moth uses the single component (6Z,9Z)-6,9-henicosadien-11-one as pheromone. Evolutionary factors are responsible for the difference in numbers of components of various taxa (Kost,

2008).

Multiple species within a single genus may utilise distinct enantiomers (Kost, 2008). For example, all *Nasonia* species pheromones contain (4R, 5S)-5-hydroxy-4-decanolide (RS) as a primary constituent, while only male *Nasonia vitripennis* produce its epimer, (4R, 5R)-5-hydroxy-4-decanolide (RR). Short chain dehydrogenases/reductases convert RS to RR (Niehuis et al., 2013; Ruther et al., 2019).

Competition for communication channels within a taxon is avoided by the derivation of new components from the chemical modification of existing ones and adding to the blend or slightly altering the ratios (Kost, 2008). These may occur by enzymatic acetylation, epimerization, decarboxylation, reduction or oxidation. Hence, compounds used by closely related species are usually similar in composition, with no switch in production pathways (Boddum, 2013). For instance, almost all known female sex pheromones in moths are fatty acid derivatives that are either created by modifying polyunsaturated fatty acids in their diet (Type II pheromones) or synthesized de novo using acetyl-CoA units (Type I pheromones) (Ando et al., 2004). Type I pheromones are biosynthesized de novo from acetate as building blocks without a direct connection to dietary material (Miller et al., 1976). They comprise of C10-C18 alcohols, aldehydes and esters (Francke & Schulz, 2010). Type II pheromones are linked to diet, i.e. a direct utilization and simple conversion of the diet (Hendry, 1976) and are usually hydrocarbons or epoxides (Francke & Schulz, 2010). The mevalonate pathway is used to synthesis isoprenoids, which make up the great majority of aggregation pheromones in bark beetles as well. Many species of bark beetles from various genera share many of these chemicals (Blomquist et al., 2012; El-Sayed, 2018). In Ruther et al. (2019), it was demonstrated that an insect of the *Urolepis* genus, which is closely associated to the *Nasonia* group produced sex pheromone in the same gland as *Nasonia* species. However, while *Nasonia* species studied so far, produce fatty acid-derived hydroxylactones, *U. rufipes* produced, a monoterpenoid derived from the mevalonate pathway. This suggested "a biosynthetic switch between the fatty acid and isoprenoid metabolism in the *Nasonia* group". This agrees with (Kost, 2008) that different organisms may use completely different molecules to convey qualitatively similar message.

2.1.2 Allelochemicals

The semiochemicals involved in interspecific chemical communication are known as allelochemicals (Reddy et al., 2020). These are pheromones which are produced by one species and are exploited by other organisms (Stenberg, 2017) thus acting as allelochemicals. If for instance, predators

and parasitoids use the pheromones of prey herbivore to locate them, mass release of herbivores' sex pheromones for biological control would be explored as an allelochemical rather than a mating disruptor.

The first verified allelochemical was juglone, a 1,4-naphthoquinone, from the black walnut, *Juglans nigra* (Soderquist, 1973). It was said to produce allelopathic interference with understory plants nearby (Chui-Hua et al., 2019).

They are classified into kairomones, allomones, synomones and apneumones. This classification is based on a cost–benefit framework (Kost, 2008) or functional role (Bakthavatsalam, 2016).

A kairomone benefits the receiver only; it is actively or passively released by a prey organism and is used by a predator to localize its prey (Bakthavatsalam, 2016; Kost, 2008). The same compound or component of release can serve multiple purposes; hence kairomones are simply originated from pheromones of the emitter and are learned by the receiver. For example, aphid alarm pheromones have two known biological effects: (i) disrupting aphid feeding on their host plants, and (ii) acting as kairomones, attracting natural enemies of the aphids (Stenberg, 2017).The alarm pheromone 6-methyl-5-hepten-2-one of the meat ant *Iridomyrmex purpureus*, for example, is used by the ant-feeding spider *Habronestes bradleyi* to locate its prey (Kost, 2008). Thus, the compound(s) that serves in one species as a priming pheromone acts at the same time as a kairomone in another one (Kost, 2008).

Allomones benefit the emitter only; for example, it can be used by a species to deter a predator (Bakthavatsalam, 2016; Kost, 2008). Synomones benefit both sender and receiver. A classic example of synomones is floral odours involved in pollinator attraction: the pollinator benefits from the nectar – pollen and the plant benefits from the transfer of pollen (Bakthavatsalam, 2016; Kost, 2008), resulting in fertilization. Occasionally, associated microorganisms like fungi and bacteria may be involved in these volatiles production rather than the organism in question (Frago et al., 2017). Volatiles from host plants may also combine with pheromones to serve an attractive or other type of effect. In bark beetles (Scolytidae), for example, a mixture of host-tree's herbivore-defence monoterpene, myrcene, with female sex pheromone (þ)-exo-brevicomin and male pheromone ()-frontalin combine to produce a highly attractive aggregation pheromone that attracts both sexes in high numbers (Schlyter & Birgersson, 1999).

Allelochemicals have also been implicated in IPM programs.

2.2 Sources of Semiochemicals

2.2.1 Headspace volatiles

The vapor above a condensed phase or surrounding a sample or organism is known as the headspace (HS) (Barbosa-Cornelio et al., 2019). Headspace contains the volatiles released by the sample or organism itself (Barbosa-Cornelio et al., 2019) in nature. They are found in aerial emissions as trails. Aerial trails are known from a number of flying insects from the orders Orthoptera, Homoptera, Coleoptera, Mecoptera, Lepidoptera, Diptera, and Hymenoptera, where they often function as sex attractant or aggregation pheromone (Kost, 2008). These have been sampled and analysed in various studies to provide insights into their composition and activity.

(Censier et al., 2014) investigated volatile compounds emitted by live *H. marginata* females by Solid Phase Micro-Extraction coupled to Gas Spectrometry coupled to Mass Spectrometry (SPME-GC-MS). The major pheromone was identified as 1-methyloctyl butanoate (non-2-yl butanoate). The sex pheromone component of the Japanese mealybug, *Planococcus kraunhiae*, was obtained by (Sugie et al., 2008), using HS sampling. The chemical structure was determined to be 2-isopropyliden-5methyl-4-hexen-1-yl butyrate by GC-MS and NMR analyses. The sex pheromone of the longtailed mealybug, identified as 2-(1,5,5-trimethylcyclopent-2-en-1-yl) ethyl acetate, a monoterpenoid was also studied by Millar et al. (2009), using HS sampling.

An example of HS sampling in plants is volatiles from lima bean leaves infested with the two-spotted spider mite *Tetranychus urticae* reported to be attractive to the predatory mite *Phytoseiulus persimilis*. Those prey-induced volatiles were identified as linalool, methyl salicylate (53), (E)-ß-ocimene and DMNT (Honda et al., 2013).

2.2.1.1. Constitutive volatiles

Volatile organic compounds emitted from plants while undamaged are referred to as Constitutive Volatile Organic Compounds (Ayelo et al., 2021). These emissions can be increased by external damage. Some insect species also produce and store pheromones in glands and tissues, as compared to being produced on demand. These are then stored as constitutive components of the organism. Thus, Constitutive volatiles are produced by both plants and animals, or other organism, in a stress-free environment. Alarm signals for example are not constitutive, but produced when the organism is stressed. In arthropods, constitutive volatiles are found in glands, tissues, on cuticles, etc., and are discussed in the subsequent paragraphs. In plants, constitutive volatile compounds are continuously released from leaves, flowers and

roots at low levels. Volatile compounds can also be released during fruit ripening as well. Plant volatiles usually contain green leaf volatiles (GLVs) such as C6 alcohols, aldehydes, esters, terpenoids and phenolics (Xiu et al., 2019; Zitzelsberger & Buchbauer, 2015)

2.2.1.2. Herbivore-Induced Plant Volatiles (HIPVS)

Plants perceive their environment and are sensitive to changes (Dias et al, 2016). Herbivores are said to increase or alter the blend of naturally released plant odour emissions compounds (constitutive volatiles) (T. C. J. Turlings et al., 1991). Damaged plants emit a foliar volatile profile comprised of compounds that are constitutively present or are in response to insect feeding, acting as cues for the herbivore's natural enemies (Dias et al., 2016; Zitzelsberger & Buchbauer, 2015). This strategy to call on enemies of their enemies when their first line of defence (toxic metabolites such as alkaloids) is not effective is common in plants. Therefore, herbivore-induced plant volatiles (HIPVs) comprise part of the indirect defence of the plant, which can boost natural enemy incidence on it (Dias et al., 2016). Volatiles may also trigger neighbouring plants to enhance their defence mechanisms (Kost, 2008). Response-induced volatiles are a consequence of the rupture of cells; thus, oviposition also induces volatiles and other changes in plant metabolites (Zitzelsberger & Buchbauer, 2015). Deposition of insect eggs induces plant volatiles that attract egg parasitoids in elm, pine and beans (Honda et al., 2013).

The process of host/prey location begins with the location of the correct habitat, followed by location of the host/prey. Kairomones are important for shorter-range location and acceptance of the host/prey, while longer-range location is accomplished by using cues associated with the habitat in general (light, colours, temperature humidity) than with the host/prey itself (kairomonal cues) (Kost, 2008). Herbivore-induced plant volatiles (HIPVs) mostly function as kairomones, mediating attraction of feeding and ovipositing herbivores, as well as allomones repelling the herbivores, or attracting herbivore's natural enemies or pollinators (Boddum, 2013).

Herbivore-induced plant volatiles emissions occur within hours to days after damage (Zitzelsberger & Buchbauer, 2015). Within artificial set-ups, attraction of carnivorous arthropods such as predators and parasitoids to HIPVs are initiated within hours (Zitzelsberger & Buchbauer, 2015). Herbivore-induced plant volatiles are released after metabolic changes in plants caused by mechanical injury of the herbivore, along with saliva coming into contact with the injured plant tissues (Hilker & Meiners, 2010). Variations in feeding mode and saliva constitution ensure highly specific metabolic changes, resulting in HIPVs signal that act as fingerprint for each damage type (Arimura et al., 2005). It can thus be said that

plants recognize their enemies with the help of compounds in their oral secretions and choose an appropriate behavioural pattern (Zitzelsberger & Buchbauer, 2015). This means HIPVs can selectively attract or repel a particular natural enemy (Weber et al., 2017). HIPVs can also attract nematodes and fungi (Xiu et al., 2019).

A Meta-analysis by Rowen & Kaplan (2016) established that HIPVs are tightly regulated and adapted depending on factors such as plant genotype, herbivore identity, and abiotic conditions. Therefore, phytohormonal signalling mechanisms would be shared among plant families, eliciting the same volatiles production pathways. The review moreover explained that different feeding behaviours elicit different quantities or qualities of plant emissions. Chewers are those who consume tissue whole, and consist mostly of defoliators like caterpillars and beetles. Sap-feeders have piercing-sucking mouthparts like true bugs, aphids and whiteflies and feed on fluids, primarily those traveling through the phloem and/or xylem. Cell-content feeders such as thrips and mites feed on mesophyll within plant cells. The reviewers, (Rowen & Kaplan, 2016), proved that chewing and cell-content feeding increased the total quantity of volatiles produced, as well as GLVs, monoterpenes and sesquiterpenes, while sap feeders induced fewer volatiles. Cell-content feeding also appeared to induce the highest benzenoid emissions, compared to sap-feeders arthropods (Rowen & Kaplan, 2016). In cotton, of the Malvaceae family, chewers were said to induce high concentrations of terpenes, while sap-feeding whiteflies induce very little (Rowen & Kaplan 2016). A notable find also was that certain less mobile sap feeders developed manipulative strategies to suppress plant responses, by remaining cryptic to the plant or through direct phytohormonal manipulation.

Knowledge about HIPVs can greatly improve biological control in agriculture. Crop loss might be reduced by natural enemies of herbivores, which follow plants' chemical cry for help (Zitzelsberger & Buchbauer, 2015). The manipulation of HIPVs in agricultural systems has been explicitly reviewed as a potential mechanism for enhancing biological control (Rowen & Kaplan, 2016).

(Stenberg, 2017) believes that combining pheromones with plant volatiles, which often have longer-ranges, would make for a more effective strategy as predators and parasitoids can quickly learn to disregard dishonest signals.

2.2.1.3. Microbe-induced volatiles

Some bacteria and fungi have symbiotic associations in the environment with plants or arthropods (Pineda et al., 2010). These serve their hosts various benefits such as nutrition, protection, inducing or

priming defences, etc. They as well emit volatile compounds which have semiochemical influence on certain predatory insects (Boone et al., 2008). Fungal symbionts of bark beetles have been shown to assist its parasitoids and dipteran predators (Honda et al., 2013). Females of *Lobesia botrana* can recognize the health status of their host plant; 3-Methyl-1-butanol, identified specifically from fungus-infected grapes, caused substantial reduction in both attraction to and oviposition on the host grape plant (Honda et al., 2013). (Schlyter & Birgersson, 1999) found that naturally stressed trees, infected by a fungal root pathogen had higher landing rates of and were often killed by attacking forest beetles. This could be mediated by chemical cues.

Mutualistic symbionts could affect the likelihood of their hosts' discovery in two ways. First, they might produce "semiochemicals" that attract natural enemies; For example, bark beetles carry symbiotic fungi which they use to digest wood, but the symbiont also releases volatiles that attract parasitic wasps (Boone et al., 2008). Secondly, they may interfere with the plant's ability to attract its herbivore's natural enemies, so benefiting its host (Dicke & Baldwin, 2010). (Frago et al., 2017) study shows that plants infested with aphids carrying the symbiont *Hamiltonella defensa* were less attractive to its parasitic wasp through changes in herbivore-induced plant volatiles.

This same relation has been detected for viruses, which are rather opportunistic than mutualists. Host plants VOCs induced by plant viruses, including terpenes, sesquiterpenes, green leaf volatiles (GLVs), fatty acid derivatives, aromatics, and nitrogen-containing compounds, as well as the volatile plant hormones, methyl salicylate, and methyl jasmonate have attractant, repellent, or no effects (Chang et al., 2023). Rice dwarf virus (RDV) infection of rice plants significantly induced the emission of (E)-β-caryophyllene and 2-heptanol, which influenced the olfactory behaviour of both non-viruliferous and viruliferous Green rice leafhoppers (GRLHs), and it's vector (Chang et al., 2023). The aphid parasitoid *Aphidius colemani* (Hymenoptera: Aphelinidae) known to have higher parasitism and survival rates on aphids fed on virus-infected than aphids fed on non-infected plants. This was determined to be mediated by VOCs by (Milonas et al., 2023).

2.2.2. Cuticular Hydrocarbons (CHCs)

Signals in ants, bees, wasps, or termites present a bouquet of complex multicomponent bouquets on the epicuticle, allowing easy detection upon direct contact or short-range olfaction. The insect cuticle is commonly covered by hydrocarbons, consisting most often of n-alkanes, methyl-branched alkanes, and

n-alkenes with various numbers of double bonds (Francke & Schulz, 2010). Typically, chain lengths of about 21 to 37 carbons, as well as more polar compounds such as long-chain fatty acids, alcohols, aldehydes, wax esters, and triacylglycerides are present. This lipid layer covers the insect exoskeleton as part of the protective layer of cuticular lipids and helps prevent desiccation. Many insects utilize these non-volatile compounds as short-range or contact pheromones, referred to as cuticular hydrocarbons, CHCs. These can be biosynthesized in glands such as exocrine glands and then redistributed by grooming or by epidermal cells. There is substantial body of literature on the semiochemical functions of CHCs including but not limited to gender determination, marking territories, maintenance of castes and kairomones (Kühbandner et al., 2013). Similar to VOCs, the CHC profiles of insects can range from simple mixtures to complex blends of over 100 substances (Kühbandner et al., 2013). Cuticular hydrocarbons providing chemical cues are common in several termite species (Costa-Leonardo & Haifig, 2010), parasitoids like *Urolepsis rufipes* (Würf et al., 2020), *Cotesia* spp. (Xu et al, 2020), *Dibrachys cavus* (Ruther et al., 2011a), etc. Young females of the alfalfa leaf-cutter bee *Megachile rotundata* attract males by cuticular alkenes, while fatty acids or alkanes that are also present on the cuticle proved to be inactive. Several other pheromonal functions of cuticular hydrocarbon mixtures have been described, for example, trail following by wasps (Francke & Schulz, 2010).

2.2.3. Glands

Several compounds have been extracted from various insect glands which had attractive quality and thus semiochemical property (Hall et al., 2012). Four out of the twenty-nine orders in the class Insecta use glandular tissue from nearly any anatomical location for the synthesis, accumulation, or release of sex and aggregation pheromones (Tillman et al., 1999). These glands may produce and store the necessary semiochemicals. In some species however, only precursor compounds have been found, implying that the semiochemicals are produced on demand. A good example of this is the saddle gall midge where its pheromone precursor nonan-2-ol, was found in crushed extracts rather than the ester compound (Censier et al., 2014). Several glands have been found to be associated with semiochemical activity. In some Lepidopteran and Dipteran species, the ovipositors bear the glands (Tillman et al., 1999). In the majority of Lepidopterans and several beetles, they are found on some abdominal segments. Glands accounted in previous reports include mandibular glands for *Cataglyphus* ants, hairpencil glands of the male danaid butterfly *L. leucone* (Francke & Schulz, 2010), labial glands of some termites (Mitaka et al., 2020), Dufour glands of some ants and bees (Keeling et al., 2004), etc. In most Cecidomyiidae, females'

ovipositors bear the pheromone gland (Amarawardana, 2009). Adult *Alphitobius diaperinus* emit pheromones from their abdominal glands (Hassemer et al., 2015). (Sevarika et al., 2022) also records the presence of antennal pheromone-producing glands in several Hymenopteran species. Pheromones is synthesized in the rectal glands of Tephritids (Blomquist et al., 1987).

2.3 Biosynthesis of semiochemicals

Semiochemicals, depending on the source (plants or arthropods) may have similar routes of biosynthesis. Pheromone component structures contain a number of characteristics that point to shared biosynthesis routes, giving enough knowledge and experience to make identifying the pheromones of different species easier (Hall et al., 2012). There is however no way to predict which kind of compound will be used by a particular species to carry a message (Kost, 2008). Since pheromones of one organism can be exploited as an allelochemical by another organism, the same principles of bio-production would apply to both. Insect pheromone biosynthesis usually utilises the isoprenoid pathway or the fatty acid pathway (Hall et al., 2012). In plants, the Jasmonic Acid and Salicylic Acid function biosynthetic pathways mediate chemical signalling caused by herbivory and pathogen infection, respectively (Arimura et al., 2005).

2.3.1. Precursors

Dietary intake provides raw materials for primary and secondary metabolism in organisms (Jurenka, 2004). Originally, studies on pheromone biosynthesis evolved from research on pheromone-related biochemical systems (e.g. fatty acid and hydrocarbon metabolism) (Tillman et al., 1999). It was found that Fatty acids, plant alkaloids and terpenes serve as insect pheromone precursors (Tillman et al., 1999). Ultimately, all precursors for pheromone biosynthesis can be traced to carbon derived through dietary intake utilised directly or altered minimally by insect enzymatic systems (Tillman et al., 1999). Many insect species contain the enzymatic activities and endocrine regulatory factors to biosynthesize their pheromone components de novo (Tillman et al., 1999).

The nutritional requirements of insects though vast depending on their lifestyle, are similar. They require approximately 30 chemicals including protein and/or 10 essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine); B-vitamin complex (biotin, folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine); and other water soluble growth factors (including choline and inositol), certain fat soluble vitamins,

cholesterol or a structurally similar phytosterol, a polyunsaturated fatty acid, minerals, and an energy source (usually provided by simple or complex carbohydrates and/or lipids) (Thompson & Hagen, 1999). Generally, non-predacious insects only require sugar and water for survival and mating, while other predacious or entomophagous insects may require a range of sugars, amino acids, fatty acids and sometimes minerals and vitamins for full development, longevity and reproduction. Floral nectars and pollens provide mainly simple sugars such as glucose, fructose, etc. Some nectars and pollens contain some free amino acids, small amounts of lipids, dextrins and beneficial vitamins. Dipteran and Hymenopteran parasitoids required similar quantitative balance of amino acids, salts, lipids, glucose, ribonucleic acids, vitamins and agar. Nonparasitic dipteran insects do not require polyunsaturated fatty acids (Thompson & Hagen, 1999).

The production of herbivore-induced plant volatiles is known to be induced by chemicals in the saliva of the attacking herbivore. Fatty acid-amino acid conjugates (FAC) are a typical example of inducing chemical. Following this is a cascade of signal transduction involving genes, enzymes and intermediary metabolites, of which ethylene, methyl jasmonic and methyl salicylic acids are key (Arimura et al., 2005). Several studies have established that mechanical tissue damage could not elicit the same responses in plants compared to herbivore attacks. Terpenoids are biosynthesized through the mevalonate pathway; some examples include linalool, β-ocimene, β-caryophyllene. Green leaf volatiles (also called GLVs, six-carbon or C6 volatiles) are produced by enzymatic activations, and they include isomers of hexenol, hexenal, hexenyl acetate. Methyl jasmonic and salicylic acids may also be emitted. The larvae of some moths and beetles in cotton species of the Malvaceae family do induce HIPVs (Arimura et al., 2005).

2.4 Properties of Semiochemicals

A pheromone of any insect is characterized by its carbon number, functional group, arrangement of double bonds, and ratio between major and minor compounds (Bakthavatsalam, 2016). For example, stearic acid is denoted as "18:0 acid, *Z*9-tricosene (*Z*9–23: Hy), *Z*9, 10-Epoxytricosane (*Z*9, 10–23: Ep), etc. This would as well apply to the allelochemicals, as they are simply exploited pheromones.

The majority of insect and mammalian pheromones are small and relatively simple molecules with low polarity (Jones & Parker, 2005). Semiochemical compounds, which are terrestrial, are typically volatile (i.e., with a high vapor pressure) have low molecular weights (300 amu or lower), and contain functional groups such as esters or lactones, alcohol, aldehyde, keto-groups (Kost, 2008). They occur at very low

concentrations, which are however sufficient to elicit biological activity. Physical and chemical properties may dictate capability, for example, signals, which delineate boundaries of territories, may be stable and non-volatile whereas compounds employed for a short-term warning of danger (alarm pheromones) may be chemically unstable and extremely volatile (Kost, 2008). Several insects have a diverse array of airborne volatiles serving several purposes like alarm, aggregation, airborne trails. Actively produced alarm signals have low molecular weights (100 and 200 u) translating to short fade-out times (Kost, 2008). Herbivore-induced plant volatiles are also airborne. Characteristics of these compounds are their lighter molecular weights (≤C20 less than 300amu), high vapor pressure, low polarity, and certain functional groups such as esters or lactones (Jones & Parker, 2005). This is important as the compounds would be required to travel several kilometres airborne.

Stereochemistry is also very essential as stereoisomers, optical isomers, and geometric isomers usually elicit different responses (Kost, 2008).

2.5 Identification of Semiochemicals

2.5.1. Behavioural assays

Usually, the first step in semiochemical identification is observation of behavioural patterns which may suggest volatile compound interaction. An example is arresting behaviour in an ectoparasitoid of some species of beetles; females produce a contact sex pheromone on their cuticles which arrests males, who respond by performing stereotypical courtship behaviour that includes high-frequency wing-fanning (Kühbandner et al., 2013).

The use of behavioural assays, choice tests, with or without the use of monitoring software such as EthoVision XT is employed. Several behavioural assays can be applied to obtain the requisite information. (Andersson et al., 2009) employed the use of Y-tube olfactometer bioassays to study synthetic pheromone blends and female gland extract of Hessian fly *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae). Olfactometer trials in (Xiu et al., 2019) showed volatiles emitted by aphids were attractive to its natural enemy lady beetle *Harmonia axyridis*. (Li et al., 2014) mentioned the use of a four-arm olfactometer to investigate the effects of HIPVs from Chinese broccoli on the foraging behaviour of two dominant natural enemy species of its herbivore pest in China. EthoVision XT video-tracking software has also been applied in semiochemical behavioural assays in various ways. In (Azandémè-Hounmalon et al., 2016), the study of the cues mediating interaction between the red spider

mite *Tetranychus evansi* and its predator *Phytoseiulus longipes* in East Africa involved monitoring insect responses with video tracking and data analyses with EthoVision software. Insects were studied in non-perforated transparent/opaque capsules. (Chiu-Alvarado & Rojas, 2011) also analysed the effects of HIPVs on parasitoids of the coffee berry borer, *Hypothenemus hampei* in coffee and maize using the EthoVision XT software. Petri dish assays are also common and a valuable technique for species that are difficult to breed under laboratory conditions (Costa et al., 2022). Petri dish methods can be coupled to EthoVision XT software for monitoring and data analyses (Antwi-Agyakwa et al., 2019).

2.5.1.1. Limitations of assays

Behavioural assays provide non-invasive techniques to allow observation of insect behaviour without disrupting natural habitats (Garai, 2024; Parker et al., 2016). They allow observation, identification and quantification of conspecific and interspecies interactions, providing insight into ecological relationships (Vickers, 2000). A variety of assay designs can be tailored to specific species or questions, maximizing applicability (Vickers, 2000). In converse, assays can have a limited scope and also introduce artefacts. Animal behaviour is influenced by a myriad of factors , including prior experiences (such as mating), social dynamics, and environmental context (Garai, 2024). This complexity makes it challenging to isolate the effects of specific chemical cues in behavioural assays. The controlled nature of experiments do not account for the numerous factors that influence organism behaviour in nature (Parker et al., 2016; Saunders et al., 2025). There may be focus on specific behaviours, overlooking broader ecological interactions, effects or processes that are long-term (Evenden & Konopka, 2024; Perl & Baird, 2023). Thus, results obtained in the controlled laboratory environments may not accurately reflect natural behaviours, leading to artifacts that could misrepresent ecological dynamics (Garai, 2024). Dicke (1998) provided a good instance, examining chemical cues in plant-herbivore interactions. He noted that while immediate feeding responses of herbivores to plant volatiles were documented, the broader effects on plant community dynamics were not investigated; the narrow focus on specific behaviours overlooked potential long-term ecological impacts. Moreover, designing, conducting, and analysing behavioural assays can be resource-intensive and time-consuming (de Bruijn et al., 2018). Each method can provide information on behaviour for limited (sometimes one) parameters. A y-tube assay is designed to study primarily, olfactory cues (Barbosa-Cornelio et al., 2019), thus it is limited in assessing multimodal decision of insects. Thus, extra efforts are required to ensure uniformity of settings and avoid distractions from visual or other cues at all angles.

2.5.2 Extraction of Semiochemicals

Sampling of volatile semiochemicals can be based on their extraction (often using solvents) or their collection (air or headspace trapping) (Barbosa-Cornelio et al., 2019). Insect pheromones are mostly lipophilic in nature. Thus, non- or less polar solvents are utilized for their extraction.

2.5.2.1. Headspace collection

A classical way of extracting insect pheromones is Volatile Trapping (Headspace collection) from live insects (Jones & Parker, 2005). Adsorbent materials such as activated charcoal and organic polymers like Porapak-Q and Tenax are used and subsequently desorbed before analyses. There are two main classifications of HS sampling: static (SHS), in which the analytes on the vapor phase are accumulated passively on capture material by sorption; and dynamic (DHS), which employs a gas flow to assist with the extraction and concentration of volatile compounds on the adsorbent phase (Barbosa-Cornelio et al., 2019).

Jiang et al. (2023) employed headspace sampling in HIPVs studies. Millar et al. (2009) identified sex pheromones in Long-tailed mealybug by headspace extractions. In (Dublon, 2009), headspace extraction was crucial for studies on the aggregation pheromones of the Western Flower Thrips.

Solid-phase microextraction (SPME) also employs volatile trapping, albeit with little disturbances to the organism and the environment (Jones & Parker, 2005). HS-SPME sampling has been a widely utilized method for collecting VOCs from different materials, such as grains at different conditions, as healthy grains , grains damaged by insects, grains infected with fungus as well as from nectar, nuts, intact leaves or those affected by herbivores, bark, flowers, essential oils, roots, fresh fruits, insect excrement, whole plants, living insects, and more examples which can be found in the available literature (Barbosa-Cornelio et al., 2019). Its non-destructive nature allows repeat sampling from the same organism and in very little time (Jones & Parker, 2005). Microbe-induced volatiles, as well as non-constitutive semiochemicals can be obtained by this method.

2.5.2.2. Cuticular extracts

Cuticular hydrocarbons (CHCs) are molecules on the cuticular surface of adult insects, generally serving as anti-desiccation compounds (De Pasqual et al., 2021). They are less or even non-volatile, compared to the other pheromone types. Cuticular hydrocarbons (CHCs) play an important role in insect communication, such as nest-mate and caste recognition in social insects, attraction as seen in termites and ants (Mitaka et al., 2020) and selection of potential mates (De Pasqual et al., 2021). They include

straight or branched alkanes and alkenes with chain length ranging from 15 to 55 carbons (Tillman et al., 1999). The pheromone components of the housefly are modified cuticular lipids; the major hydrocarbon components in newly emerged males and females are (Z)-9-alkenes with the C27 homolog predominating (Blomquist et al., 1987). Some studies report oxygenated derivatives such as aldehydes, ketones and epoxides. An example is the common house fly, *Musca domestica* which has the epoxide and ketone Z9,10–23:Ep and Z14–23:Ke appearing on the female cuticle simultaneously with Z9–23:Hy (Tillman et al., 1999). (Guo et al., 2022) also found that cuticular deposits of tetradecanal (14: Ald) and 2-heptadecanone (2-Hep) on female parasitoid *Campoletis chlorideae* elicited a sequence of mating behaviours in males.

Long-chain CHCs generally have low volatility, thus, are obtained by solvent extraction.

2.5.2.3 Gland extracts

Most insect species produce sex pheromones in glandular structures and can control the release of pheromone behaviourally by extruding or exposing the gland during "calling behaviour"(Schlein et al., 1980). Location of glands on various organisms' bodies is diverse. It is reported that in both the housefly and tsetse fly the pheromone is produced by a unicellular gland on the legs of the female (Blomquist et al., 1987). In several *Aphelinus* species, the presence of antennal pheromone-producing glands has been recorded (Sevarika et al., 2022). Plants also possess semiochemical producing glands. Glands in several *Gossypium* species are well recorded. Cultivated plants of *Gossypium hirsutum* Cav. (cotton) produce and store terpenoid volatile organic compounds in pigment glands which are continuously emitted at low levels, and further induced by damage (Clancy et al., 2023).

To obtain gland contents, the target insect can be crushed whole or specific glands or body parts may be excised. Constitutive semiochemicals and precursors for non-constitutive ones can be profiled by this method.

2.5.2.4. Pros and cons of extraction methods

Headspace extraction is a non-destructive method which allows for the analysis of volatiles without modifying the sample (Agilent Technologies, n.d.). Thus, it allows determination of volatile products that are constitutive of the organism. Typically cleaner samples are produced, as it minimizes interferences from the natural or even a solvent mix matrix (Ettre, 1993). This factor is even more enhanced when coupled with thermal desorption, which eliminates the need for solvent completely. Headspace collection is generally faster and simpler to implement compared to solvent extraction (Agilent Technologies, n.d.)

when all required tools are available. Thus, a main limitation is the specialized equipment required which limits general accessibility and practice. Another limiting factor is sensitivity. Some volatile compounds may be present at extremely low concentrations, making detection challenging (Pawliszyn et al., 1997). This could result in the need for multiple or extended extractions to acquire sufficient concentrations for analyses. The method is also sensitive to environmental conditions, such as temperature and atmospheric pressure, which can affect results (Ettre, 1993). Moreover, if insects are under duress due to adverse change of conditions, from field to laboratory, different and undesirable volatile profiles may be produced.

Cuticular and gland extractions would fall under solvent extracts. This method has higher sensitivity. It is capable of extracting a wide range of compounds, including those present at trace levels, while also targeting less volatile compounds (Fiehn, 2008). It is suitable for a wide variety of samples, including those whose matrices are complex (Khan et al., 2018). Solvent extraction can be adapted to different solvents and conditions to target specific compounds (Lord & Pawliszyn, 2000). On the other hand, it runs a higher risk of introducing artifacts and contaminants from processing and even solvents (Pawliszyn et al., 1997). The extraction process can be lengthy, or complex, impacting the speed of analysis (Anastassiades et al., 2003). The use of organic solvents moreover raises environmental and safety issues (Lord & Pawliszyn, 2000) as the focus in recent times shifts towards greener chemistries.

2.5.3 Analysis of volatiles

Techniques that have high-resolution and high-sensitivity are required as semiochemicals occur naturally in nanogram or even picogram amounts and are usually multicomponent mixtures. Majority of insects semiochemicals are volatile or semi-volatile, hence are suited to GC–MS analysis (Jones & Parker, 2005). They are also thermally stable, i.e., volatize below 300 °C, (Jones & Parker, 2005). Gas Chromatography is the most commonly used technique for VOC analysis because it allows simultaneous separation and analysis of volatile components with great sensitivity, robustness, reproducibility, and versatility (Barbosa-Cornelio et al., 2019). Gas chromatography effects separation by volatility, polarity, and molecular weight (Jones & Parker, 2005). Nonpolar polysiloxane column phases such as 100% polysiloxane or 5% polyphenylmethyl siloxane have a broad range for low polarity compounds such as pheromones, are thermally stable, and durable. For more polar compounds (carboxylic acids, oxo-acids, lactones, ketones, and polar heterocyclic molecules) a column with a polyethylene glycol phase can be

used. Better separation of complex mixtures where isomers of molecules are present is achieved with chiral columns (Jones & Parker, 2005).

Gas chromatography columns can be connected to several detection systems, facilitating compound identification. The most frequently used detectors for GC analysis of semiochemicals are the mass spectrometry detector (MSD) for molecular identification of compounds, flame ionization detector (FID) for quantification, and the electroantennography detector (EAD) for in-line analysis of biologically active molecules (Brezolin et al., 2024). Flame ionization detector (FID) uses a hydrogen flame, which creates a current between two electrodes when an analyte passes through, producing a signal. It can detect as little as 1 ng of compound. Retention indices from retention data, can be used to obtain compound structural information in a standardized system. Kovat's indices can be calculated for by extrapolation of total carbons in a molecule.

Mass spectrometry detector is a much more sensitive detection equipment, with ability to detect nanogram or picogram amounts. Electron ionization (EI-MS) is commonly used, where a stream of electrons bombards the sample molecule causing radical ions to be formed, and the molecule to fragment. Structural information is obtained as a characteristic fragmentation or 'fingerprint' which is specific for groups of compounds. By comparing this to spectral libraries and standards, sufficient identification of compounds is achieved.

Electroantennographic detection (EAD) is an electrophysiological technique that is specifically used in the chromatography of insect pheromones involving the use of an excised antenna or whole insect and much recently, a single olfactory cell (single sensillium recording or SSR) as a biodetector. Electroantennographic detection measures the change in potential between the tip and base of the antenna as odours are passed over it giving a relative measurement of the number of chemoreceptors stimulated (Barbosa-Cornelio et al., 2019). The GC effluent can be split between the EAD and an FID allowing the correlation of biological response to identified compounds (Barbosa-Cornelio et al., 2019). As little as 15 pg of compound has ever been detected. The use of GC–EAD allows the rapid screening of compounds and extracts in order to identify those eliciting a response and eliminate those not detected by the insect (Jones & Parker, 2005). Individual component molecules appear to have individual receptor sites on antennae (Flint & Doane, 2023). The usual small quantities are not sufficient for recovery and utilization in other assessments, thus limiting, in many cases, the experimental size and scope of behavioural experiments, when VOCs are extracted (Barbosa-Cornelio et al., 2019).

Microreactions such as derivatization and functional group modification are also useful in structural elucidation both in GC and MS. Exact positions and geometry of double bonds and other functional groups can easily be determined by epoxidation (using m-chloroperoxybenzoic acid), catalytic hydrogenation, reduction reactions, derivatization reactions (usually with dimethyl disulfide), methylation (especially of carboxylic acids, using boron trifluoride), amongst others(Jones & Parker, 2005). Non-volatile pheromones are elucidated by use of high-performance liquid chromatography (HPLC). NMR is employed when more complex structures are encountered.

2.6. Applications of Semiochemicals Research

The review by (Flint & Doane, 2023) highlights the several recent successes and economic importance of pheromones. It was established that almost all Lepidopteran species at least, have some commercially available pheromone products. Most have been used in trapping insects for detection and monitoring studies (determination of temporal distribution) (Flint & Doane, 2023). Another use reviewed involves application of large amounts to hide trails of females, leading to mating disruption. Also, insect navigational blindness could occur. This is due to habitation effect occurring at antennal receptor sites or the insect's central nervous system from the inundation of signals from commercial pheromone products. This disrupts their activities, such as feeding, finding mates. The mode of activity of various pheromones will determine their application in pest management programs. Kairomones and synomones are known to boost the efficiency of natural enemies (Chauhan & Punia, 2022). The use of Semiochemicals would be an immerse contribution towards the integrated management of cocoa insect pests.

2.6.1. Cocoa

Theobroma cacao is a tropical tree native of Mexican pre-Colombian territory, belonging to the sub family Sterculioidea and family Malvaceae. It has an elaborate root system, trunk, branches, leaves and flowers which develop into pods 5 – 6 months after flowering. Its harvestable product is the fruit (pods) from which beans are obtained to produce products such as cocoa butter, chocolates, cocoa powder, amongst others. In 2020, global cocoa bean production reached 5.8 million tonnes, with Ivory Coast leading at 38% of the total, followed by Ghana and Indonesia (FAOSTAT, 2020). The young fruit is called cherelles. The cocoa ecosystem has been touted as one of the most diversity-rich, harbouring many insects, birds, rodents, microorganisms and soil organisms. Cocoa is attacked by several insect pests,

with the major ones being cocoa mirids, stink bugs, cocoa stem borer's, mealybugs and coreid bugs. The minor ones are leaf defoliators, termites, grasshoppers, aphids, pod borers, and psyllids. The cocoa mealybugs also double as vector of the Cocoa Swollen Shoot Virus Disease (CSSVD), a devastating disease affecting cocoa in West Africa. Initial infestation of the cocoa plant takes up to 2 years before symptoms are expressed (Asogwa et al., 2024). Extreme cases of cocoa swollen shoot virus (CSSV) infection can result in drastic yield reductions (25%–50%) and complete yield loss within 3–4 years (Asogwa et al., 2024; Ameyaw et al. 2014). Cocoa swollen shoot virus currently causes an estimated annual loss of 50,000 tonnes of global cocoa production (Asogwa et al., 2024)

2.6.2. The Cocoa Mealybugs

Formicococcus njalensis (Laing) and *Planococcus citri Risso (Homoptera: Pseudococcidae)* are the most established mealybugs associated with cocoa and cocoa swollen shoot virus (CSSV) (Domfeh et al., 2016). Their cryptic nature and waxy coating makes their control difficult (Ameyaw et al., 2014; Hanna et al., 1955). Their males undergo complete metamorphism into winged adults whiles their females undergo incomplete.

In the three species of mealybugs found in Cocoa; *Formicococcus njalensis*, *Planococcus citri* and *Ferrisia virgata,* reproduction can be asexual (Padi, 1997). *Formicococcus njalensis* are particularly able to produce hundreds of crawlers asexually. They have a symbiotic relationship with under-canopy ants (Hymenoptera: Formicidae) (Mohan et al., 2016). Ants of the genus Crematogaster (in bush areas) as well as the genus Pheidole, (in areas of well-maintained cultivated cocoa) showed exponential population relationship with mealybugs, along with their predators and parasites (Cudjoe et al., 1993; Cornwell, 1957).

2.6.2.1. Biological Control of Mealybug

Due to their cryptic nature and adaptations, the control of mealybugs have been more successful with biological means, rather than classical chemical control methods (Sevarika et al., 2022). Encyrtids are the most successful at predating mealybugs through parasitism and parasitoid tendencies (Franco et al., 2009).

Ackonor (2002) reared natural enemies from colonies of *Planococcus citri* from randomly selected cocoa farms in Ghana. The reared population comprised adult females, third instar nymphs and mummified

individuals harbouring parasitoids. He observed the following predators*; Coccodiplosis coffeae* Barnes (Diptera: Cecidomyiidae)*, Hyperaspis egregia* Mader *and Scymnus sp*. (Coleoptera: Coccinellidae), and an unidentified lepidopteran. Key Hymenopterans of the Encyrtidae family recorded included *Aenasius abengouroui* Risbec*, Leptomastix dactylopii* Howard, *Anagyrus beneficians* Compere*, Anagyrus amoenus* Compere. Also recorded were a hyperparasitoid*, Cheiloneuras carinatus* Compere, parasitising *C. coffeae, Xyphigaster pseudococci* Risbec and six rare, undetermined parasitoids. *Coccodiplosis coffeae* was the commonest natural enemy, occurring throughout the year and infesting 72.8% of *P. citri* colonies. The author found parasitism levels were generally low, the highest mean being 4.6 ± 2.6% for *A. abengouroui.* Simultaneous parasitism by more than one species was common and this gave rise to mean monthly parasitism levels ranging from 0.8 to 4.5%.

In spite of protection from parasitoids and predators, (Pérez-Rodríguez et al., 2021) recorded that sugar provisioning reduced attendant ant tending, resulting in an increase of mealybug parasitism rates. Therefore, the researchers suggested that artificial sugar provisioning could improve pest management strategies against mealybugs.

2.6.3. Overview of Semiochemicals of Parasitoid Families/Genus Associated with Cocoa Mealybugs

2.6.3.1. Cocoa, Malvaceae family

Reports on constitutive volatiles with semiochemical impact in cocoa are limited to floral emissions which attract pollinating species. The study by (Tavera et al., 2023) picked up, among other minor constituents, β-caryophyllene in cocoa pods serving as a kairomone for *Helopeltis bakeri*, a mirid pest. They also reported finding "compounds that belong to the Terpenoids and Fatty acids pathways" as constitutive plant volatiles in pods.

Herbivore-induced plant volatiles (HIPVs) have the potential to be used for conservative biological management because they are widespread and significantly contribute to the attraction of mealybug natural enemies (Subramanian et al., 2021). In general, fewer species of dipteran parasitoids are reported to respond to HIPVs compared with parasitoid wasps (Honda et al., 2013). These include the green leaf volatiles ((*Z*)-3-hexen-1-ol, (*E*)-2-hexenal), linalool, terpenes, etc.

In (Jiang et al., 2023), aphid-infested *Gossypium hirsutum* Cav. (Malvales: Malvaceae) (wild cotton) were found to release 4 volatiles 1: butyl acrylate, 2: 1,2-diethylbenzene, 3: p-diethylbenzene, 4: 4-ethyl-

o-xylene, which were attractive to the multicoloured Asian lady beetle *Harmonia axyridis*. According to (Rose & Tumlinson, 2004), the same cotton species released terpenes; (*E*)-β-ocimene, linalool, (*E*)-β-farnesene, (*E,E*)-α-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, isomeric hexenyl butyrates, 2-methylbutyrates, indole and (*Z*)-3-hexenyl acetate, when infested by corn earworms. Numerous investigations on *G. hirsutum* described volatile terpenoids, primarily mono- and sesquiterpenoids which are produced and stored in pigment glands in the leaves, stems, seeds, and flower buds and released consistently at low levels in undamaged plants (Clancy et al., 2023). Usually, a different blend is released in the absence of damage. Some parasitoids respond to volatiles from undamaged plants as well (Clancy et al., 2023).

Some parasitoids can distinguish HIPVs from various plants that harbour their hosts. For instance, when *Heliothis virescens* larvae attacks of plants from Malvaceae, Solanaceae and Poaceae families, its parasitoid wasp *Cardiochiles nigriceps* is attracted by the produced HIPVs, but not those caused by the related *Helicoverpa zea*, a non-host (Dias et al., 2016).

2.6.3.2. Cocoa mealybugs

Since mealybugs are the most invasive pest species, classical biological control has been frequently employed against them (Mani & Shivaraju, 2016a). One of such is the use of natural enemies; parasitoids and predators. Naturally occurring parasitoids of the mealybug include encyrtids and aphelinids, and predators include coccinellids, lacewings, cecidomyiids and drosophilids (Mani & Shivaraju, 2016b).

All known pheromones of Pseudococcidae are terpenoid derivatives with many of them constituting esters of terpene alcohols (Zou & Millar, 2015). Most reported pheromones are obtained by headspace volatile collection. Perhaps due to the dispensability of males in reproduction, studies have not investigated pheromones of *F. njalensis*. *P. citri* on the other hand was identified as a cyclobutane-containing ester of a monoterpenol; ((1R,3R)-cis-2,2-dimethyl-3-isopropenylcyclobutanemethanol acetate) by (Bierl-Leonhardt et al., 1981).

Mealybugs in other crop systems produce volatile cues attractive to parasitoids, such as in *Phenacoccus manihoti* with its parasitoid *Apoanagyrus lopezi* (Bertschy et al., 2001). Some pheromones of genus close to *F. njalensis* include o-caffeoyltyrosine of the cassava mealybug *Phenacoccus herreni*, [(R)-2,2-dimethyl-3-(1-methylethylidene)-cyclobutyl] methyl(S)-2-methylbutanoate for pink hibiscus mealybug *Maconellicoccus hirsutus*, (E)-((1S,3R)-2,2-dimethyl-3-(2-methylprop-1-enyl) (cyclopropyl) 2-methylbut-2-enoate of the striped mealybug *Ferrisia virgata*. Mealybugs appear to create unique

pheromone channels per species, hence related species do not share signalling compound structures (Zou & Millar, 2015). On the contrary, there is a suggestion to use "generic" mealybug lures to attract several species, particularly in crops that are infested with multiple species simultaneously (Zou & Millar, 2015). An interesting study by (Franco et al., 2011) indicated that wasp parasitoids of Vine mealybugs had increased parasitism rates on *P. citri* when sex pheromones of vine mealybugs was present.

O-caffeoylserine isolated from the body surface of *Phenacoccus herreni* (Homoptera: Pseudococcidae) elicited strong drumming behaviour, thereby acting as a contact host-recognition kairomone in the parasitoids *Acerophagus coccois* and *Aenasius vexans* (both Hymenoptera: Encyrtidae) (Ayelo et al., 2021).

Other herbivores induced host plant volatiles attractive to their natural enemy, an example is *Aenasius vexans* and *Apoanagyrus diversicornis* parasitoids on *Phenacoccus manihoti* in South America (Bertschy et al., 2001). In cassava crop, another mealybug-hosting plant, showed that the encyrtid wasp parasitoids were more attracted to the herbivore-induced emissions of infested plants, ascertained by the fact that "infested leaves were preferred over the combination of healthy leaves and a removed mealybug complex" (Bertschy et al., 2001).

Honeydew, a by-product of mealybugs, has been identified as important for symbiotic ants (Pérez-Rodríguez et al., 2021), as well as parasitoids and predators. The chemical substances in honeydew induce arrestant behaviour for parasitoids to stimulate close-range prey-searching behaviour, and at times elicit long-range attraction (Bertschy et al., 2001; Thompson & Hagen, 1999).

2.6.3.3. Encyrtids

The Encyrtidae (Hymenoptera) encompasses a large group of parasitic wasps widely used in biocontrol programs of scale insects (Hemiptera: Coccoidea) (Sevarika et al., 2022). They are a family of wasps, usually parasitoids and have been excellent models to study pheromone communication in insects (Ruther et al., 2019). They are able to oviposit in eggs, larvae or different sized instars of its host adult host (Thompson & Hagen, 1999). Despite this, reported sex pheromones are rare for Hymenoptera parasitoids compared to other orders (Guo et al., 2022). Many hymenopteran parasitoids are small body sized usually solitary and widely dispersed (Guo et al., 2022). Semiochemicals of hymenopterans are chemically diverse. Biosynthesis likely includes aromatic, fatty acid, and terpenoid pathways as well as simple modifications of host-derived precursors (Keeling et al., 2004). Very few (less than 10) semiochemical lures have been developed, despite the diversity and agricultural benefits of the Encyrtid family (Keeling

et al., 2004). It has been argued that host cues may play a less important role in the foraging behaviour of parasitoids due to their minute quantities, thus they use plant-provided cues (Bertschy et al., 2001). Nonetheless, African systems in reviewed studies reported a positive response of the female parasitoid *A. lopezi* to the cassava mealybug *P. manihoti*, (Bertschy et al., 2001). *Leptomastix dactylopii* known primarily as the citrus mealybug parasite wasp (Mansour et al., 2012) can occur wherever its host mealybugs occur (University of California, 2023), including cocoa. It has already been successfully employed along with the mealybug destroyer lady beetle to control citrus mealybug in greenhouses and interior spaces (University of California, 2023). Endoparasitoid wasps in the genus *Anagyrus* Howard (Hymenoptera: Encyrtidae) are also commonly used in the biological control of mealybugs (Hemiptera: Pseudococcidae) worldwide (Andreason et al., 2019).

Some semiochemical activity identified so far include HIPV and host pheromones with kairomonal effect, cuticular hydrocarbons (CHCs) and headspace emission. Many larval parasitoids often recognize volatiles from the damaged host plant and/or host larval frass volatiles (Keeling et al., 2004). *Aenasius vexans* Kerrich and *Apoanagyrus diversicornis* Howard (Hymenoptera: Encyrtidae) are examples of larval parasitoids of the cassava mealybug, *Phenacoccus herreni* which rely on odour emissions by host-infested plants (Bertschy et al., 2001) to locate their hosts. In *Cotesia Leptopilina Nasonia* species, airborne sex pheromones are common from both females and males (Guo et al., 2022). S-lavandulyl seneciote of vine mealybugs are also known to attract *Anagyrus spp* (J. G. Millar et al., 2002). Cuticular hydrocarbons (CHCs) serve as contact sex pheromone in the parasitoid wasp *Urolepis rufipes* (Würf et al., 2020) as well as *Lariophagus distinguendus*, a parasitic wasp (Kühbandner et al., 2013).

2.6.3.4. Midges

The Cecidomyiidae family, also known as gall midges, is a fast-diverging family of the Dipteran order containing more than 5000 described species (Gagné, 2004). Many gall midges are important crop pests (Hagen et. al, 1999). The life span of adult midges can be as short as 1-2 h, but is commonly 1-2 days (Harris & Foster, 1999). Within this limited time, the midges have to locate a conspecific partner for mating and the females have to locate a suitable oviposition place (Harris & Foster, 1991; Molnar et al., 2018). The utilization of sex pheromone-monitoring traps is a crucial element in integrated management strategies for midges in the UK (Hall et al., 2012). Among the three subfamilies of Cecidomyiidae, only species within Cecidomyiinae have been documented to manufacture sex pheromones (Hall et al., 2012).

Cecidomyiid midges species are not entirely phytophagous; their diverse feeding habits also include zoophagy and mycetophagy (Hall et al., 2012). In contrast to general Diptera family, only females in Cecidomyiidae family have been found to produce pheromones (Amarawardana, 2009). The females emit a species specific sex pheromone that attracts the male (Hall et al., 2012) and use volatiles emitted by the host for localization of oviposition site, whiles the males use the female produced sex pheromone for mate localization (Boddum, 2013). Data by Molnar et al., (2018) show that males are responsive to host plant volatiles hence they may also be attracted to the host plant. Several species of Cecidomyiid midges have been shown to be attracted to the volatiles in their host plants (Hall et al., 2012). *Aphidoletes aphidimyza*, the only zoophagous midge in the study by Molnar et al. (2018) responded to several plant compounds and to herbivore induced plant volatiles (HIPV), indicating that they may use plant volatiles to localize their prey aphid colony.

Sex pheromones have been identified for 16 gall midge species (Hall et al., 2012). All identified pheromones are chiral, straight, odd numbered carbon chains (7-17 carbon atoms) with an oxygenated function (acetoxy-, butyroxy- or keto-functionality group) on the second carbon, the S-stereoisomer is most common (Hall et al., 2012). Decarboxylation is the key event contributing to the odd number of carbons. The stereochemistry at the second carbon is important for attraction (Amarawardana, 2009). The molecules can be saturated or unsaturated and all gall midge sex pheromones identified so far have at least one chiral centre. Di-functionalities occur on C7 whilst double bond functionalities are found in the eighth position (Amarawardana, 2009). Despite their similarities, the female sex pheromones are species-specific, only attracting conspecific males (Hall et al., 2012). Fatty acid coenzyme A derivatives serve as the starting point for proposed biosynthesis pathways to the pheromone components of cecidomyiid midges, which are completed by either chain elongation or chain shortening (Boddum, 2013). Foster et al. (1991) elucidated a biosynthetic pathway for the *Mayetiola destructor* midge. The female M. destructor's pheromone gland contained the fatty acyl moiety (9E)-9-dodecenoate, which they suggested was an intermediary in the biosynthesis of the main pheromone component (2S, 10E)-2-acetoxy-10-tridecene. As a result, the dodecenyl precursor's two-carbon homologation with malonyl coenzyme A (CoA) yielded a 14-carbon, β-ketoacyl CoA (Foster et al., 1991). The pheromone component would be obtained by hydrolysing, decarboxylating, and stereoselectively reducing the resulting methyl ketone, then acetylating it (see figure 2, pathway a) (Hall et al., 2012). In other cecidomyiid species, the positions of double bonds in pheromone components suggest generation by means of chain shortening of fatty acid precursor, instead of chain elongation (see figure 1, pathway b) (Hall et al., 2012). β-oxidation of oleyl-CoA produces 2-acyl-8-heptadecenes, followed by decarboxylation producing a methyl ketone

(first chain shortening), then reduction and acetylation (second chain shortening) (Hall et al., 2012). The final product is the 2-C shorter 17-carbon monoacetate (2R, 6Z)-2-acetoxy-6-pentadecene component (Hall et al., 2012). Homo-conjugated dienes could be generated from a linoleate precursor in a similar manner (Hall et al., 2012). An extra oxygenation within the chain can explain a second stereogenic centre, which is a further structural variability (Hall et al., 2012). In unsaturated molecules, the double bond is always followed by the second oxygen down the chain (Hall et al., 2012). The pathways for di-functional compounds are difficult to predict (Amarawardana, 2009).

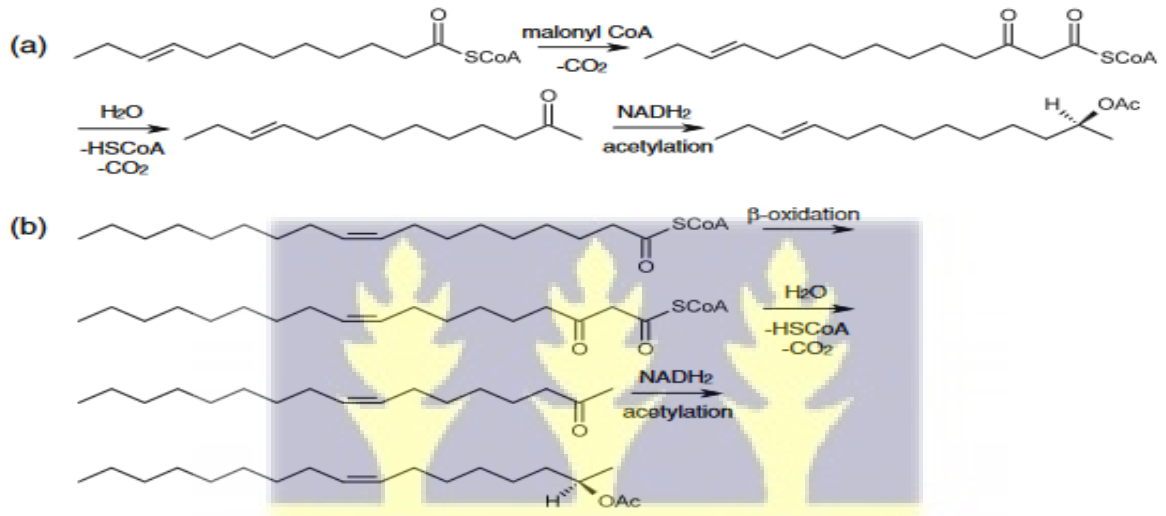

Figure 2: Proposed biosynthetic pathways of cecidomyiid midge pheromone components, beginning with derivatives of fatty acid coenzyme A, by (a) chain elongation or (b) chain shortening (Hall et al., 2012)

Pheromones of Cecidomyiids have been separated by solvent-based extraction of either excised glands or entire insect bodies (Hall et al., 2012) as well as headspace volatile collection of females. (Censier et al., 2014) identified sex pheromone of the saddle gall midge *Haplodiplosis marginata* as a racemic ester. According to (Hillbur et al., 2005), extracts from gland in ovipositors of calling *Contarinia nasturtii* females contained the required pheromone. CHCs are common in other Dipteran families and (Blomquist et al., 1987) suggest that cuticular lipids, along with their changes and associated endocrine regulation should be examined for remaining unexamined Dipterans families. Plant volatiles are vital for phytophagous gall midges and it is expected that this would apply for the other behavioural subsets (Hall et al., 2012).

In conclusion, one practical application of semiochemicals is in management programs of cocoa insect pests. Chemical cues are important to natural enemies seeking cryptic prey of which mealybugs are an example (T. C. Turlings & Benrey, 1998). Both wasps and flies are parasitoids found in cocoa mealybugs.

A number of hymenopteran parasitoids are attracted to herbivore-infested plants that emit HIPVs (Arimura et al., 2005). The natural enemies of mealybugs are also attracted to herbivore-induced plant volatiles (HIPVs), which can be employed for conservative biological control (Subramanian et al., 2021). Some plant volatiles are highly specific and are composed of compounds not found in unrelated species (Honda et al., 2013). In view of these, it is considered worthwhile to investigate for chemical cues directing the activities of these parasitoids found in the cocoa ecosystem. It would be economically beneficial to determine what chemical cues and pheromones influence parasitoid activity in parasitoids and predators of *Formicococcus njalensis.*

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1. Insect samples

3.1.1. Materials

Plastic vials with netted lids for keeping test organisms, Camel hair brushes for handling test organisms, Log Tag thermo-hygrometer, Aspirator, Hand lens, Leica microscope

3.1.2. Collection

Colonies of *Formicococcus njalensis* were sampled weekly from unsprayed (at least one month without chemical application) farms at New Tafo, Addo-Nkwanta and Tontro in the Eastern region (Figure 3). Using the hand height method, cocoa trees were examined and mealybug colonies observed were gently brushed into plastic vials using a Camel's hair brush. The collections were covered with fitted netted lids and transported to an insectary room in the Department of Entomology at Cocoa Research Institute of Ghana in the Eastern Region. Pods that were heavily infested and had mummies were harvested.

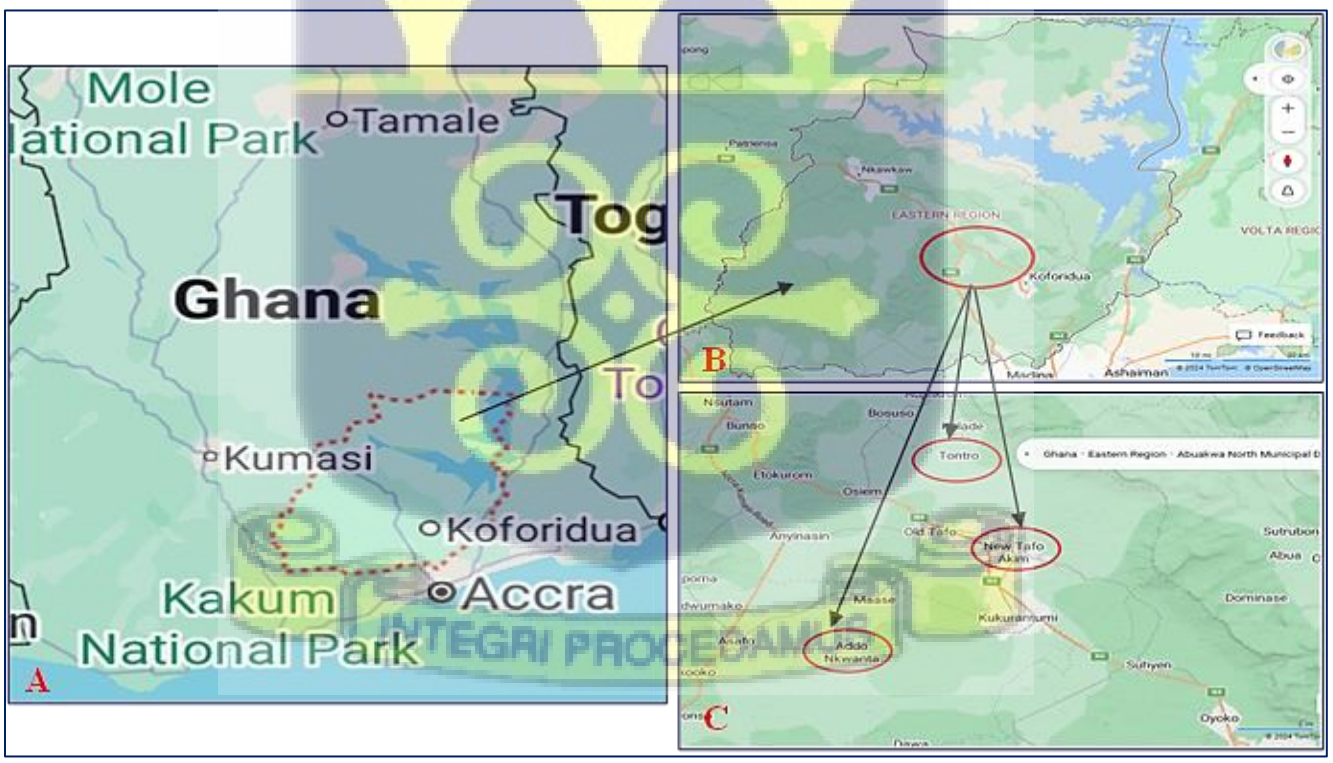

Figure 3: Map showing insect collection locations.
(A) location of Eastern Region of Ghana [red dashed outline]. (B) Close-up view of Eastern Region showing regional capital [Koforidua] and proximity to study area [red circle]. (C): Location of cocoa farms [red circles; Tontro, New Tafo – Akim, Addo-Nkwanta] where insects were collected. Study period; 2023-2024. Base maps adapted from OpenStreetMap (https://www.openstreetmap.org/copyright, retrieved October 2024).

3.1.3. Rearing

Mealybug collections were maintained at room temperature 28 ± 2°C, 60 ± 10 % relative humidity (RH), and 12:12 (L:D) hour photoperiod and observed for the emergence of natural enemy species. Wasp species of the Encyrtidae family, including *Anagyrus* spp. and *Leptomastix* spp. were collected. Also, a predator fly of Cecidomyiidae, *Coccodiplosis coffeae* was obtained.

3.1.4. Sexing and identification

Organisms that emerged were collected using an aspirator, identified under a Leica microscope and a digital hand lens with the aid of published materials by Harris (1967), University of California (2023) and Andreason et al. (2019) and sexed. Sexing was done by observation of the structures of antennae and lower abdomen. Males generally are smaller-sized, possess more conspicuous "hairs" on the antennae, and present relatively shorter abdomens.

3.2. Extracts

3.1.1. Materials

Nitrogen gas generator from Nitrox Ltd. model DON 400-1, DA7C Charles Austen pump with air and vacuum outlets, Activated Charcoal filters, Porapak Q tubes (4 mm internal diameter), VAS Portable Volatile assay device, Hot Air Oven, Glass chambers, sizes 34/35 and 19/26 with connectors 19/26 and 24/29, Flow meter (0–10L/min), from Hemel Hempstead Herts, Transsonic T310 sonicator, HF-frequency 35 kHz, Retort stands, 200µl glass syringe, Amber glass vials, -18 freezer, N-Hexane (GC grade), De-ionised distilled water, Acetone, Non-scented non-coloured liquid soap, Distilled water.

3.2.2. Insect headspace volatiles

All reuseable glassware were washed with non-scented liquid soap, rinsed in distilled water and dried in a Hot Air oven before use. Teflon tubes were conditioned with acetone and nitrogen gas. Chambers were conditioned by washing and drying at 80°C for 12 hours. Before use, Porapak tubes were also conditioned by eluting with several rinses of hexane and dried under gentle stream of nitrogen. Volatile organic compounds (VOC) collection was conducted using Porapak Q. Separate sexes of each insect were placed in separate glass chambers. Insects were left to settle in the chambers for at least 30 min prior to starting collection. A gentle stream of activated charcoal filtered air was pushed into each chamber at a flow rate of 0.7l/min. Volatiles were trapped onto fitted Porapak Q tubes. Volatiles were collected for 24 hours. Collections were also made from empty glass chambers, serving as control or blank. The experiments

were conducted under 26±1 °C and 12:12 (L: D) hour photoperiods. The obtained volatiles were eluted with 1ml hexane. This was then concentrated to 0.2 ml under a gentle stream of nitrogen. The samples were stored in amber glass vials in a -18°C freezer until use.

3.2.3. Cuticular and whole-body extracts

After emergence, males and females were transferred to separate vials. Whole body extracts were prepared by immersing obtained batches in hexane, 20 microlitres per individual for 15 minutes (Hillbur et al., 2005) with the application of ultrasound (Barbosa-Cornelio et al., 2019). Cuticular extracts were prepared first, by immersing obtained batches in hexane, 20 microlitres per individual for a shorter 2 minutes at room temperature. The supernatant was then filtered and concentrated by evaporating the hexane under a gentle stream of nitrogen. The extracts were kept in sealed amber glass vials at -18°C until use.

3.3. Bioassays

3.3.1. Materials

Y-tube olfactometer 15cm per arm, 2cm internal diameter, Micropipettes 20, 200 and 1000 µls, DA7C Charles Austen pump with air and vacuum outlets, Activated Charcoal filters, Hot Air Oven for drying and conditioning, Glass chambers, sizes 34/35 and 19/26 with connectors 19/26 and 24/29, Flow meter (0–10L/min), from Hemel Hempstead Herts, De-ionised distilled water, Acetone, Ethanol, Non-scented non-coloured liquid soap for washing of glassware, Distilled water.

3.3.2. Y-tube olfactometry

The activities of extracts were assayed using Y-tube olfactometry.

For Y-tube experiments, following the procedure described by Bertschy et al. (2001), tubular glass chambers of 3 cm diameter were connected to the two arms of the olfactometer. Filter paper was used as substrate for odour sources of 10µl in the glass chambers of one arm. Solvent in filter paper was the control in the other arm. Activated charcoal-filtered air entered the two upper arms of the olfactometer at 0.5l/min rate of flow. Insects were then introduced into the base of the olfactometer. To initiate movement of insect, a dark material was used to cover the entry point of the olfactometer. Behaviour of organisms was recorded within 5 minutes. The positions of choice were classified as treatment, control and no-choice if the organism moved into the treatment arm, control arm and stayed in the stem of the

olfactometer, respectively. The experiment was repeated for additional replicates using different insects. The lowest replicates obtained was 5, the highest was 39.

3.3.3. Statistical analyses

A Pearson's Chi-squared test of independence was applied to assess choice for a particular odour across insects and sex. As the data (time spent in arms) were not normally distributed, a non-parametric Kruskal-Wallis H test was used to determine the statistical difference in overall choices. Post-hoc pairwise comparisons were derived, following a generalized linear mixed model fit by maximum likelihood (GLMM), from estimated marginal means (EMMs) via the 'emmeans' R package. *P*-values for comparisons were adjusted using the Holm method to control the family-wise error rate. Effect sizes were then quantified using Hedges' *g*. Estimated probabilities and effect sizes are reported with 95% Confidence Intervals (CI).

3.4. Instrumental analyses

3.4.1. Materials

Shimadzu Single quadrupole Gas Chromatograph-Mass Spectrometer, 2010 version, with auto sampler AOC201, equipped with a VF-5MS column (30 m x 0.25µm ID x 0.25 µm), in splitless mode.

3.4.2. Gas Chromatography – Mass Spectrometry programming

GC analyses were carried out using a temperature program of 35°C for 5 min with an increase to 300°C at 10°C/min for the VF-5MS. Helium was employed as carrier gas at 1 ml/min rate of flow. Analytes were injected by direct injection at a temperature of 250°C. Compounds were detected by mass spectrometry with ion source temperatures of 280°C and interface temperature of 300°C, scanning between 40-450*m/z*.

3.4.3. Compounds identification

Compounds were identified according to their mass spectrum and chromatography using National Institute of Standards and Technology (NIST 20) compound library. Confirmation of compounds was done using synthetic standards.

CHAPTER FOUR

4.0. RESULTS AND DISCUSSIONS

4.1. NATURAL ENEMY COLLECTIONS AND IDENTIFICATION

Coccodiplosis coffeae [CoSp]

The total number of midges, *Coccodiplosis coffeae* (CoSp), a predator, obtained in the study period was 978. There were abundantly more females than males, which was the case for several species obtained (Table 1). Despite the populations, their fragile nature made volatile collection challenging. Set-ups had to be discarded after the midges collected dropped dead a few hours after start time. At other times, they dropped dead before end time, and since it could not be detected the exact time of death, they had to be discarded. Insects are known to sometimes have a change in volatile profile under stress (Cattaneo et al., 2025) and this was to be avoided as our goal was to obtain attractants of healthy organisms. Unlike in other works carried out, since the exact glands or production sites and period of release of active volatiles on the anatomy of the insect was yet to be determined, whole sampling methods were used; volatiles were collected over a 24-hour span, capturing a full day's profile. Extractions were also made on whole organisms. Due to its short life span of 1 to 2 days, they were observed to begin copulation within hours of emergence. The females were also seen dragging the tips of their abdomen on the surfaces where they crawled with some even laying eggs after doing this. Thus, it was expected that sufficient information could be obtained from volatile collection method used same day of emergence. Identification of males and females was done using information provided in documents by Harris (1967). The following features were used to identify the sexes the sexes:

Both sexes are bright orange in colour with transparent wings. The females are generally larger in body size than the males (Figures 5 and 4). The second key difference is the antennae. While both are very long, male antennae segments appear compressed. A closer inspection reveals they exist as a chain of connected "balls and sticks" and densely covered by setae (hair-like structures). In females the antennae appear more extended, resembling tiny cylinders linked together at the tips like a chain. Setae are sparse in females. The third distinguishing factor is the genitalia. That of males is strongly curved basally and prominent.

Figure 4: Male CoSp

Figure 5. Female CoSp

Anagyrus beneficans [AnSp];

For AnSp, a parasitoid, the total number obtained was 534. The population dynamic exhibited here was more females than males (Table 1). This is similarly the case in CoSp. The AnSp also had higher survival rates than CoSp, sometimes lasting up to 4 days when not fed. However, for consistency of results and due to small numbers, volatile collection was done at 1 to 2 days after emergence. Some species of wasps are known to have different volatile activity along their lifespan in accordance with their maturity rates. For example, a consideration made was that antenna and leg grooming behaviours were observed. Such tendencies usually convey the idea of detection of some interesting conditions linked to their activities within the vicinity. Moreso, grooming is a way of distributing their semiochemicals as they are produced for some organisms. Another challenge in AnSp was the size of the organism and their escapist behaviours. Some males were smaller than 0.5mm. This small bodily size is usually the result of host size from which it was emerged rather than other unfavourable conditions. Both sexes also had the tendency to suddenly jump, making them easy to lose sight of and enhancing their escape. It was generally observed that higher temperature and bright light conditions increased this behaviour. Identification was confirmed using Cambridge University Press (1943). The following key features were used:

Sexual dimorphism is displayed in adults (Figures 6 and 7). Females are 1.5-2.0mm in size, dull orange to brownish. Head is not dorsoventrally flattened. The basal segment of the antennae is black and the distal portion is white. Compound eyes are greyish brown. Legs are white to yellowish. The wings are hyaline. The ovipositor is short and only slightly extended. For the males, they are much smaller than

females, usually 0.7-1.25mm and black in colour. The antennae are white, threadlike thin, noticeably hairy and without the thick black base.

Figure 6. Male AnSp

Figure 7. Female AnSp

Leptomastix dactylopii [LeSp]

The LeSp, the second parasitoid, were a much larger bodily size of wasp to track and work with. They too had the same tendency to jump suddenly. This species was the fewest in numbers (53) collected over the period from the same colonies that the previous two were obtained (Table 1). Similar behaviours were observed as the AnSp. Their identification was confirmed by University of California (2023). And the following key features were used throughout the period: There is sexual dimorphism in the adults (Figures 8 and 9). Both have dark bulging eyes with hyaline wings. The females are brighter-coloured orange to brown and larger at 2 – 2.5mm in length. Antennae appear smoother with tiny setae. The males are darker in colour usually brown and less than 2mm in length. Antennae appear hairy as longer setae are distributed along the antennae.

Figure 8. Male LeSp

Figure 9. Female LeSp

Table 1 : Obtained numbers of females and males of natural enemies emerging from collections of mealybugs

Collection Date	Source	Midges (*Coccodiplosis* spp.)		Wasp (*Anagyrus* spp.)		Wasp (*Leptomastix* spp.)	
		Male	Female	Male	Female	Male	Female
December, 2023	New Tafo	2	21	0	0	0	1
January, 2024	New Tafo	38	170	13	21	4	5
February, 2024	New Tafo	3	32	38	79	4	15
March, 2024	New Tafo	1	61	40	89	4	11
April, 2024	New Tafo	1	28	36	47	0	1
May, 2024	New Tafo	6	14	10	20	2	6
June, 2024	New Tafo	10	50	37	84	0	0
July, 2024	New Tafo	135	406	8	12	0	0

Table 2 gives the details of plant material used in the study.

Table 2. Cocoa plant parts used for bioassays and volatile collection

Plant Material	Label	Selection Date	Selection State	Use Date	Use State
Pod 1	Pod 1	June 2024	4.2cm length	July 2024	8.5cm length
Pod 2	Pod 2	June 2024	2.7cm length	July 2024	10cm length
Pod 3	Pod 3	June 2024	4.8cm length	July 2024	10.5cm length
Pod 4	Pod 4	June 2024	4.6cm length	July 2024	10.5cm length
Pod 5	Pod 5	June 2024	3.9cm length	July 2024	Withered
Pod 6	Pod 6	June 2024	44cm length	July 2024	Discarded
Seedlings	Labelled with use	Planted in May and usage begun from June, thus 1- to 3-month-old			

4.2. SEMIOCHEMICAL COLLECTION

The batches of volatile and extract samples were collected from insects of 1-to-2-day old age. This was done due to the conditions described under sampling, in order to maximise results from varying numbers obtained. Below is a breakdown of the sets of replicates used across the two sexes of the parasitoids, as well as the host insect and host plant numbers (Table 3).

Table 3

Table 3. Inventory of samples used in volatile collections and bodily extractions made from the various organisms in the study

Type of Collection	Sample Id	Numbers Used					
		CoSp Male	CoSp Female	AnSp Male	AnSp Female	LeSp Male	LeSp Female
Headspace volatiles	Rep 1	0	35	7	9	0	0
	Rep 2	0	17	23	22	0	0
	Rep 3	0	15	25	36	0	0

	Rep 4	0	0	4	9	0	0
	Rep 5	0	0	13	23	0	0
Cuticular extracts	Rep 1	49	50	21	43	6	14
	Rep 2	172	50	57	56	6	30
	Rep 3	0	50	50	56	0	5
	Rep 4	0	0	38	0	0	0
	Rep 5	0	0	0	0	0	0
Whole body extracts	Rep 1	49	50	21	43	6	14
	Rep 2	172	50	57	56	6	30
	Rep 3	0	50	50	56	0	5
	Rep 4	0	0	38	0	0	0

Type of Collection	**Sample Id**	**Mealybug Female**	**Cocoa Pod Detached**	**Cocoa Pod Attached**	**Cocoa Seedlings**
Headspace volatiles	Rep 1	100	1	1	1
	Rep 2	100+	1	1	1
	Rep 3	0	1	0	1
	Rep 4	0	1	0	1
Cuticular extracts	Rep 1	50	0	0	0
	Rep 2	50	0	0	0
	Rep 3	50	0	0	0
Whole body extracts	Rep 1	50	0	0	0
	Rep 2	50	0	0	0
	Rep 3	50	0	0	0

4.3. Y-TUBE EXPERIMENTS

Each insect used in the experiment was given 5 minutes, that is, 300 seconds to make a choice. Generally, there was no arresting effect of extracts observed in the experiments. All insects moved naturally and actively searched the various arms along with grooming behaviours, as if exploring for some interesting odour. Thus, analysis was based on total time spent searching within the two arms of the olfactometer. The midges were very dainty to work with in the y-tube as they mostly drop dead in the stem of the olfactometer. Moreso, the insects spent a long time acclimatizing in the stem, requiring re-inoculation of

extract and blank solvent. Using the previous observation that they were attracted and agitated by brighter lights, a dark cloth was used to cover the end of the stem and arms to speed up their activity. A period of 10 minutes was allowed before this became necessary for sedentary test subjects.

The behavioural responses of female and male parasitoids to the various extracts used in the y-tube olfactometer bioassays (Figures 10 to 14) along with statistical analyses are elucidated below. The charts in the figures are based on percentage total time spent in extract arm versus the control blank arm.

4.3.1. Significance test

For the several categories separated by parameters "control" vs "choice", *t*-test or its equivalent non-parametric was computed to compare means of the two groups. This is to determine if there is a significant difference in time spent between treatment choice and the control blank for the experiments conducted.

The null hypothesis states that there is no significant difference between the two arms.

For each category, H0: $\mu treatment = \mu control$.

Where $\mu treatment$ is the mean time spent in treatment arm for a specific extract

$\mu control$ is the mean time spent in control arm for the same extract

If the *p*-value obtained from the *t*-test is less than the chosen significance level (0.05), we reject the null hypothesis and conclude that there is a significant difference between treatment and control for that category. The probability, from Generalized linear mixed model fit by maximum likelihood (GLMM), estimates for proportion of total time (0 to 1) that insects spent in a specific treatment (odour) arm provided direction of choice between treatment and control. Probability (or prob) of 0.5 was the expected neutral point, indicating no preference. Above 0.5 is preference of odour treatment while below is avoidance (choice of control). 95% confidence interval (CI) around probability estimates was used to determine deviation from expected neutral.

Effect sizes were also obtained to visualise the average spread of the data showing significant difference or not. A small effect size (0.2) means the two groups are mostly overlapping, thus, choice for treatment by test insects isn't much different from the control blank arm. A medium effect (0.5) means there is a clear shift; the average test insect has higher than about 69% preference of treatment. A large effect (0.8) means the test insects distinctly (higher than 79%) prefers the treatment over the control blank. Negative values indicate a skew towards control choice instead.

The test results are summarized below;

Overall, Pearson's Chi-squared (with continuity correction) test suggested that all the different experimental conditions (odours) produced essentially the same outcome regarding odour time (X^2 = (800), p =0.20). However, the pattern of odour times is dependent on the sex of the insect (X^2 = (100), p =0.00017).

Kruskal-Wallis H tests conducted revealed significant differences in odour choice time across sexes (Kruskal-Wallis X^2 = 11.6, df = 1, p =0.0007), and insects (Kruskal-Wallis X^2 = 8.48, df = 2, p =0.014), but there was no difference across the various treatment groups, (Kruskal-Wallis X^2 = 5.38, df = 8, p = 0.716).

Generalized linear mixed model fit by maximum likelihood (GLMM) revealed that strong significant differences were occurring across treatments and sexes (p<0.0001). AnSp insect, female (F) sex and female cuticular extracts were used as comparison baselines for these fittings. Female volatile treatments had the lowest significant difference value (p = 0.022; p<0.05*). Results for CoSp treatments exhibited high significant differences (p=0.007; p< 0.001***), while that of LeSp (p=2e-16; p<0.0001****) was extremely significantly different from AnSp experiments

When adjusted for sex and insect type, it revealed a significant preference for female whole-body extracts generally. The estimated marginal mean proportion of time spent in female whole-body extracts was 0.797(probability, 95% CI: 0.534, 0.931), which is significantly different from the neutral proportion (0.5). This indicates that odour time preference significantly exceeded that of blanks across female whole body extracts treatments. This made female whole-body extracts to be the most explored odour across treatments and sexes. The effect size of this observation was significantly close to large (Hedges' g = 0.77, 95%CI: [0.37, 1.16]). Male whole-body extracts (probability = 0.74, 95% CI: 0.45, 0.91) and male cuticular extracts (probability = 0.74, 95% CI: 0.45, 0.91) also indicated preference, but the deviations were not significant, reference to confidence intervals is made.

Insect-derived odours generally appeared more explored (probability > 0.5) as compared to slight avoidance in kairomonal odours including mealybug volatiles, herbivore-induced plant volatiles and constitutive plant volatiles (probability < 0.5). These deviations are however not on significant levels as the neutral point value (probability = 0.5) falls within their respective confidence intervals.

Midges (CoSp)

For CoSp females, most extract categories show very high significant difference (p<0.0001) in choice of treatment over control arm, compared to female cuticular extracts. This was with the exception of mealybug extracts which skewed more towards control comparatively. GLLM analyses showed no significant difference for female whole-body extracts (p=0.77) against female cuticular extracts as baseline, this is likely because they are both equally significantly explored choices foe CoSp females. A significant deviation, indicating strong preference is confirmed by post hoc comparison, at female cuticular extracts (probability = 0.98, CI; [0.55,0.99]), female whole-body extracts (probability = 0.98, CI; [0.54, 0.99]) and herbivore-induced plant volatiles (probability = 0.99; CI; [0.89, 1]). The preferences for these respective odours were substantial, with large effect size for female cuticular extracts (Hedges' g = 0.81, 95%CI: [-0.26, 1.8]) and female whole-body extracts (Hedges' g = 0.96, 95%CI: [0.18, 1.7]), then medium to large for herbivore-induced plant volatiles (Hedges' g = 0.71, 95%CI: [-0.32, 1.67]). There however is high variability in herbivore-induced plant volatiles treatments data possibly due to small sample size.

From these observations, it can be seen that female CoSp were strongly attracted to female body extracts (Figure 10). This implies some aggregation activity of the body extracts. They were however moderately attracted to female's volatiles and male body extracts (Figure 10). Aggregative volatiles are once again inferred. Exploring the kairomonal aspect, female CoSp appeared less interested in mealybug volatiles as they spent less than 50% of the time frame exploring that area (Figure 10). It was however expected that the reverse would be true, since oviposition is carried out on mealybugs. On the other hand, they were strongly attracted to the related host plant's volatiles, as well as volatiles emitted by mealybug-infested plants (Figure 8). It is possible that the plant volatiles may exert longer range attractive cues, while the mealybugs would have shorter range effect. Another explanation could be that mealybugs may not be their only source of oviposition site. Hence, the female CoSp depend on the plant volatiles to locate the mealybugs, rather than the mealybugs themselves.

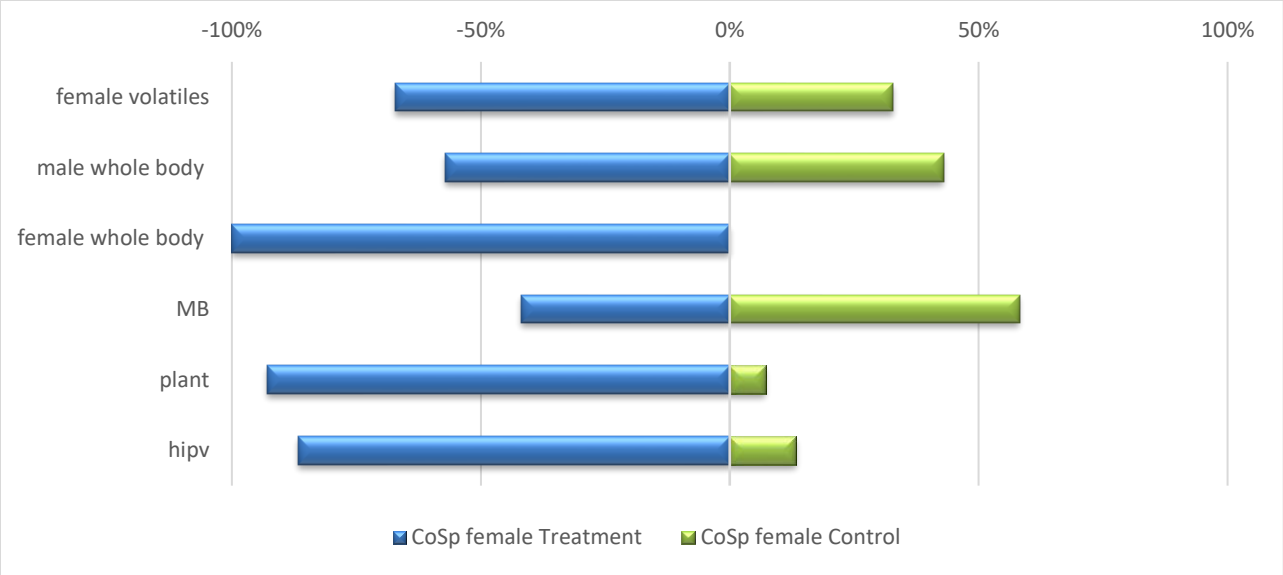

Figure 10: Stacked diverging bar chart showing y-tube assay responses to various extracts by female *Coccodiplosis coffeae* (CoSp)
MB – mealybug volatiles; plant – plant volatiles; hipv - herbivore-induced plant volatiles / mealybug-infested plant volatiles.

Wasps (AnSp)

In the replicates completed, GLLM analyses showed no significant difference compared to female cuticular extracts, for female whole-body extracts (p=0.42), male cuticular extracts (p=0.50), and constitutive plant volatiles (p=0.055). The remaining extract treatments were comparatively highly significant at the 0.0001 level. Very high significant difference was noticed between sexes (p=1.29e-6). The herbivore-induced plant volatiles group demonstrated a very strong significant difference (p=5.95e-07). Preference was however in the opposite direction, or slight avoidance, with a probability of 0.42 (95% CI [0.17, 0.71]). This deviation was not significant and had small effect size, with uncertain precision (Hedges' g = -0.11, 95%CI: [-0.43, 0.22]). This is because the confidence interval crosses zero (goes from negative to positive), indicating that the results were not statistically significant and the true direction of the effect remains uncertain]. The strongest estimated probability of preference was with male whole-body extracts (p=2e-16; prob=0.87, 95%CI [0,65, 0.96]). This deviation is significant, warranting further investigation into factors contributing to this effect. Effect size for the male whole-body extracts' observation was very large (Hedges' g = 1.48, 95%CI: [0.66, 2.26]). The same applied to female whole-body extracts (Hedges' g = 1.03, 95%CI: [0.33, 1.69]) and male cuticular extracts (Hedges' g = 0.83, 95%CI: [0.15, 1.47]).

Strong significant differences existed between males and females' responses (p<0.1.29e-06). Thus, if females were strongly attracted to one odour, the opposite effect may be seen for males. This can be seen in the percentage time spent figures. For AnSp males, female volatiles and hipvs show significantly more time spent in treatment arm, while there was no preference for female cuticular and female whole-body extracts. In contrast, for AnSp females, all extracts show significant attraction except female cuticular extracts, constitutive plant extracts and herbivore induced plant extracts which showed more skew towards the control.

Thus, male AnSp strongly attracted to female AnSp volatiles, implied pheromonal components (Figure 11). They were also moderately attracted to mealybug volatiles and volatiles from mealybug-infested plants than to plant volatiles alone (Figure 11). Thus, their kairomonal activity is linked to the mealybug host. This could be because they would expect to find food source or mating partners within that vicinity, ensuring the survival of their progeny.

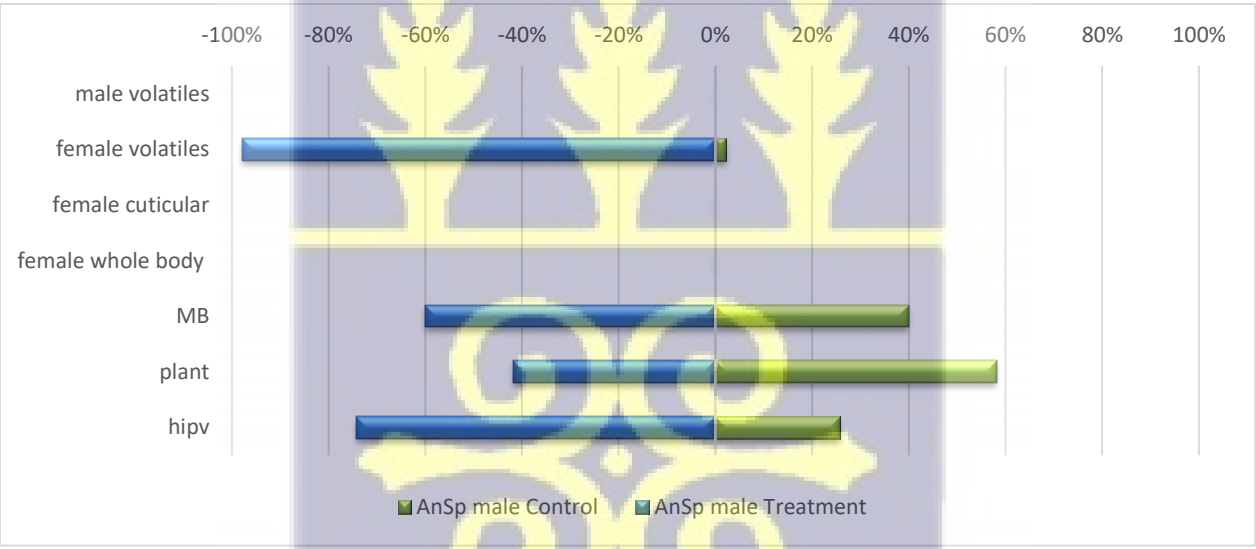

Figure 11: Stacked diverging bar chart showing y-tube assay responses to various extracts by male *Anagyrus* spp. (AnSp). MB – mealybug volatiles; plant – plant volatiles; hipv - herbivore-induced plant volatiles / mealybug-infested plant volatiles

For the female AnSp, they were observed to be strongly attracted to male cuticular extracts, male and female whole-body extracts (Figure 12). This implies some aggregative property in these extracts. In some Encyrtidae species, the males however produce the pheromones for the attraction of the female (Kurtanovic et al., 2022). An exhaustive electroantennography (EAG) of the extracts using female AnSp would provide valuable information on the bioactivity of the various components of these extracts. There was however moderate attraction to all the other extracts with the exception of crushed females and mealybug-infested plant volatiles. Kairomonal cues are obtained from both the solitary mealybugs and

the plant. This is expected for oviposition purposes. Their link to plant volatiles could also be for the sourcing of other resources such as food. Unlike the CoSp who are ephemeral, the AnSp have a longer lifespan and thus require active feeding.

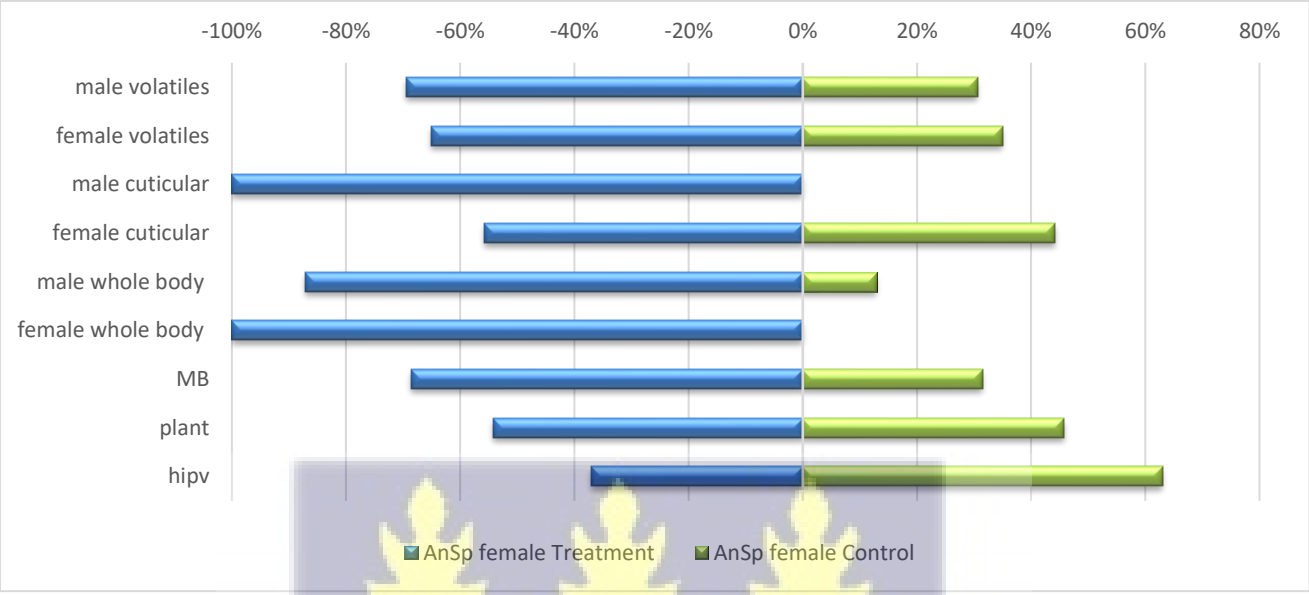

Figure 12: Stacked diverging bar chart showing y-tube assay responses to various extracts by female *Anagyrus* spp. (AnSp). MB – mealybug volatiles; plant – plant volatiles; hipv - herbivore-induced plant volatiles / mealybug-infested plant volatiles

Wasps (LeSp)

Among the LeSp, GLLM analyses across both sexes found strong significant differences between sexes and in all odours (p<0.0001) except female whole-body extracts (p=0.55) against baseline female cuticular extracts. Once again, this is because they are of equal significance levels. Only mealybug volatiles (p=2e-16) (probability =0.38, 95%CI [0.21, 0.59]) and constitutive plant volatiles (p=2e-16) (probability=0.46, 95%CI [0.26, 0.66]) exhibited avoidance, but their deviations were not significant, with reference to their confidence interval. The effect size of this observation is medium to small (Hedges' g = 0.37, CI; [0.06, 0.68]). Strong effect sizes were observed for male cuticular extracts and male whole-body extracts (Hedges' g = 0.88, CI; [0.12, 1.60]). Female cuticular extracts and female whole body extracts showed medium effect sizes (Hedges' g = 0.52, CI; [-0.05, 1.07]), For herbivore-induced plant volatiles (Hedges' g = 0.08, CI; [-0.52, 0.67]), for mealybug volatiles (Hedges' g = -0.19, CI; [-0.68, 0.31]) and constitutive plant volatiles (Hedges' g = -0.47, CI; [-1.09, 0.16]), small or medium to small effect sizes were obtained, with high variability in confidence intervals.

The strong significant differences between sexes were an indication that sexes are likely to react in opposite fashion to same exposed odours. This is evident in the overall percentage preference figures.

For LeSp males, there is significant preference for only male cuticular extracts and male whole extracts. Constitutive plant volatiles exhibited significant avoidance. In contrast, for LeSp females, most extract categories showed significant preference skew towards treatment, except for the mealybug volatiles.

The males showed stronger attraction towards their own sex's bodily extracts as compared to every other extract (Figure 13). This is indicative of aggregation compounds present in these extracts. The kairomonal effect was not very strong. Moreover, they seem to be repelled from constitutive plant volatiles.

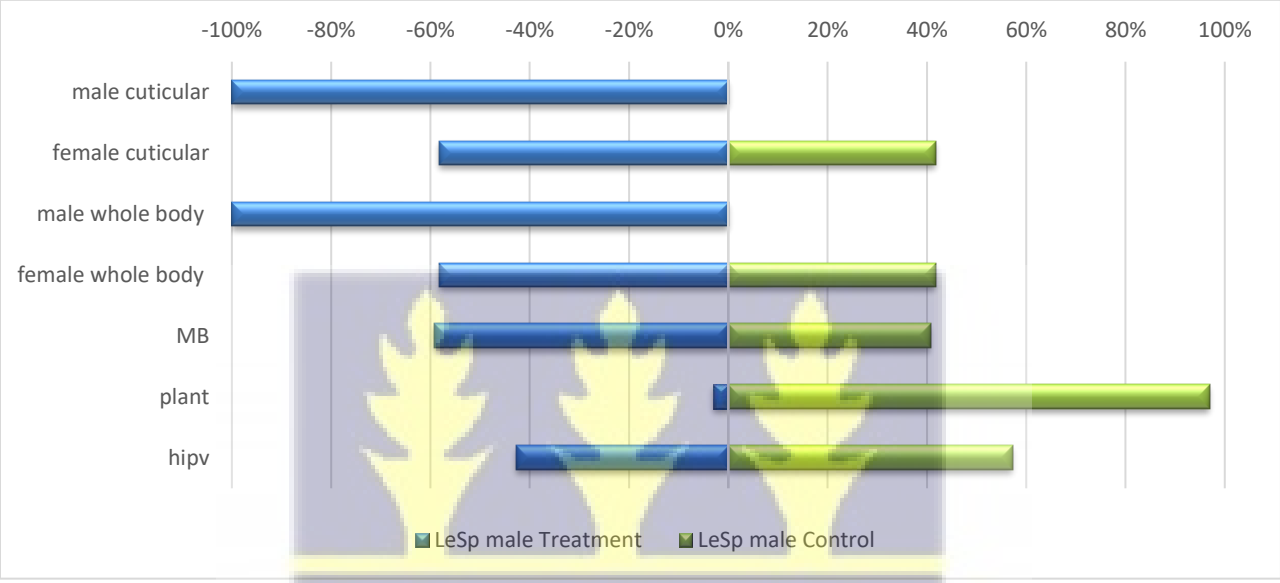

Figure 13: Stacked diverging bar chart showing y-tube assay responses to various extracts by *Leptomastix* spp. (LeSp) male. MB – mealybug volatiles; plant – plant volatiles; hipv - herbivore-induced plant volatiles / mealybug-infested plant volatiles

LeSp females were observed to be significantly attracted to all extracts used except for the volatiles of the host mealybugs (Figure 14). Similar to the CoSp females, they may rather depend and be guided by plant volatiles to find the mealybugs on which they oviposit. Or else, mealybugs may not be their only source of oviposition resource.

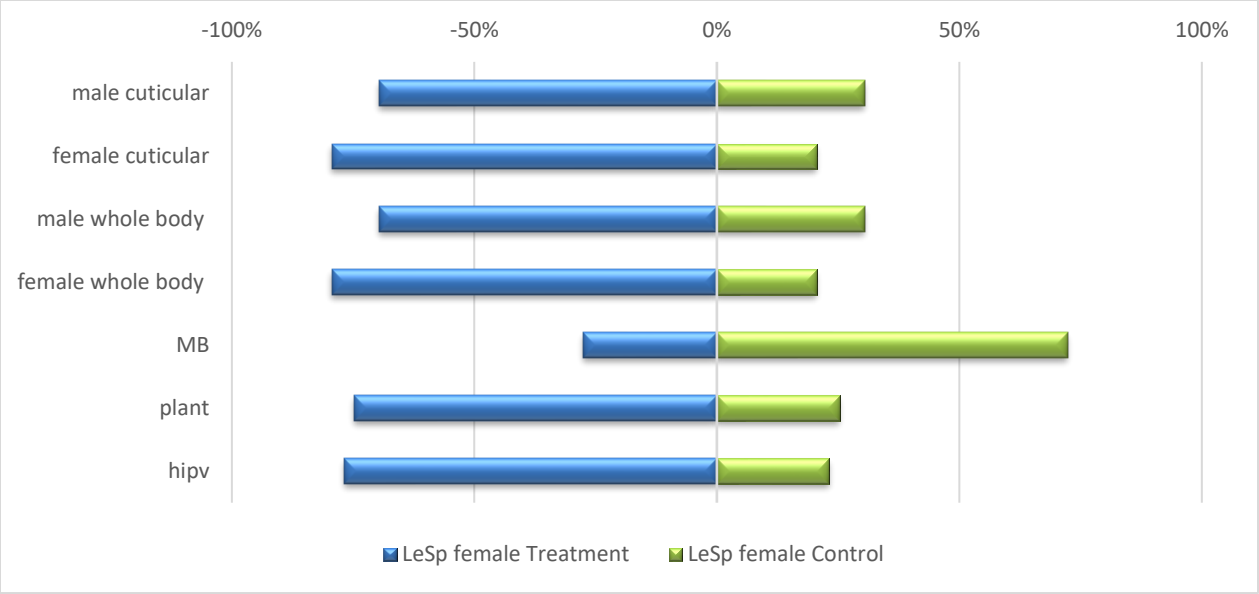

Figure 14: Stacked diverging bar chart showing y-tube assay responses to various extracts by *Leptomastix* spp. (LeSp) female. MB – mealybug volatiles; plant – plant volatiles; hipv - herbivore-induced plant volatiles / mealybug-infested plant volatiles

Below is an overview of the data points (Table 4).

Table 4. Summary of Y-tube olfactometry experiments.

Extract No.	Parameter	Results *					
		CoSp male	CoSp female	AnSp male	AnSp female	LeSp male	LeSp female
1	Male volatiles	Die or weaken upon intro.	-	-	1785/787	-	-
	No. of Reps	0	0	0	12	0	0
2	Female volatiles	Same as above	972/475	1214/87	1525/820	-	-
	No. of Reps	0	6	7	8	0	0
3	Male cuticular	Same as above	1028/773	-	1420/0	770/60	597/261
	No. of Reps	0	14	0	12	5	5
4	Female cuticular	Same as above	780/131	0/0	1415/1120	472/338	1301/338

	No. of Reps	0	5	5	10	7	7
5	Male whole body	Same as above	1028/773	-	2947/437	758/32	597/261
	No. of Reps	0	14	0	13	5	5
6	Female whole body	Same as above	735/113	0/0	1980/0	490/320	1301/338
	No. of Reps	0	5	5	8	7	7
7	Mealybug volatiles	Same as above	580/809	665/443	3920/1802	451/311	358/938
	No. of Reps	0	6	12	26	6	10
8	Plant volatiles	Same as above	920/120	414/576	2930/2475	39/1038	417/141
	No. of Reps	0	6	6	24	6	5
9	Herbivore-induced volatiles	Same as above	1764/271	736/255	1888/3216	528/707	497/150
	No. of Reps	0	10	7	29	6	5

*Total time(s) spent in treatment arm/ Total time(s) spent in control arm

It is well known that organisms employ multimodal integration, i.e., a combination of cues to make behavioural decisions. Various non-chemical cues such as, visual signals (colour), auditory signals, and tactile interactions, significantly influence behavioural decisions in insects (Dafni, 1992; Dyer et al., 2007). These cues can interact with chemical signals or may act independently to shape ecological interactions (Rundle & Nosil, 2005). Reser et al. (2012) examined how honeybees integrate visual and chemical cues during flower selection. The bees were shown to prefer flowers that matched their previous experiences with both colour and scent, demonstrating that visual and olfactory cues work together to shape foraging behaviour. Parasitoid wasps are especially known to integrate multiple cues in search for resources (Giunti et al., 2015), with many species relying on adaptation through learning. Y-tube olfactometry is not designed to elicit complete elucidation of chemical signalling.

Artificial cues were controlled by sealing off window views and using uniform white backgrounds in assays. Not much has been documented on the effect of white colour for the insects used in this study, but it is generally deemed to have some attractive effects. While wasps are drawn to lighter colours including white for foraging (Dafni, 1992) and possibly mating and aggregation (Baker & Baker, 1983), midges prefer darker colours but, can also exhibit responses to lighter colours, which may include white (Chittka & Menzel, 1992; Prokopy & Owens, 1983). There appears to be a lack of direct research specifically addressing colour attraction related to the genera studied. There is also no dedicated research that investigates how colour affects *Coccodiplosis coffeae*. A notable gap in focused studies on the colour preferences of *Leptomastix* spp., *Anagyrus* spp., and *Coccodiplosis coffeae* exists. Future research may delve into how these parasitoid wasps and predator fly respond to colour, enhancing our knowledge of their ecological roles and interactions.

Sufficient efforts were made to minimise the influence of anthropogenic factors in the y-tube assays. Insects were of the same state, unmated, to ensure uniform prior experience. Midges were isolated according to sexes immediately upon emergence, usually at daybreak. The wasps were similarly isolated. Room conditions were kept approximately constant for all subjects both during rearing and assays (room temperature and average humidity). to ensure similar volatility of chemical cues and similar trap rates. This also ensured fit physiological state for test insects. A constant airflow for all assays ensured even dispersion of odours at all times. All rooms used were void of detectable odours, perfume use was avoided during the assays, and access was limited to external users. This served three purposes. First is the elimination of chemical noise and distraction in behavioural assays. Smadja & Ganem (2008) highlight that uncontrolled background odours in the experimental setup may distract subjects, leading to inconsistent behavioural responses that do not accurately reflect preferences for specific chemical cues as the interaction. Therefore, responses accurately reflect preferences for specific chemical cues. Secondly, external volatiles eliminated meant less chance of different odour molecules interacting, thus confounded the interpretation of individual compounds' attractiveness in Y-tube assays (Dotterl, 2008). Third, minimising the chances of test subjects developing preferences or aversions based on previous encounters with specific odours, which can distort the results. Research by Farina et al. (2007) found that honeybees exhibited learned associations with flower odours, which influenced their choices in Y-tube assays. Preferences based on prior learning can result in skewed data regarding innate behavioural responses to chemical cues. Several studies indicate parasitoid wasps can be trained on visual as well as chemical cues, meaning their behaviour can be affected by prior exposure to cues (Desouhant et al., 2010; Fedorova et al., 2023; Lucchetta et al., 2008; Samková et al., 2020). Cecidomyiids are known to be primarily

influenced by olfactory or chemical cues, but some visual cues have been found to assist their foraging (M. O. Harris & Rose, 1990). The precautions taken were therefore vital for sound results from assays

There could also be genetic background influence. Hence, some individual variations may exist in olfactory sensitivity or response timing. Churgin et al. (2025) demonstrated that individual variation in the olfactory sensitivity of fruit flies significantly impacted their preferences in several separate behavioural assays. Specific neural correlates and genetic factors influenced olfactory processing.

The natural enemies used in this study may have different phases of maturity, despite emergence into adult stage, especially with wasps (Kurtanovic et al., 2022; Ruther et al., 2011b). This can also significantly impact their olfactory sensitivity and behavioural responses in Y-tube assays. To go beyond the 1–2-day limit used in this study, a study of diel mating patterns would be required. This would provide a complete profile of the life stages of the insect species obtained, thus, determining the optimum timing for specific behaviours and signalling.

The most limiting factor in this study is sample sizes. Data deficits are not uncommon in olfactometry experiments, due to unforeseen circumstances (Barbosa-Cornelio et al., 2019). It is already established through previous studies, as well as this one, that parasitism successfully occurs on *Formicococcus njalensis* howbeit at low rates. Data balancing, by deletion or extrapolation, as in other types of studies, are usually not feasible for chemical ecology studies (Wajnberg & Haccou, 2008). More data is desired, with the promising trends already outlined. It would require accumulation of samples over a longer period of time, or rather more sensitive instrumentation.

4.4. GAS CHROMATOGRAPHY – MASS SPECTROMETRY ANALYSES

Analyses of the obtained mass spectra were indicative of mostly carboxylic acids, ester, straight chain and branched alkanes, haloalkanes and alcohols. These compounds were expected as they usually have semiochemical activity or are precursors to semiochemicals. Some could however be general metabolites, depending on the species. The same chromatographic conditions were also used for analyses of extract collections and the standards. Thus, retention times and indices were comparable. Non-isothermal retention indices, as defined by Van den Dool and Kratz was favoured under the chromatographic conditions used. The mass spectra of the various replicates are given in the appendices. In cases of absence of molecular ion, the chain length and the presence of other functionalities in the molecule was determined by comparing retention indices with that of known pheromone compounds.

***Coccodiplosis coffeae* [CoSp]**

No male volatile compounds were analysed for CoSp. The test insects dropped dead within hours of being introduced into the collecting chamber.

Male cuticular compounds and diagnostics

The compound occurring at 9.19 /9.46 minutes (figure 15) generated key fragment ions at *m/z* 59 (alpha-cleavage- at hydroxy side resulting in a highly stable acylium cation) , 101 (another possible alpha-cleavage- at hydroxy side removing a methyl radical), 59, 58, 83 (terminal -OH results in dehydration and allylic cation) , 98 (small weak M-18 peak resulting from -OH dehydration) and 43 as base peak (a result of alpha cleavage at carbonyl side, producing an acylium ion), corresponding to the compound 4-Hydroxy-4-methyl-2-pentanone.

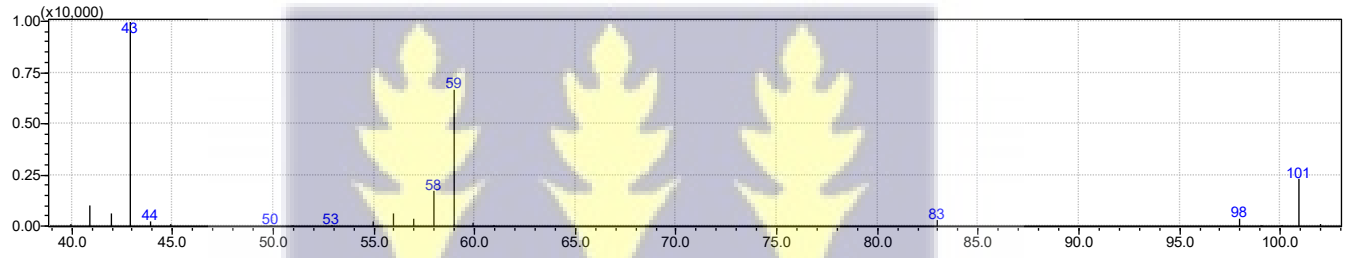

Figure 15: CoSp male cuticular compound (i)

The peak appearing at 18.95/18.952 minutes (figure 16) contained odd numbered major fragments with mass 14 differences, indicating hydrocarbon species. Fragment ions 71 (highly stable sec-pentyl cation when cleavage occurs at c9-c10, also c4-c5 cleavage to generate an isopentyl cation/ c6-c7 cleavage resulting in an isopentyl cation), 43(c2-c3 cleavage resulting in a stable isopropyl cation or c10-c11 cleave to produce a propyl cation / c9-c10 or c2-c3 cleavage resulting in stable isopropyl cation) , 85, 99, 113, 55, 41 and 57(c3-c4 cleavage releasing an isobutyl cation /cleavage at c3-c4 to produce a stable isobutyl cation which stabilizes by hydride shift and rearrangement to tert)) as base peak, corresponds to a multiple- methyl branched compound, such as 2 6 10 trimethyl tridecane/ 3 ethyl 2 6 10 trimethyl undecane.

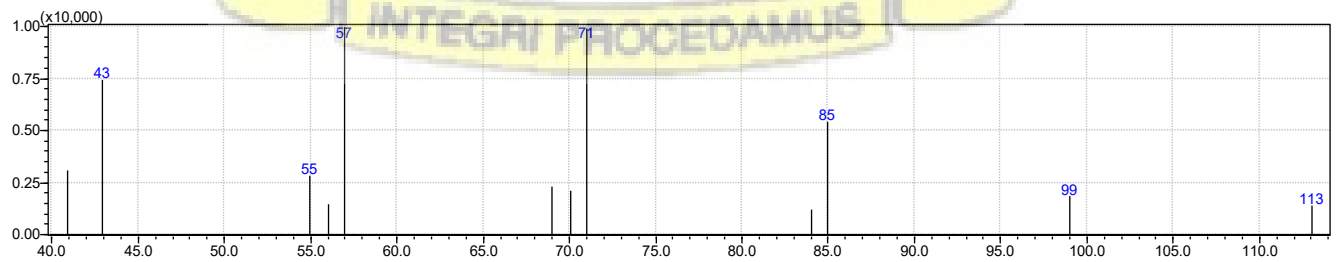

Figure 16: CoSp male cuticular compound (ii)

The analyte appearing at 20.783/21.066 minutes (figure 17), produced a molecular ion of 355 with odd numbered main fragments in increments of mass 14 indicating a hydrocarbon. fragment ions 43, 71, 85, 99 and 57 as base peak as shown in figure 17 above. This peak is denoted an unknown hydrocarbon.

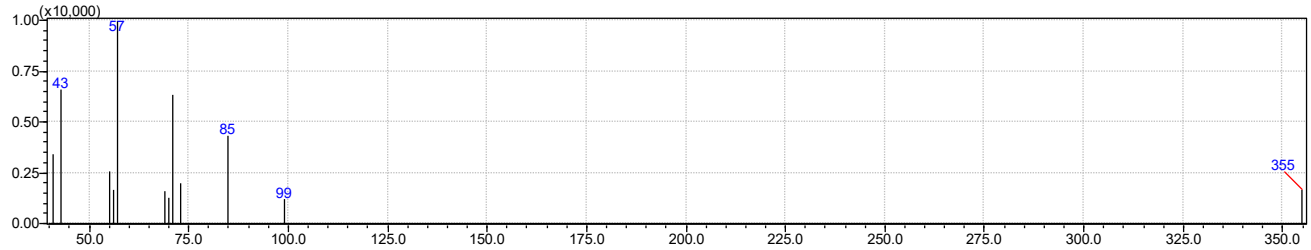

Figure 17: CoSp male cuticular compound (iii)

At 26.556/26.761 minutes (figure 18), the peak occurring produced fragment ions at 69, 83, 97 43, 222, 180 *m/z* with 55 as base peak and 265 as parent peak. This compound was identified to be a hydrocarbon with either an alkene group or an oxygen functionality due to the presence of even fragments at *m/z* 222 and 180, and the abundance of *m/z* 55.

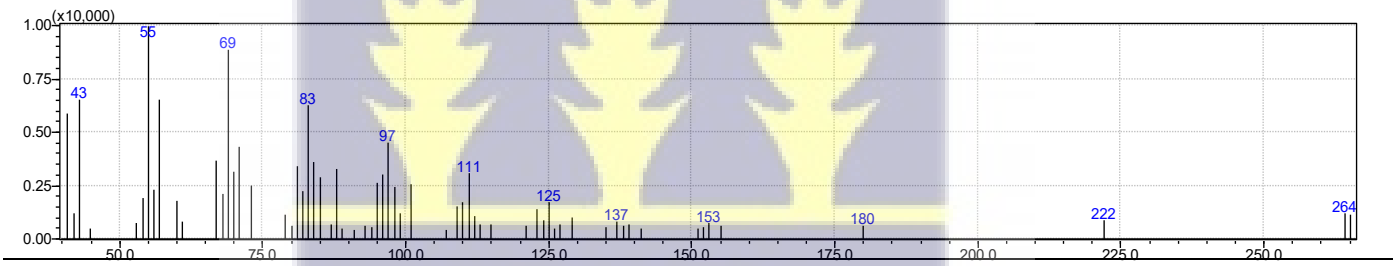

Figure 18: CoSp male cuticular compound (iv)

The key fragment ions in figure 19 indicate a long chain alkane and 57 for base peak formed from cleavage at c3-c4 forming a stable isobutyl which was observed at 31.809/31.965 minutes on the GC chromatogram with molecular ion *m/z* 380 [M]+, was matched to the compound 2-methylheptacosane.

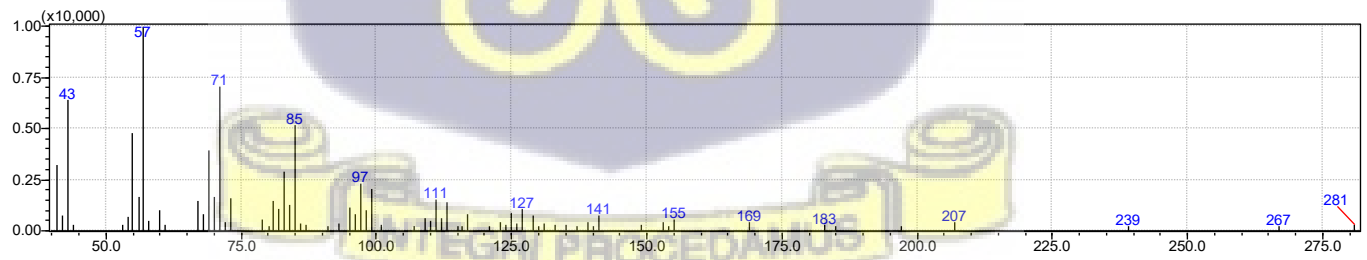

Figure 19: CoSp male cuticular compound (v)

The remaining compounds did not occur in the replicated samples.

Male whole-body compounds and diagnostics

The analyte appearing at 9.469/9.098 minute (figure 20), produced generated key fragment ions corresponding to the compound 4-Hydroxy-4-methyl-2-pentanone. at *m/z* 59 (alpha-cleavage- at hydroxy side resulting in a highly stable acylium cation), 101 (another possible alpha-cleavage- at hydroxy side removing a methyl radical), 83 (terminal -OH results in dehydration and allylic cation), 98 (small weak M-18 peak resulting from -OH dehydration) and 43 as base peak (a result of alpha cleavage at carbonyl side, producing an acylium ion).

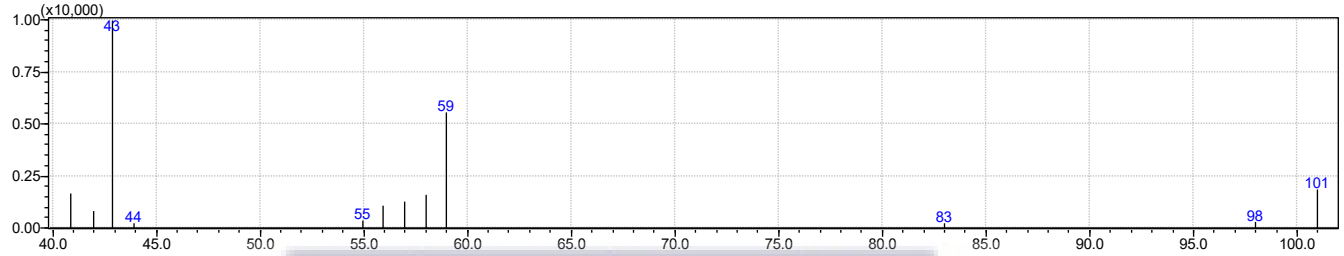

Figure 20: CoSp male whole-body compound (i)

The compound peak showing at 18.95/ 18.942 minutes (figure 21) produced base peak *m/z* 69, which is indicative of an alkene or a nitrile compound. other key fragment ions are odd numbered, at *m/z* 57, 71 and 43 thus a hydrocarbon. *m/z* 55 and 83 also support alkene functionality.

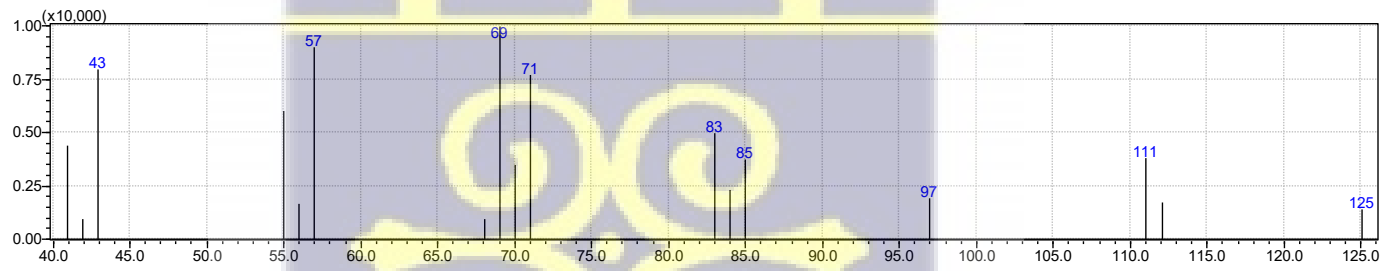

Figure 21: CoSp male whole-body compound (ii)

Here again (figure 22) at 21.294/21.066 minutes, the peak occurring produced fragment ions odd numbered major fragments with mass 14 differences, indicating hydrocarbon. The matches are 2 6 10 trimethyl tridecane or 3 ethyl 2 6 10 trimethyl undecane. Fragment ions 71 (highly stable sec-pentyl cation when cleavage occurs at c9-c10, also c4-c5 cleavage to generate an isopentyl cation/ c6-c7 cleavage resulting in an isopentyl cation), 43(c2-c3 cleavage resulting in a stable isopropyl cation or c10-c11 cleave to produce a propyl cation / c9-c10 or c2-c3 cleavage resulting in stable isopropyl cation) , 85, 99, 113, 55, 41 and 57(c3-c4 cleavage releasing an isobutyl cation /cleavage at c3-c4 to produce a stable isobutyl cation which stabilizes by hydride shift and rearrangement to tert)) as base peak, corresponds to a multiple- methyl branched compounds.

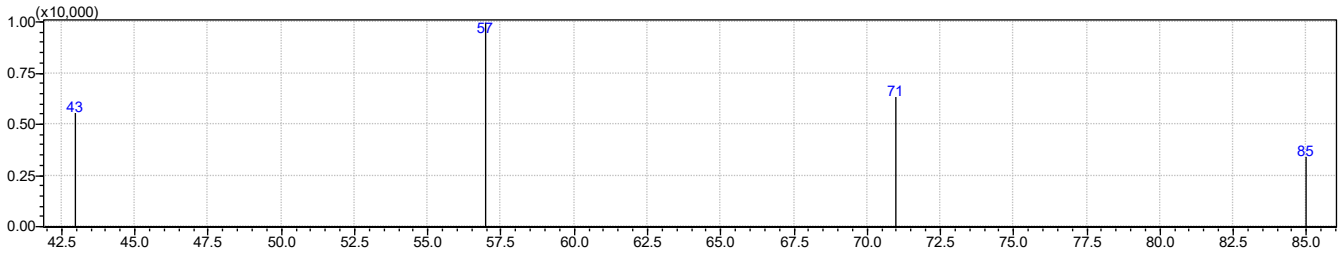

Figure 22: CoSp male whole-body compound (iii)

The peak appearing at 32.8 and 32.6 minutes (figure 23) indicate a long chain alkane and 57 for base peak formed from cleavage at c3-c4 forming a stable isobutyl which was observed at 31.809/31.965 minutes on the GC chromatogram. this compound could be attributed as a long chain hydrocarbon.

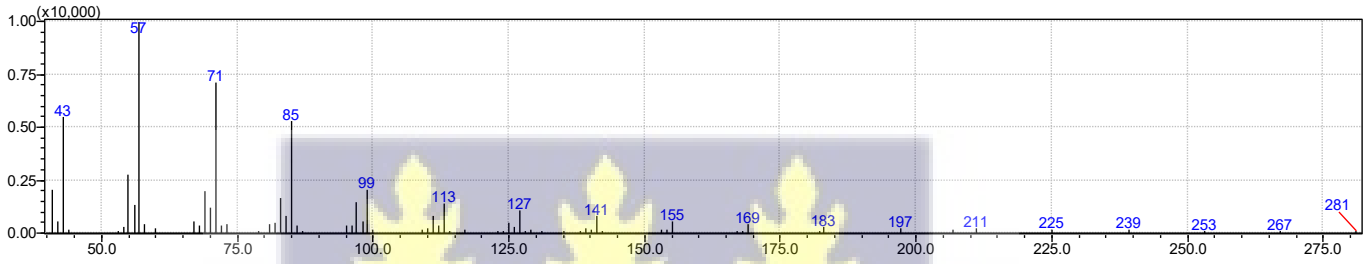

Figure 23: CoSp male whole-body compound (iv)

The analyte appearing at 33.16 minutes (figure 24), produced fragment ions conforming to the pattern of a long chain alkane compound. With base peak at *m/z* 57, the compound would correspond to a long chain alkane.

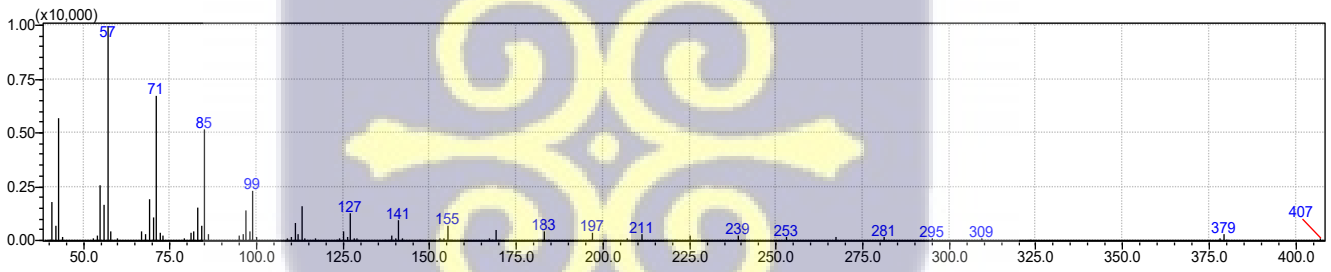

Figure 24: CoSp male whole-body compound (v)

The remaining compounds did not occur in the replicated samples.

Female volatile compounds and diagnostics

The compound occurring at 9.19 /9.46 minutes (figure 25) generated key fragment ions 58 (resulting from a McLafferty rearrangement when carbonyl is at c2), 5758 (resulting from a McLafferty rearrangement when carbonyl is at c2 or an acylium ion when carbonyl is at c3), 71 and 43 base peaks resulting from an acylium ion when the carbonyl is at position c2. The pattern corresponds to the

compound 6,10-Dimethylundecan-2-one. Thus, requires further investigation. The fragment peak at *m/z* 55 indicates a cyclic saturated ketone.

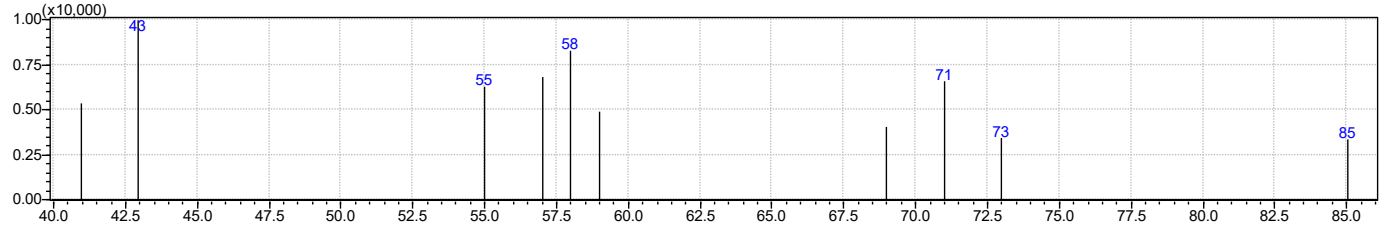

Figure 25: CoSp female headspace volatiles compound (i)

At 31.2 minutes (figure 26), the peak occurring produced fragment ions at 71, 85, 43, 281 *m/z* with 57 as base peak corresponding to fragmentation pattern of a compound containing long chain alkane. Proposed structures were not compatible with observed pattern. The obtained RI was 2758.

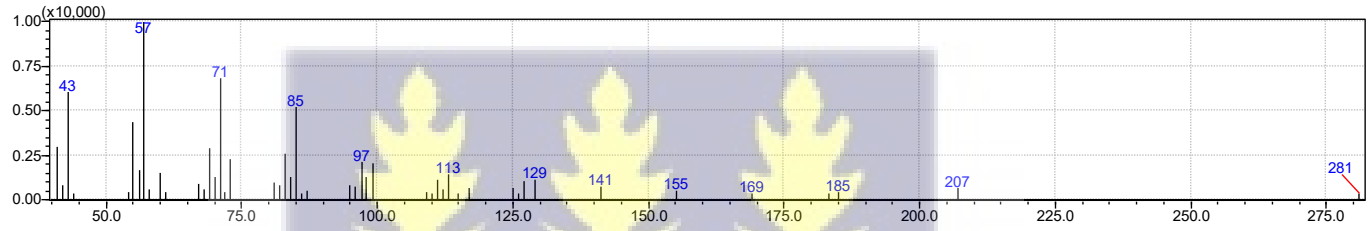

Figure 26: CoSp female headspace volatiles compound (ii)

The remaining compounds did not occur in the replicated samples.

Female cuticular compounds and diagnostics

The compound occurring at 9.19 /9.46 minutes (figure 27) generated key fragment ions at *m/z* 57, 71, 85, 43 and 99. The base peak was *m/z* 57 with *m/z* 71 following closely, corresponding to the compound 4, 8 dimethyl dodecane. Cleavage of a stable butyl fragment affords the base peak, whiles fragment of a 2-pentyl cation produces the *m/z* 71. Both of these occur at the carbon branch site, which is most common fragmentation pattern for branched alkanes.

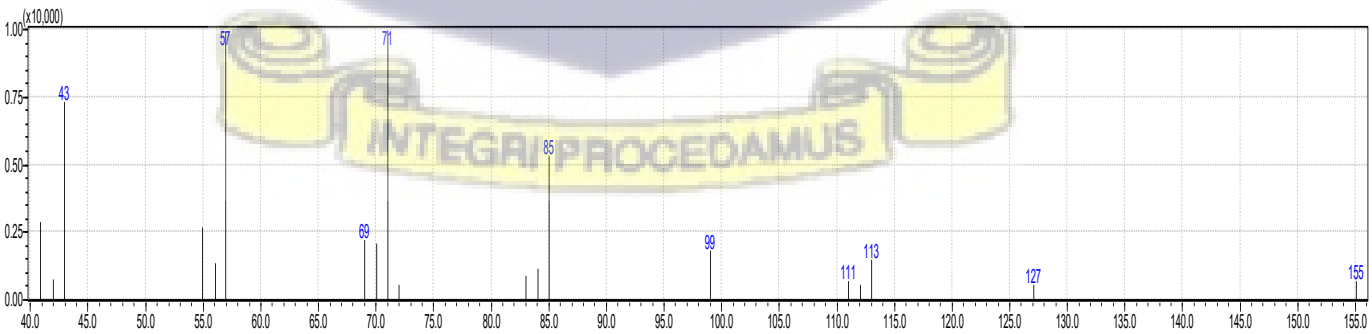

Figure 27: CoSp female cuticular compound (i)

At 20.9 minutes (figure 28), the compound identified had *m/z* 57 for its base peak. This compound is matched to a -diol compound whose chain length is too short to be found at this position. The pattern shows 14n+1 fragment ions, and an *m/z* 55, which indicates an alcohol or alkene.

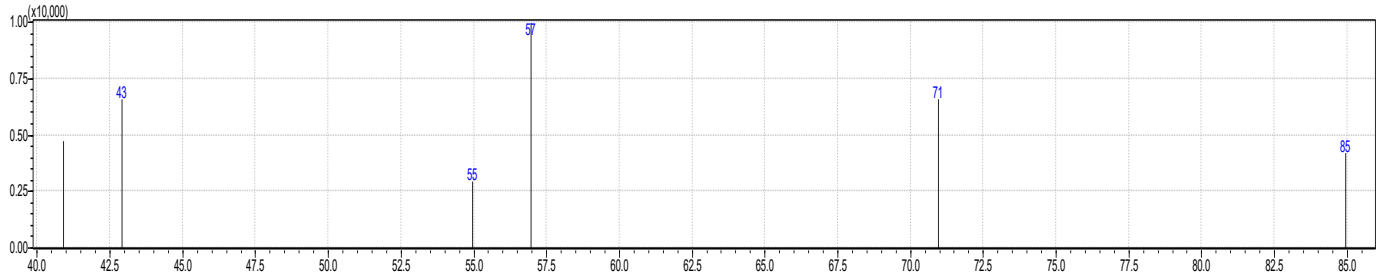

Figure 28: CoSp female cuticular compound (ii)

For the peak occurring at 24.8 minutes (figure 29), *m/z* 74 was the base peak, which connotes an McLafferty rearrangement of a methyl ester. The match is made to Methyl 12-methyltetradecanoate.

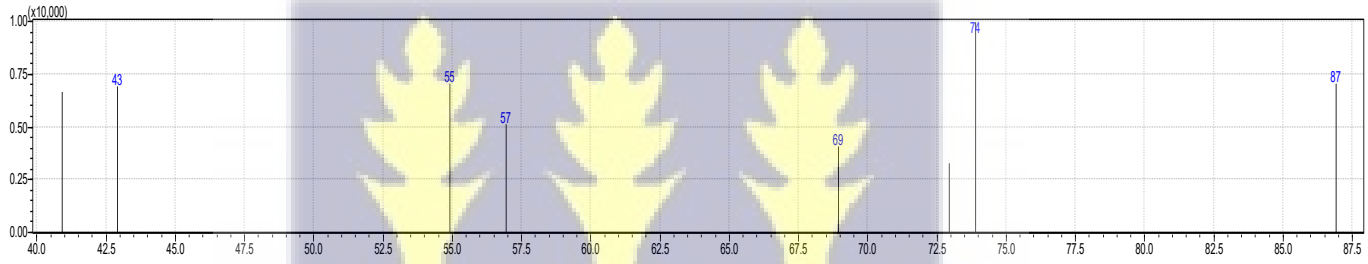

Figure 29: CoSp female cuticular compound (iii)

At 25.9 minutes (figure 30), the peak was designated as octyl 10-undecenoate. An alkyl-branched 10-undecenoic acid is definitely warranted as the carboxylic acid functionality can be described by *m/z* 60, 45, whiles *m/z* 55, 69, 73 are the result of alkene functionality.

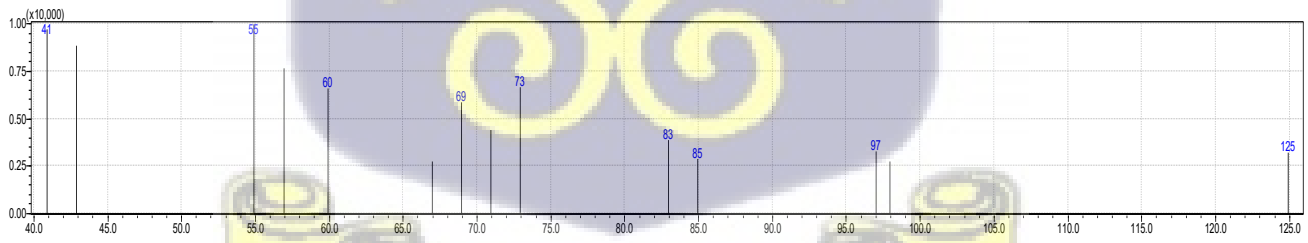

Figure 30: CoSp female cuticular compound (iv)

The fragmentation pattern (figure 31) for the peak at 26.5 minutes has *m/z* 67 for a base peak, with *m/z* 55 and 81 being the next most abundant. This profile is attributed to 9,12-Octadecadienoic acid (Z, Z). while *m/z* 60 relays the carboxylic acid functionality, *m/z* 55, 73 allude to alkene functional groups.

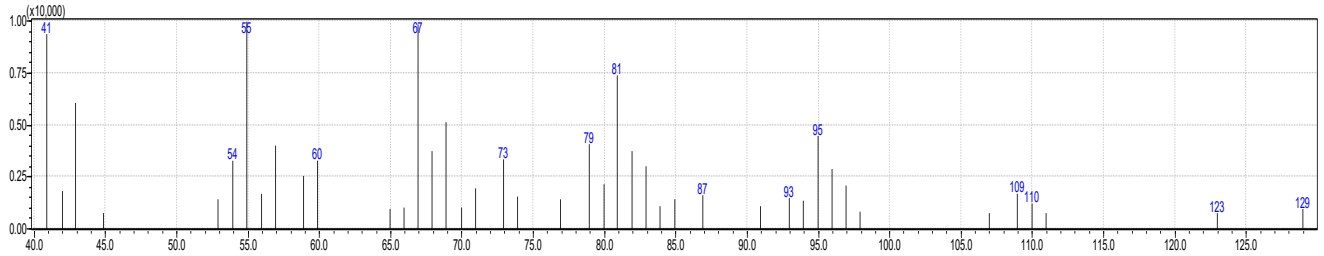

Figure 31: CoSp female cuticular compound (v)

While *m/z* 60 present in this spectrum (figure 32) indicates a carboxylic acid from a McLafferty rearrangement, *m/z* 73 can also be obtained from cleavage at c3-c4. *m/z* 55 would be provided by an allylic cation from an alkene. This compound is denoted 9-Eicosenoic acid, (Z)-.

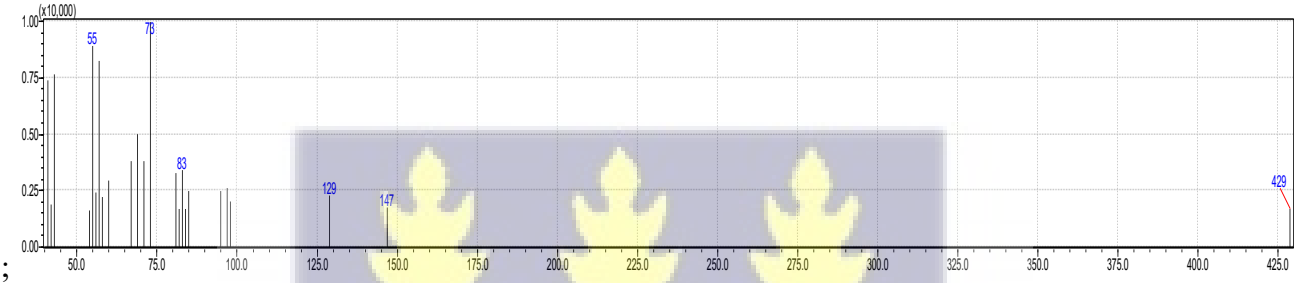

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Figure 32: CoSp female cuticular compound (vi)

Figure 33 The proposed compounds for the peak at 32.27 minutes (figure 33) are Methyl 2-oxooctadecanoate or 2-Hexyl-1-decanol. The base peak here is *m/z* 57. While the fragmentation pattern follows the general 14 amu characteristic of long chain alkanes, *m/z* 60 would result from a McLafferty rearrangement of a carboxylic acid which is absent in both. Also, *m/z* 55 and 81 and 69 would denote an alkene or alcohol.

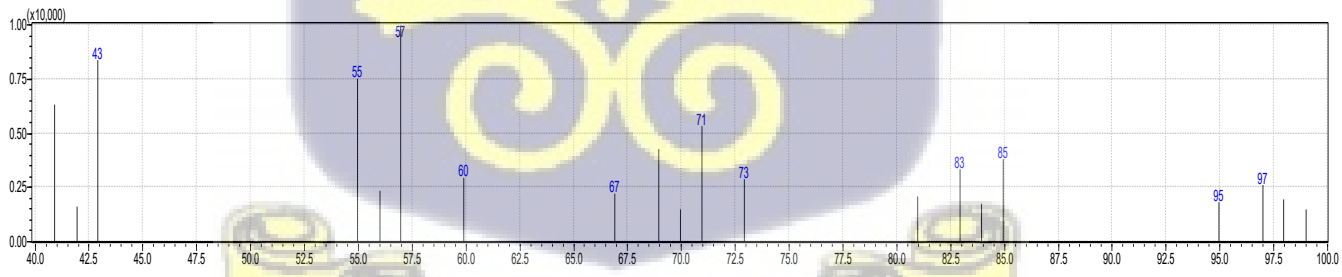

Figure 33: CoSp female cuticular compound (vii)

With fragmentation pattern in the general pattern of 14n+1, the species at 22.3 minutes is denoted 2-methylnonacosane, a methyl-branched alkane (figure 34).

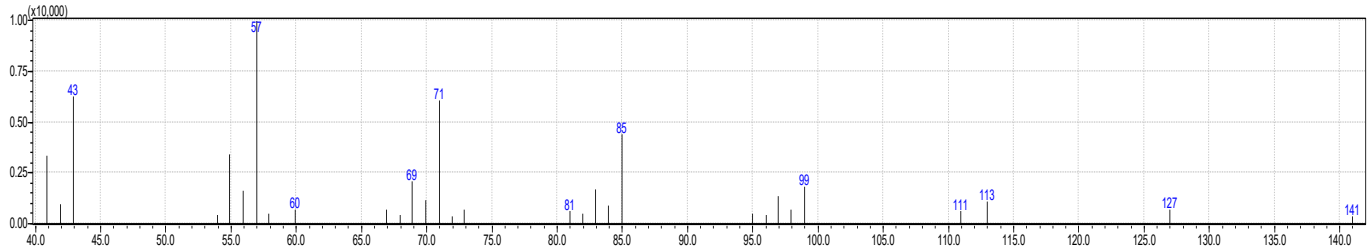

Figure 34: CoSp female cuticular compound (viii)

Female whole-body compounds and diagnostics

The compound (figure 35) occurring at 18.9 minutes generated key fragment ions at *m/z* 57, 71, 85, 43 and 99. The base peak was *m/z* 57 with *m/z* 71 following closely, corresponding to the compound 4, 8 dimethyl dodecane. Cleavage of a stable butyl fragment affords the base peak, whiles fragment of a 2-pentyl cation produces the *m/z* 71. Both of these occur at the carbon branch site, which is most common fragmentation pattern for branched alkanes.

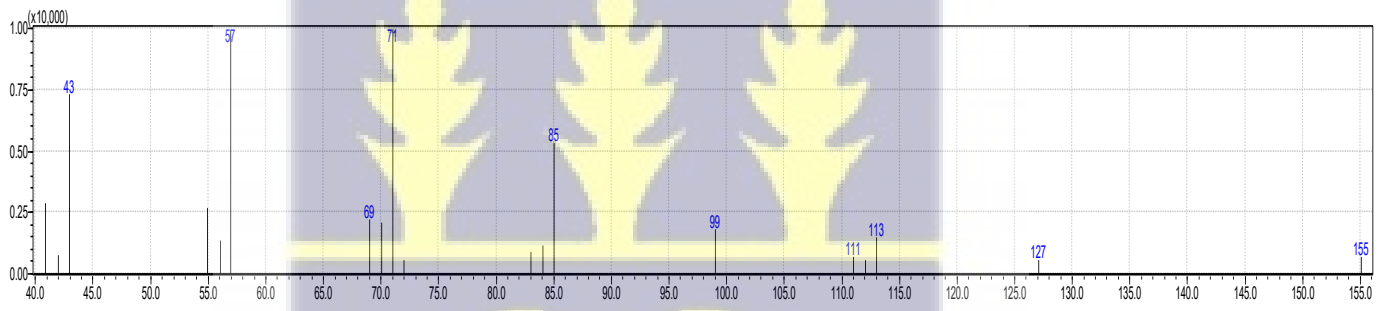

Figure 35: CoSp female whole-body compound (i)

At 20.9 minutes, the compound (figure 36) identified had *m/z* 57 for its base peak. This compound is matched to a -diol compound whose chain length is too short to be found at this position. The pattern shows 14n+1 fragment ions, and an *m/z* 55, which indicates an alcohol or alkene.

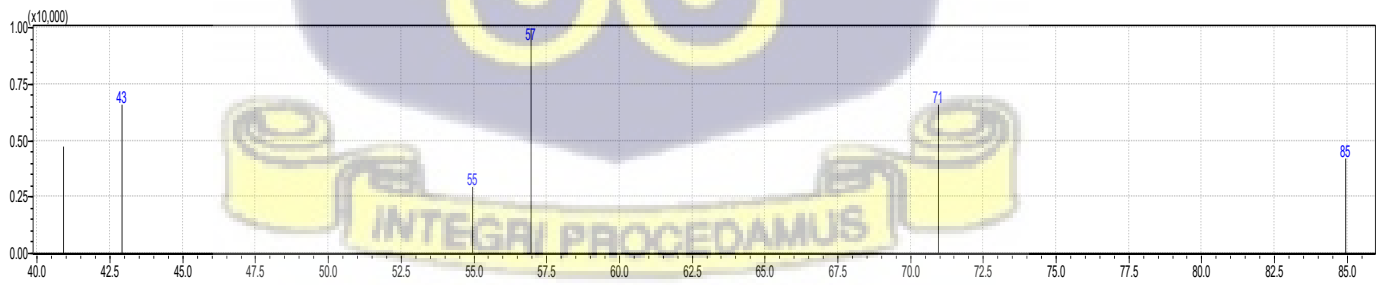

Figure 36: CoSp female whole-body compound (ii)

For the peak occurring at 24.8 minutes, *m/z* 74 was the base peak, which connotes an McLafferty rearrangement of a methyl ester. The match is made to Methyl 12-methyltetradecanoate (figure 37).

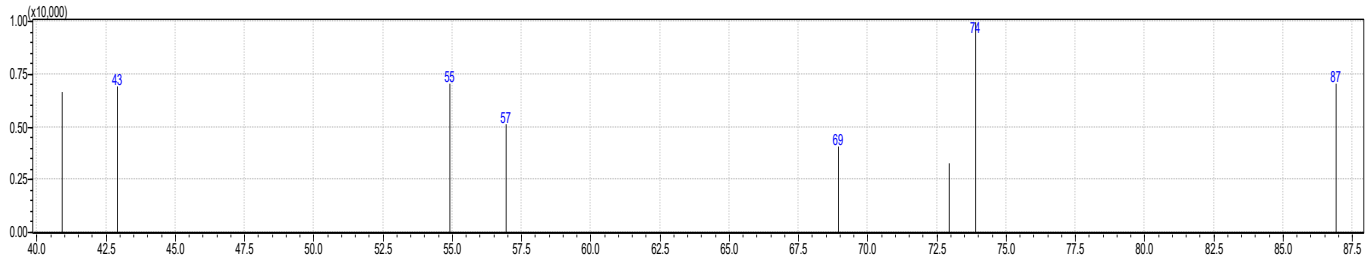

Figure 37: CoSp female whole-body compound (iii)

The fragmentation pattern (figure 38) for the peak at 26.5 minutes has *m/z* 67 for a base peak, with *m/z* 55 and 81 being the next most abundant. This profile is attributed to 9,12-Octadecadienoic acid (Z, Z). while *m/z* 60 relays the carboxylic acid functionality, *m/z* 55, 73 allude to alkene functional groups.

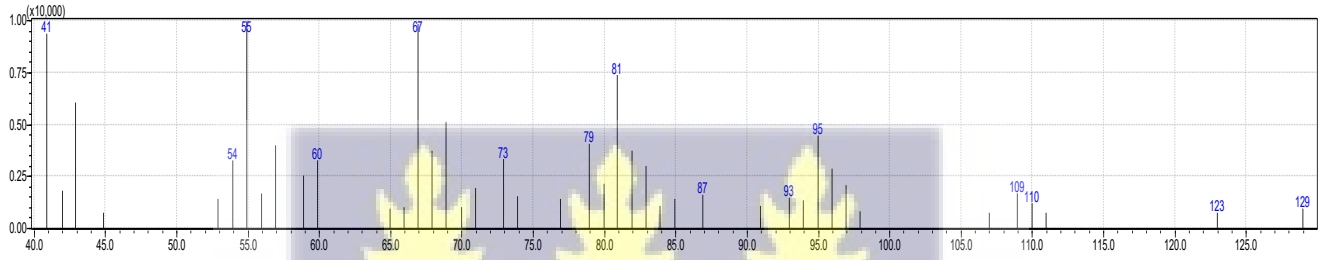

Figure 38: CoSp female whole-body compound (iv)

Figure 39 While *m/z* 60 present in this spectrum indicates a carboxylic acid from a McLafferty rearrangement, *m/z* 73 can also be obtained from cleavage at c3-c4. *m/z* 55 would be provided by an allylic cation from an alkene. This compound is denoted 9-Eicosenoic acid, (Z)- (figure 39).

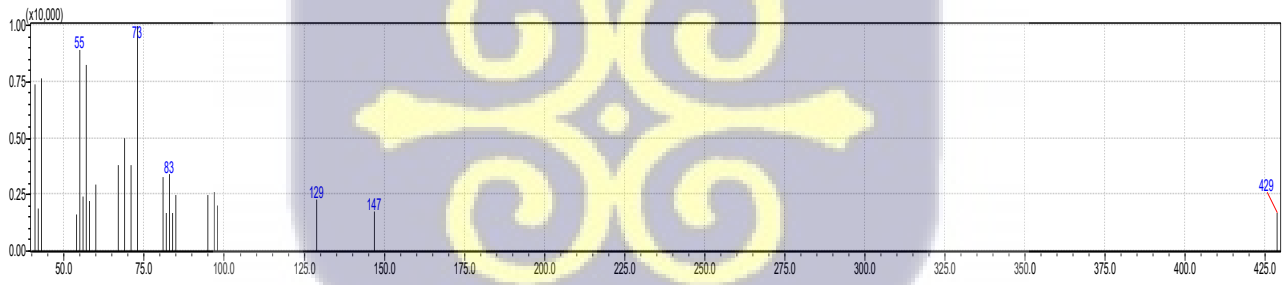

Figure 39: CoSp female whole-body compound (v)

At 32 minutes, a base peak of *m/z* 73 is obtained (figure 40). This is likely the presence of an aldehyde.

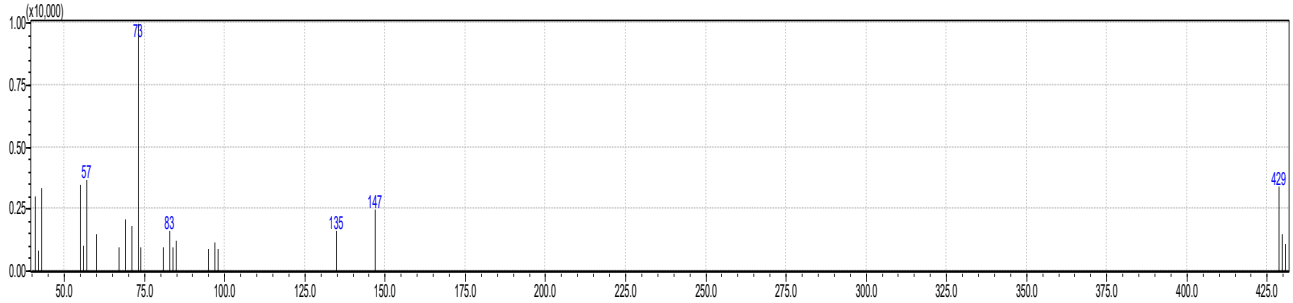

Figure 40: CoSp female whole-body compound (vi)

The proposed compounds for the peak at 32.27 minutes are Methyl 2-oxooctadecanoate or 2-Hexyl-1-decanol (figure 41). The base peak here is *m/z* 57. While the fragmentation pattern follows the general 14 amu characteristic of long chain alkanes, *m/z* 60 would result from a McLafferty rearrangement of a carboxylic acid which is absent in both. Also, *m/z* 55 and 81 and 69 would denote an alkene.

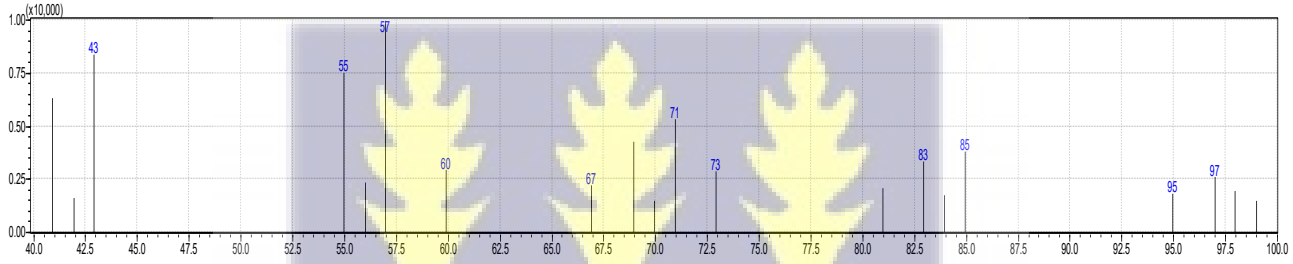

Figure 41: CoSp female whole-body compound (vii)

The remaining compounds did not occur in the replicated samples.

***Anagyrus* spp. [AnSp]**

Male volatile compounds and diagnostics

The peak appearing at 12.5 minutes, produced as base peak *m/z* 59 (figure 42). *M/z* 59 indicates presence of an ester, typically a methyl ester *m/z* 45 (an ester or a carboxylic acid). The structure matched pattern for Carbonic acid, 2-ethoxyethyl 2-methoxyethyl ester proposed. Key fragment ions could be identified 45 (ethoxy cation fragment) 72, 103.

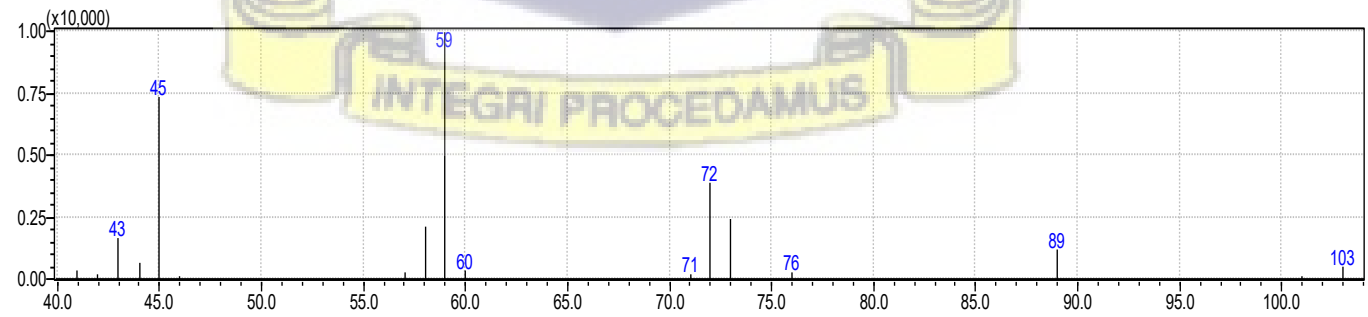

Figure 42: AnSp male headspace volatile compound (i)

The compound (figure 43) occurring at 9.19 /9.46 minutes generated key fragment ions *m/z* 43, 71, 85, 99 and 57 base peaks indicating a butyl cation fragment from an alkane. The compound is reported as 2-Methyl-n-hexacosane.

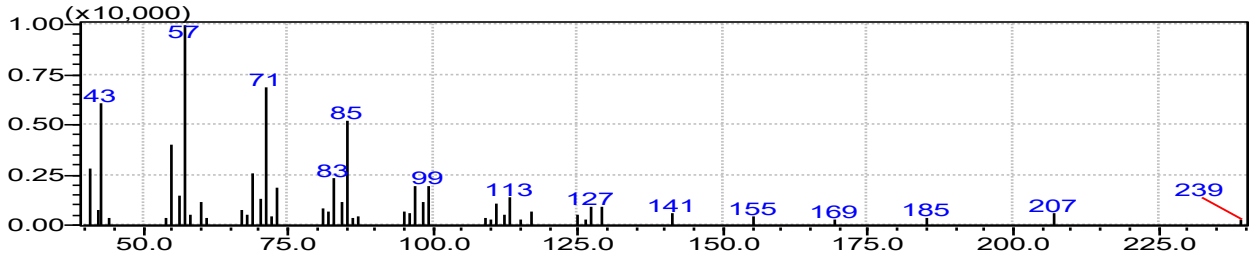

Figure 43: AnSp male headspace volatile compound (ii)

The fragment ions of 14n+1 intervals and *m/z* 57 for base peak which was observed at 32 minutes (figure 44) on the GC chromatogram was matched to the compound 2 methyl heptacosane, a long chain methyl branched alkane.

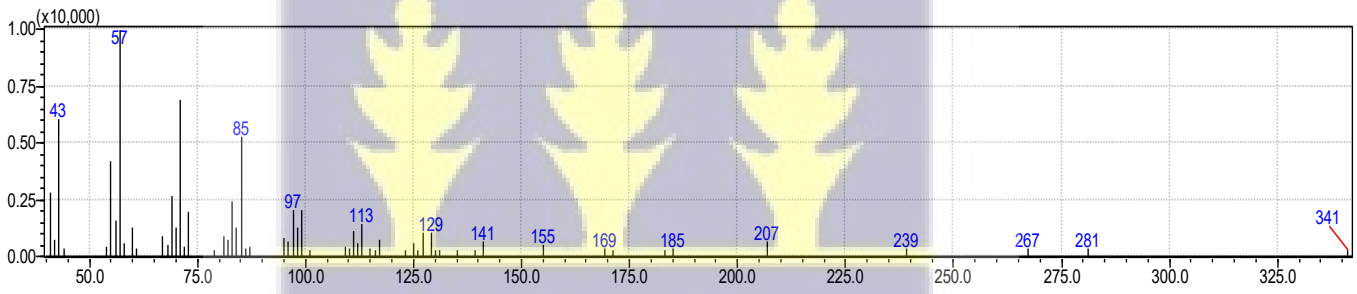

Figure 44: AnSp male headspace volatile compound (iii)

At 32.8 minutes, the peak occurring produced fragment ions at 14amu intervals (figure 45) with *m/z* 57 as base peak indicating a long chain alkane. This compound was identified to be 2 methyl octacosane.

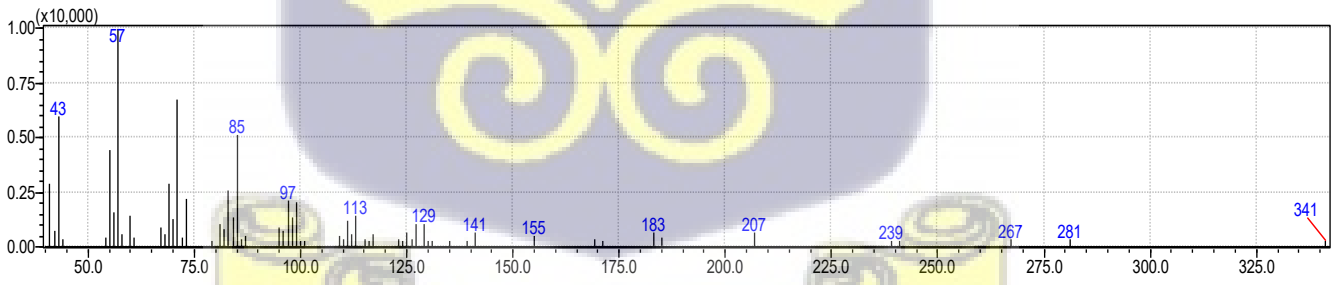

Figure 45: AnSp male headspace volatile compound (iv)

The remaining compounds had peak areas below sufficient detection range or did not occur in the replicated samples.

Male cuticular compounds and diagnostics

The analyte appearing at 26.32 minute (figure 46), produced an even-numbered molecular ion at 264 with key fragment ions occurring at 14 intervals indicating the presence of a hydrocarbon chain. The fragment peak at 55 is indicative of a cyclic or saturated ketone. This also happens to be the base peak as shown above. This proposed compound is an 18-carbon cyclic ketone with one alkene bond, or an analogue with molecular formula $C_{18}H_{32}O$. as molecular weight is 2 less than a fully saturated compound and ion clusters rather than relatively solitary ones.

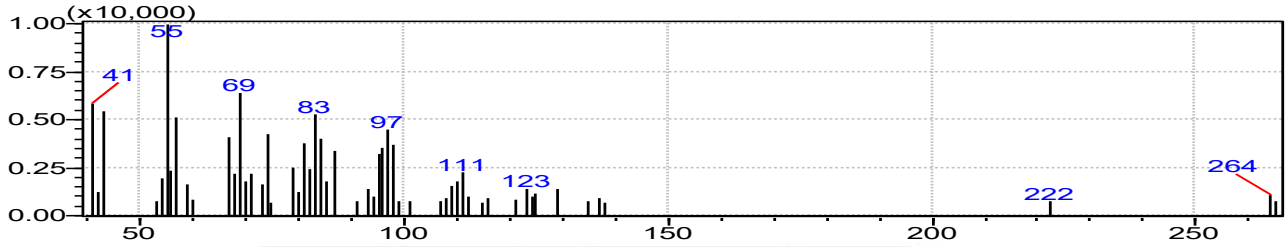

Figure 46: AnSp male cuticular compound (i)

The peak appearing at 27.88/27.93 minutes, produced main molecular ions of 14amu difference, indicative of a long chain alkane, *m/z* 57 as base peak results from n-butyl cation, 43 from n-propyl cation e, 60 is the result of McLafferty rearrangement on a terminal carboxylic acid functional group, which may be less prominent due to longer chain. The profile corresponds to the compound Eicosanoic acid (figure 47).

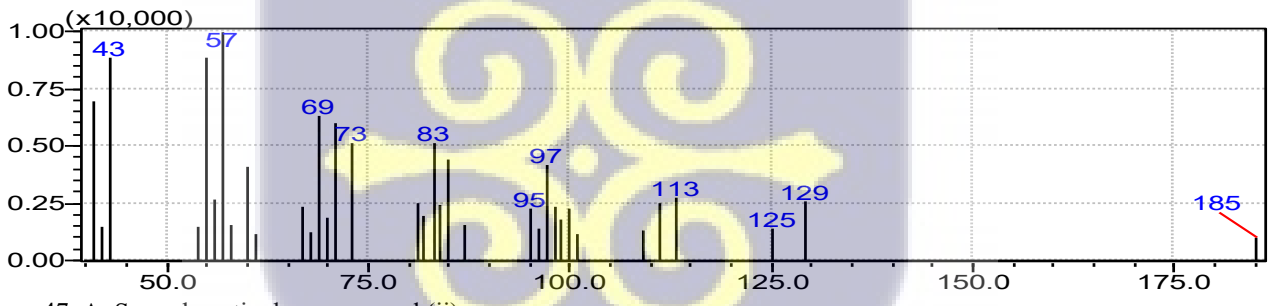

Figure 47: AnSp male cuticular compound (ii)

The compound occurring at 28.94 minutes (figure 48) generated key fragment ions similar to above (figure 47). Here however, the base peak occurs at *m/z* 55 (allylic cleavage at c16-c17), while *m/z* 59 is more prominent from a normal hydrocarbon cleavage at c3-, corresponding to the compound 18-eicosenoic acid.

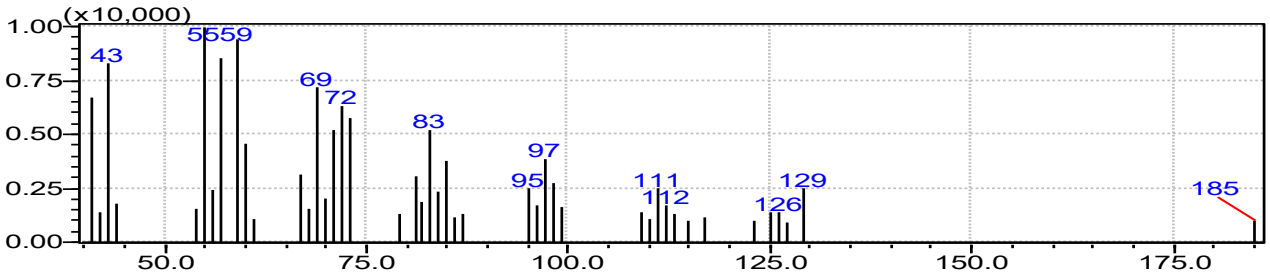

Figure 48: AnSp male cuticular compound (iii)

The analyte appearing at 30.08 minutes (figure 49), produced a molecular ion peak at *m/z* 57 which could be an acylium ion of a ketone or butyl cation fragment of an alkane chain. The large fragment ion at *m/z* 315 could indicate a loss of a methyl radical, assuming *m/z* 330 is the parent peak. This peak corresponds to a methyl ketone compound.

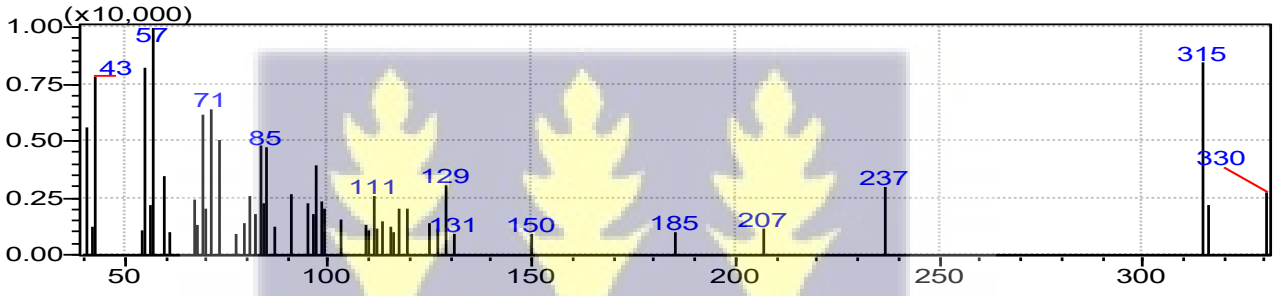

Figure 49: AnSp male cuticular compound (iv)

The peak appearing at 30.27 minutes produced fragmentation patterns with base peak *m/z* 57 (figure 50) forming from an isobutyl and n-butyl cation as well as other fragments corresponding to 2-Methylpentacosane compound.

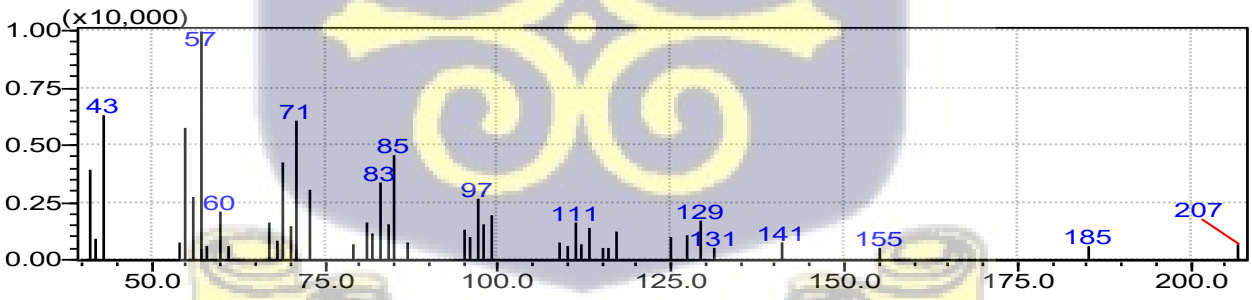

Figure 50: AnSp male cuticular compound (v)

At 30.34 / 30.4 minutes, the fragmentation pattern (figure 51) was a long homologous series of related ions 14 amu apart at *m/z* = 43, 57, 71, 85, 99, 111, etc. With base peak *m/z* 57 forming from an isobutyl and n-butyl cation as well as other fragments identical to 2-Methylhexacosane.

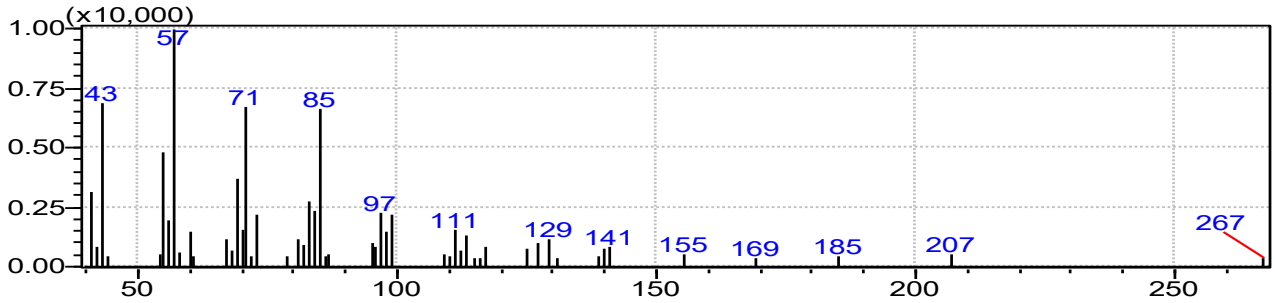

Figure 51: AnSp male cuticular compound (vi)

The analyte appearing at 30.7 minute, had fragment ions (figure 52) consistent with long chain alkane such as *m/z* 43, 57, 71, etc., as well as an aldehyde at *m/z* 43, 44, 73. This peak corresponds to an aldehyde or ketone compound.

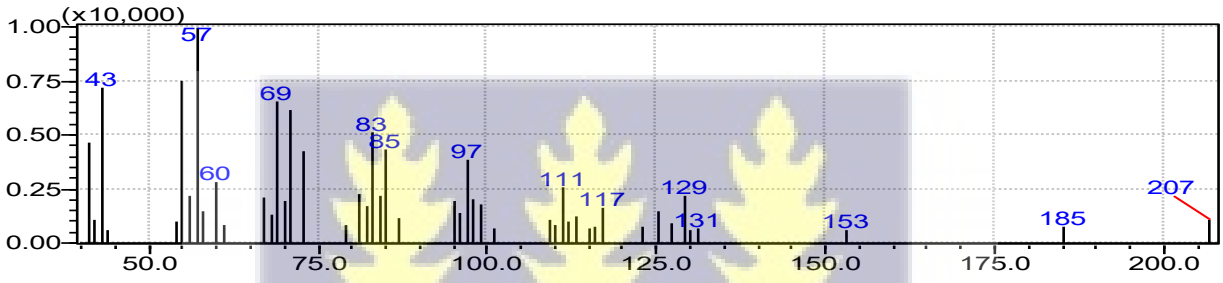

Figure 52: AnSp male cuticular compound (vii)

The peak appearing at 31.5 minutes, has fragment peaks similar once again to a long chain alkane (figure 53). 2 methylheptacosane is implicated.

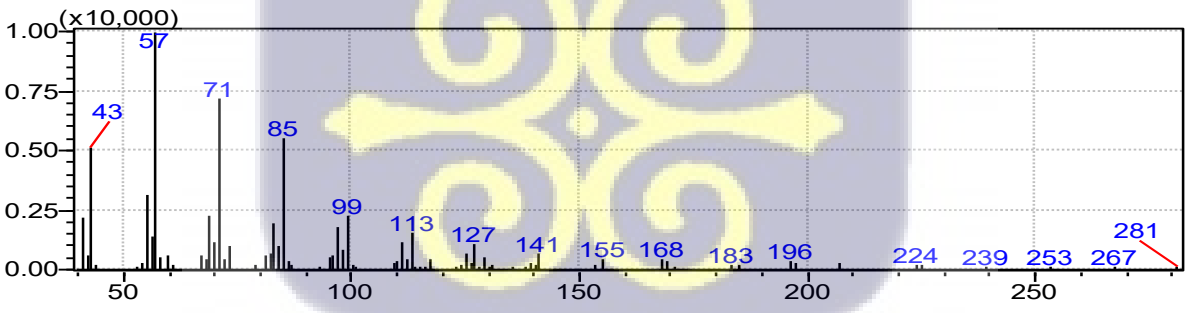

Figure 53: AnSp male cuticular compound (viii)

At 31.7/31.9.minutes, the peak occurring produces fragment ions corresponding to 2 methyloctacosane as a longer chain methyl branched alkane (figure 54).

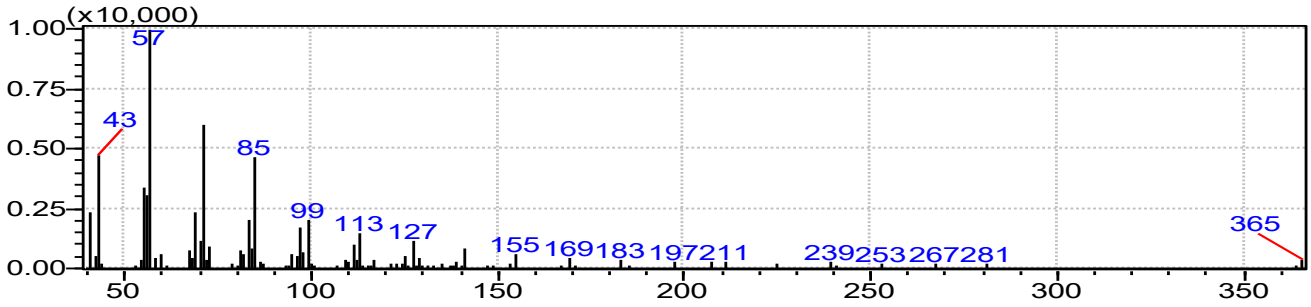

Figure 54: AnSp male cuticular compound (ix)

The remaining compounds did not occur in the replicated samples.

Male whole-body compounds and diagnostics

The analyte appearing at 26.04/26.25 minutes, produced fragment ions in accordance with an alkane or an aldehyde or a ketone (figure 55).

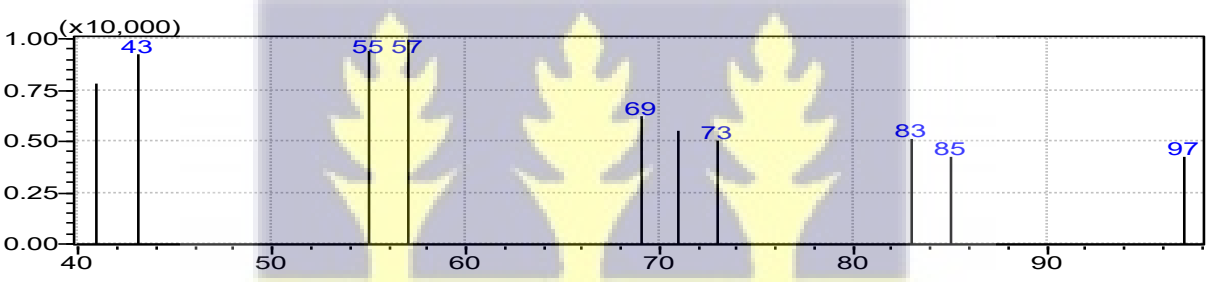

Figure 55: AnSp male whole-body compound (i)

The peak appearing at 26.2/26.3 minutes, corresponds to the compound 9-Octadecenoic acid (Z)-, methyl ester (figure 56). Fragments at *m/z* 74 occurs from a McLafferty rearrangement. Also, *m/z* 59 occurs from the alpha-cleavage of the methyl ester group. The alkene could be positioned at c16 to form the base peak *m/z* 55 which would be the allylic cation.

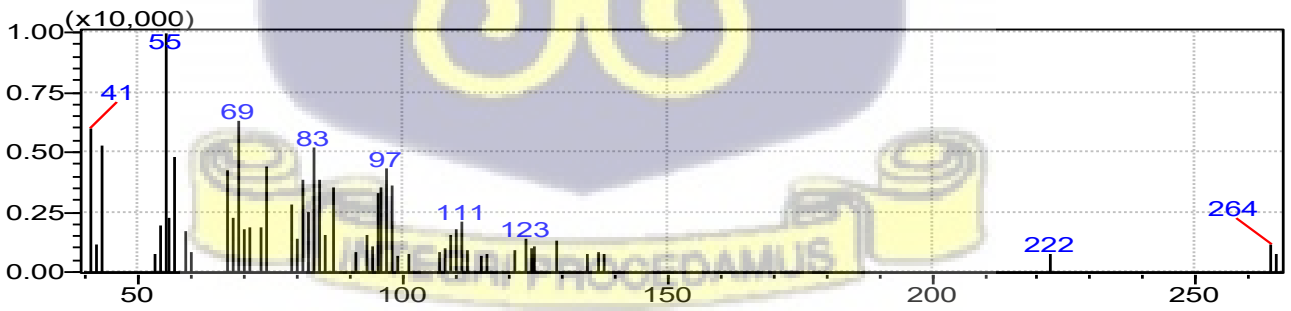

Figure 56: AnSp male whole-body compound (ii)

At 30 minutes, the main peaks occur in 14amu differences (figure 57), signifying an alkane chain. A tall *m/z* of 43 could indicate methyl ketones. The *m/z* 73 gives indications of an aldehyde functional group or

m/z 73 and 87 could be indicative of a carboxylic acid, whilst *m/z* 60 could be the result of a McLafferty rearrangement of a carboxylic acid. This compound could be a saturated carboxylic acid.

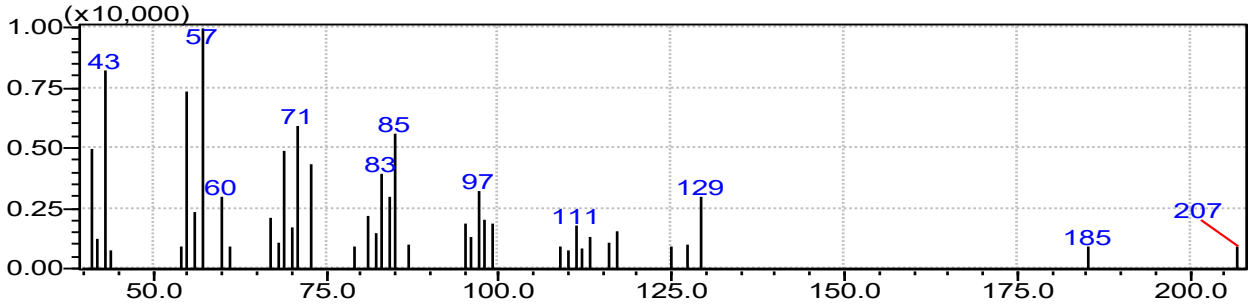

Figure 57: AnSp male whole-body compound (iii)

The compound (figure 58) occurring at 30.8 presents similar to the previous spectrum

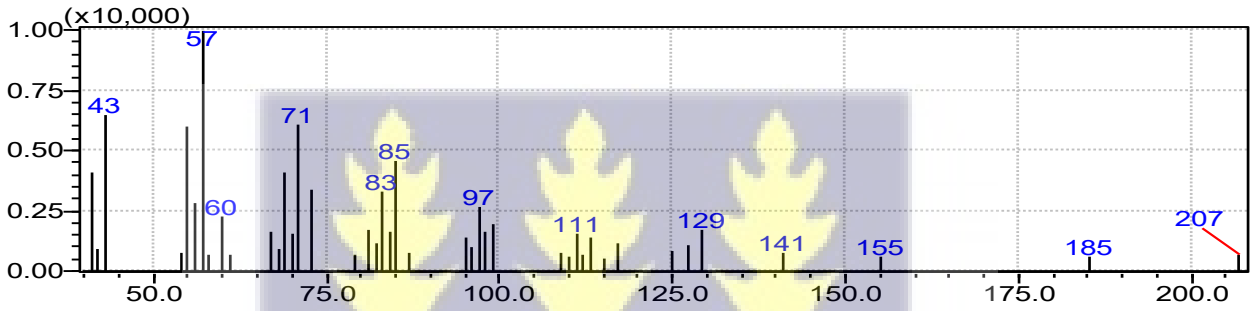

Figure 58: AnSp male whole-body compound (iv)

At 32.2 minutes, 2-Methyl Octacosane is obtained (figure 59) with the characteristic long chain hydrocarbon fragmentation pattern of CH_2 interval. The compound might as well contain one degree of unsaturation from an alkene group, as *m/z* 55 and *m/z* 41 have high abundances as well.

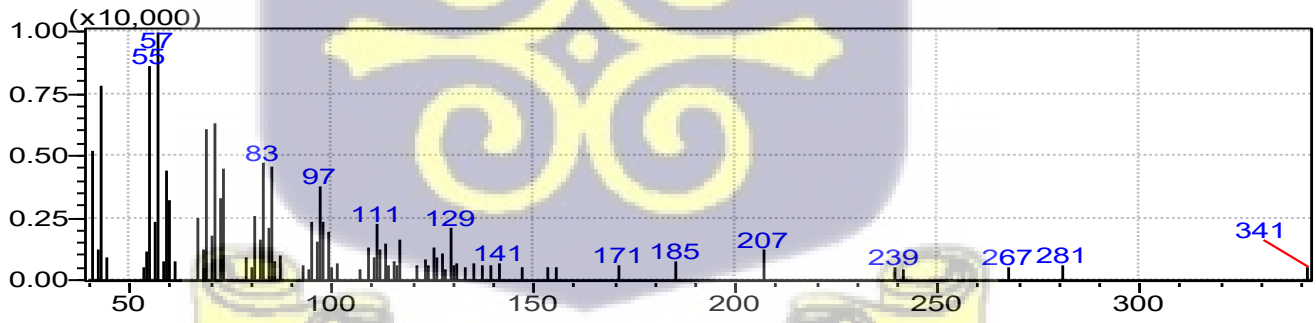

Figure 59: AnSp male whole-body compound (v)

The analyte appearing at 33 minutes, produces ions of 14amu intervals (figure 60) denoting a long chain alkane comparable to a 2 methyl nonacosane.

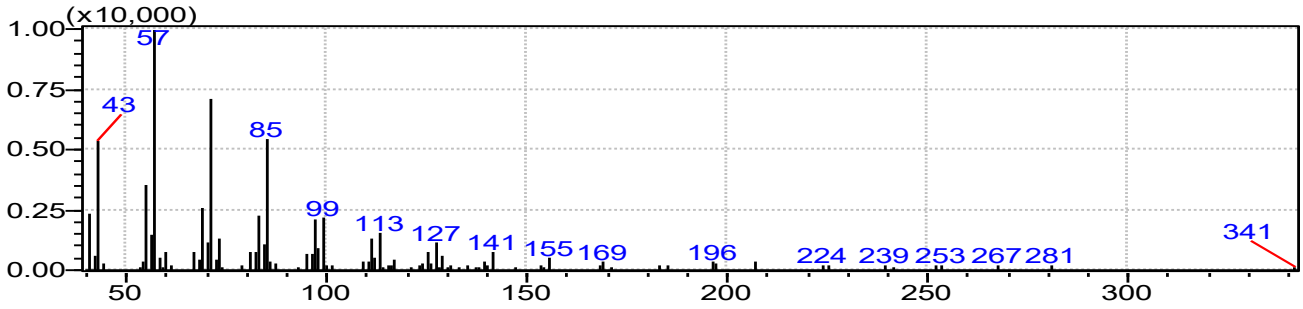

Figure 60: AnSp male whole-body compound (vi)

The remaining compounds did not occur in the replicated samples.

Female volatile compounds and diagnostics

At 9.3 minutes, the peak occurring produced an abundant fragment ion at *m/z* 43 (figure 61) which is indicative of a methyl ketone. This compound was identified to be 2-Pentanone, 4-hydroxy-4-methyl.

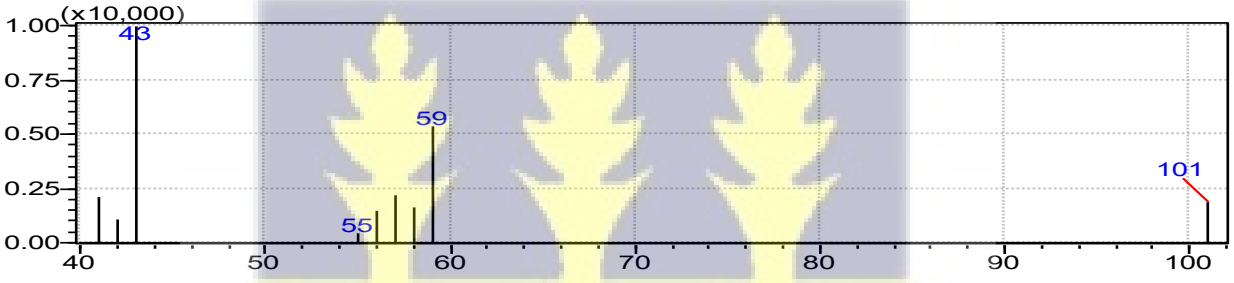

Figure 61: AnSp female headspace volatiles compound (i)

The peak appearing at 9.7/9.6 minutes, produces a base peak of *m/z* 91 (figure 62), which is a tropylium ion of an aromatic hydrocarbon. Its molecular ion [M]+ is obvious at *m/z* 106. The compound is Ethylbenzene.

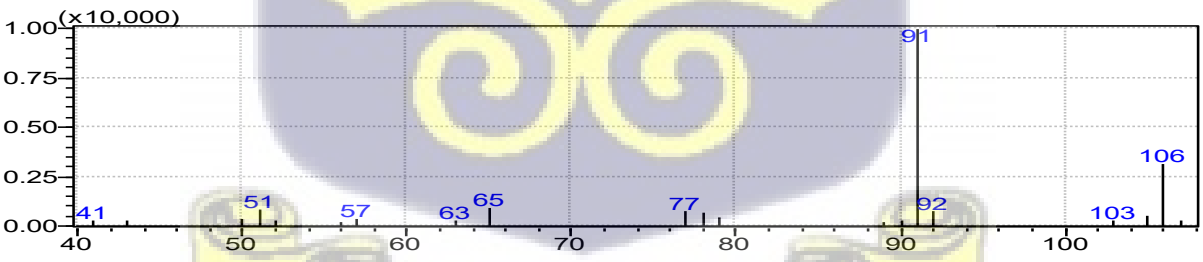

Figure 62: AnSp female headspace volatiles compound (ii)

The analyte appearing at 9.9/9.8 minute, another aromatic hydrocarbon (figure 63) as a result of the tropylium ion was identified to be a xylene.

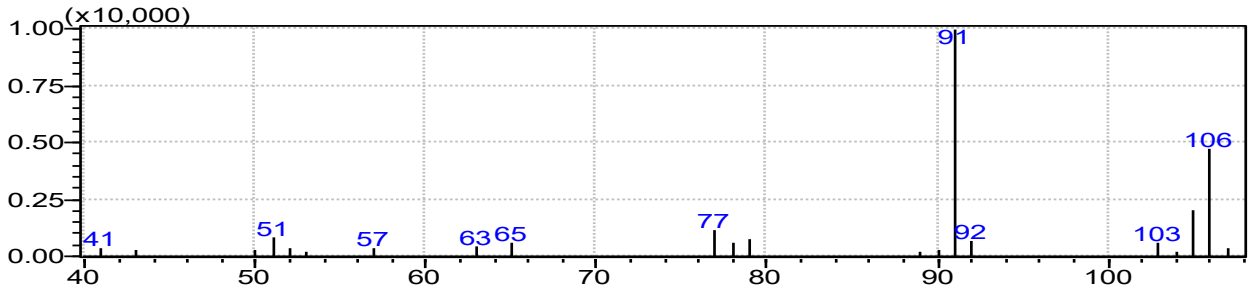

Figure 63: AnSp female headspace volatiles compound (iii)

The compound occurring at 12.9/12.6 minutes generated its base peak at *m/z* 57 (figure 64). The compound, an alcohol, could be 2-Ethyl-1-hexanol. *m/z* 98 is resulted from the loss of H_2O and CH_3. The fragment *m/z* 57 is formed by cleavage at the branch point to afford a stable n-butyl cation fragment.

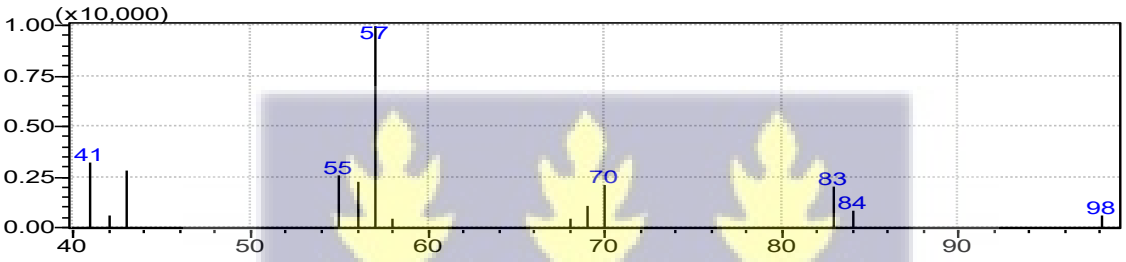

Figure 64: AnSp female headspace volatiles compound (iv)

The analyte appearing at 14.1 minutes, identified to be 3,5,5-Trimethyl-1-hexene (figure 65) produced a base peak at 57, from the formation of a stable tert-butyl cation. An isobutyl radical forms also releasing an iso-pentenyl cation at *m/z* 69. The *m/z* 55 fragment is also formed by the expulsion of the tert-pentyl radical. On the contrary, the fragments of *m/z* 55, 44, 43, 69 and 70 indicate an oxygen functionality.

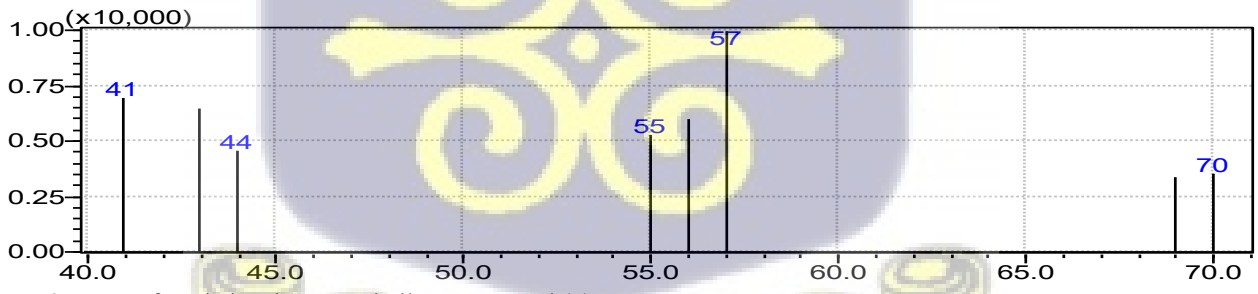

Figure 65: AnSp female headspace volatiles compound (v)

The fragment ions delivered at 17 minutes were matched to 2,2-Dimethyl-3-hexanone (figure 66). The alpha cleavages on either side produce the fragment ions at *m/z* 57 and 43. The tert butyl radical is more stable and thus affords the more abundant *m/z* 57 as base peak.

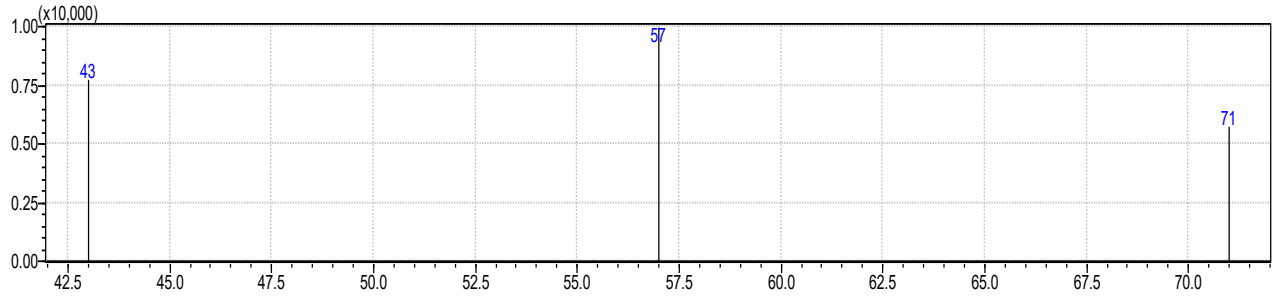

Figure 66: AnSp female headspace volatiles compound (vi)

At 18.4 minutes, the peak occurring produced fragment ions at 14amu intervals (figure 67) corresponding to a straight chain alkane. This compound was identified as 6-ethyl-2-methyl-decane.

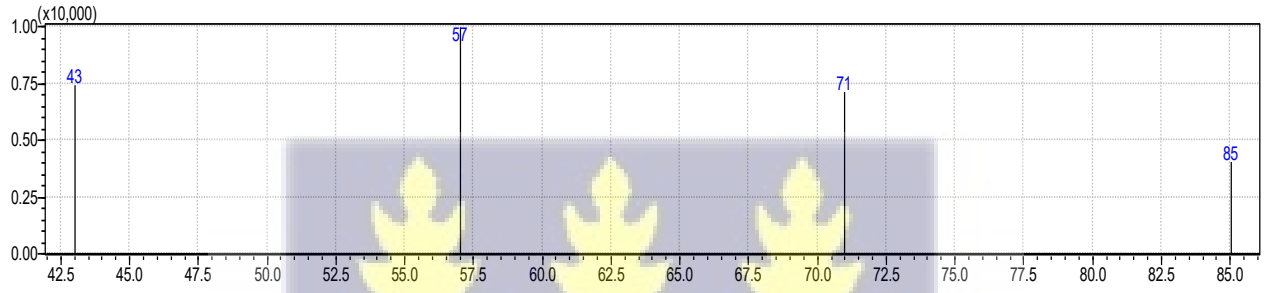

Figure 67: AnSp female headspace volatiles compound (vii)

The fragment ions for the peak at 19.7 minutes also matched to the compound 2, 2-Dimethyl-3-hexanone (figure 68). A ketone is implied, as evident from *m/z* 43.

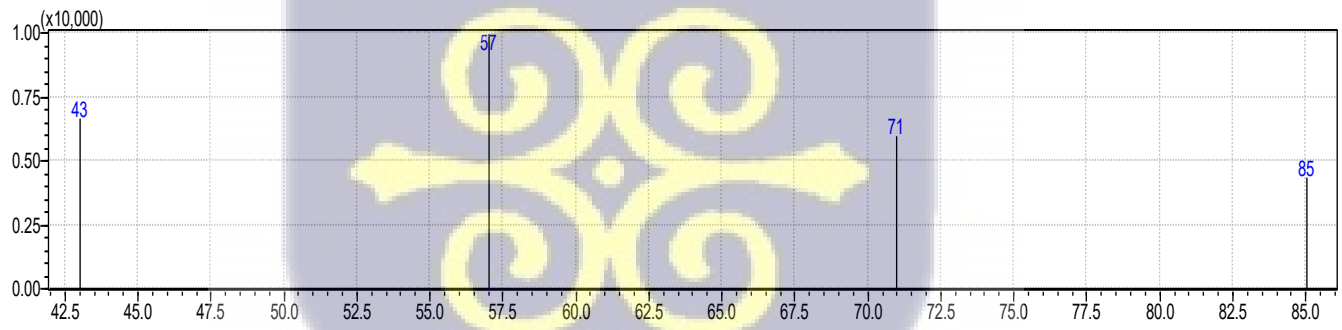

Figure 68: AnSp female headspace volatiles compound (viii)

The peak appearing at 24.6 minutes, producing base peak *m/z* 74 (figure 69), a product of McLafferty rearrangement, is identified as a methyl ester. The fragment ions at *m/z* 57 is the product of cleaving off of 2-butyl cation. The compound is Methyl 12-methyltetradecanoate.

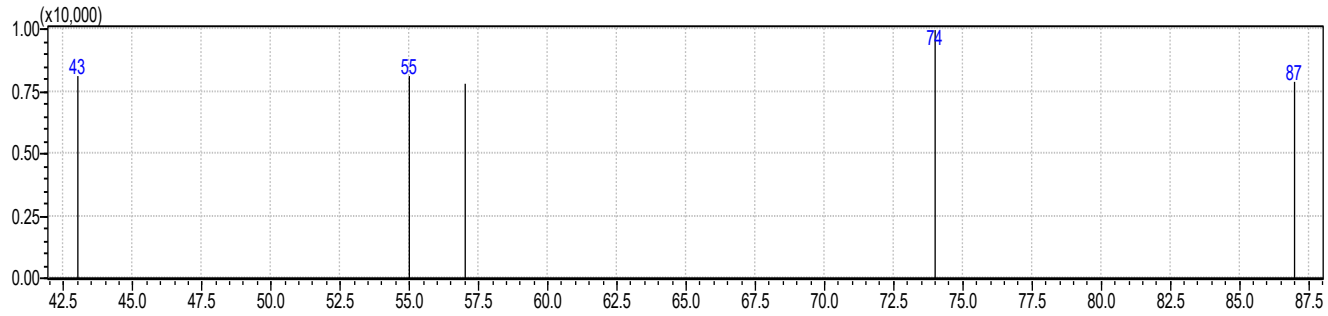

Figure 69: AnSp female headspace volatiles compound (ix)

The analyte appearing at 26.5/26.6 minutes, also produced a base peak at *m/z* 74 (figure 70), indicating a McLafferty rearrangement of a saturated methyl ester. This peak corresponds to compound Methyl octadecenoate. The alkene position is likely at c13 where we can obtain allylic cations at *m/z* 97.

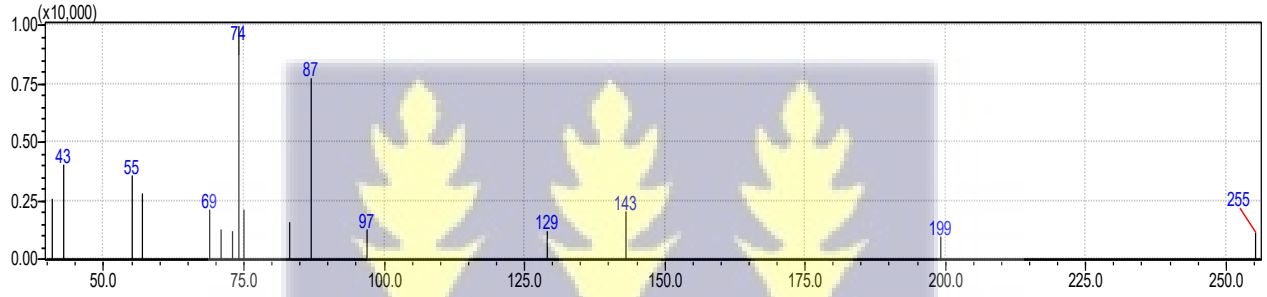

Figure 70: AnSp female headspace volatiles compound (x)

The remaining compounds did not occur in the replicated samples.

Female cuticular compounds and diagnostics

The peak appearing at 12.9 minutes, produced base peak with *m/z* 57 (figure 71). A *m/z* 70 would denote some oxygen functionality. Also, *m/z* 55 and 70 could imply an alkene functionality.

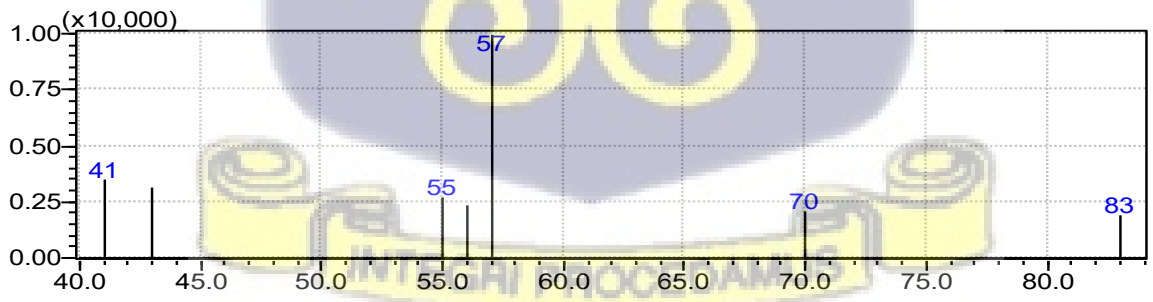

Figure 71: AnSp female cuticular compound (i)

The analyte appearing at 24.3 minutes is identified as compound Oxiran-2-ylmethyl palmitate (figure 72). It produced *m/z* 57 as base peak and some key fragments in 14amu increments, indicating a long

chain alkane. A McLafferty rearrangement of the carboxylic ester yields the fragment at *m/z* 116, while a tridecyl radical breaks off to afford the cation of *m/z* 129.

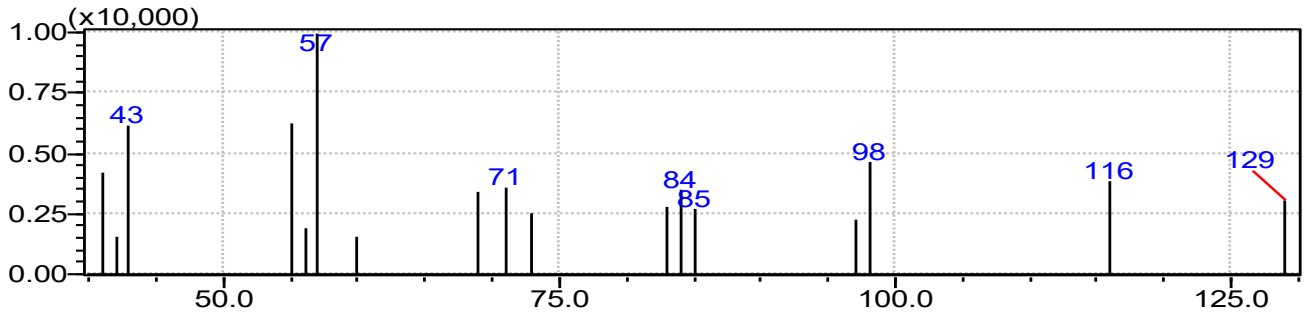

Figure 72: AnSp female cuticular compound (ii)

The compound occurring at 25.4 minutes generated key fragment ions at *m/z* 355, 147, 221, 281 and base peak at *m/z* 73 (figure 73). The peaks *m/z* 73 and 355 allude to the presence of an alkene. The fragment at *m/z* 355 could be the molecular ion for this compound.

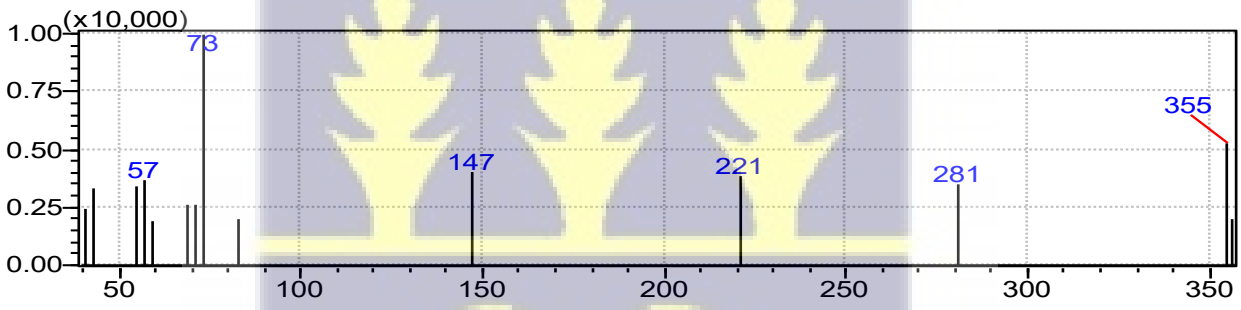

Figure 73: AnSp female cuticular compound (iii)

The fragment ions observed at retention time 26.05 had *m/z* 57 for base peak (figure 74). This could be a carboxylic acid by *m/z* 60 which is usually a McLafferty rearrangement of the functional group. This can be matched to a carboxylic acid.

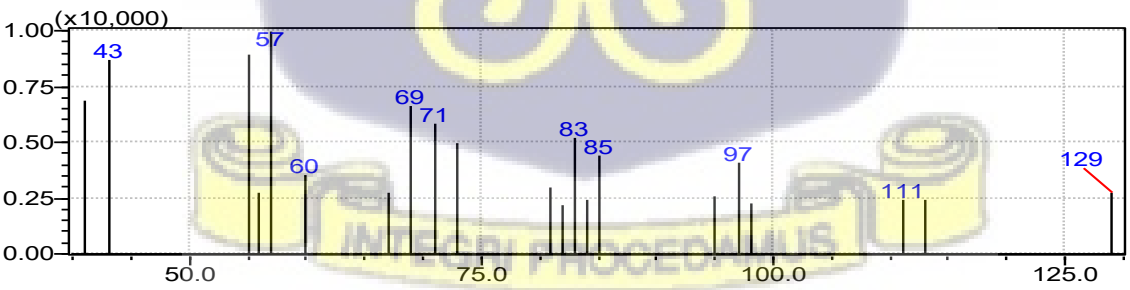

Figure 74: AnSp female cuticular compound (iv)

At 26.54 minutes, Octadecanoic acid, methyl ester (figure 75) was identified with base peak of *m/z* 74, resulting from McLafferty rearrangement. The molecular ion [M]+ was evident at *m/z* 298.

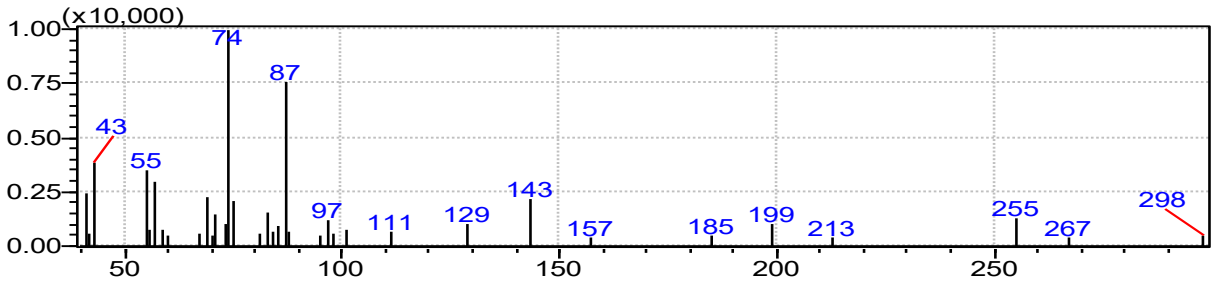

Figure 75: AnSp female cuticular compound (v)

Also at 28.95 minutes, the compound obtained, with retention index calculated as 2426.85 would have a carbonyl functionality (figure 76), either aldehyde (*m/z* 72 from McLafferty rearrangement 60, 44 from McLafferty rearrangement also) or a ketone (*m/z* 43, 55).

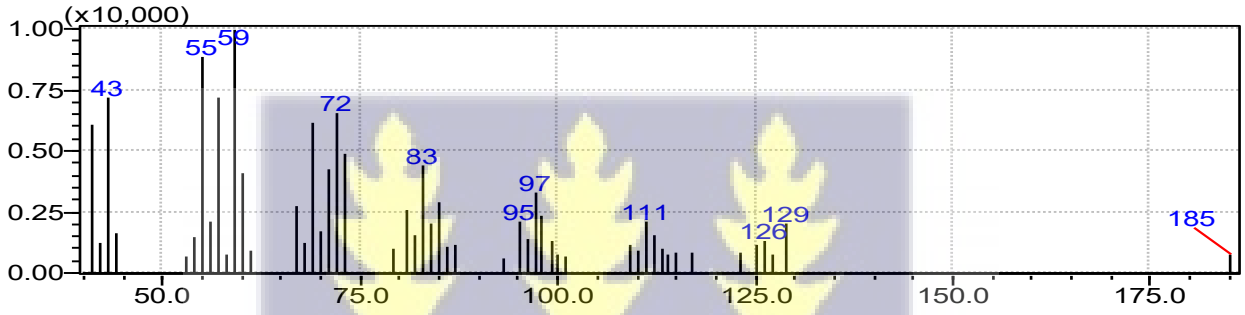

Figure 76: AnSp female cuticular compound (vi)

The peak appearing at 31.24 minutes, producing fragment ions characteristic of a long chain hydrocarbon (figure 77) and *m/z* 57 as base peak, corresponded to the compound 2-Methylhexacosane.

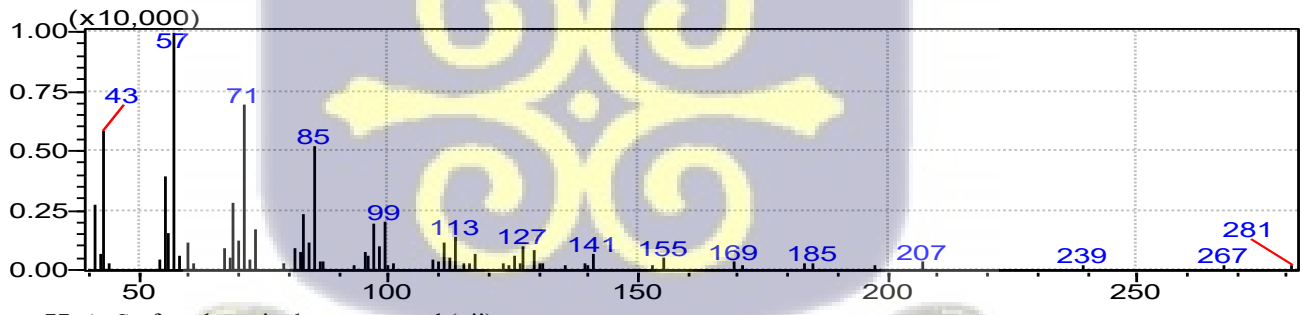

Figure 77: AnSp female cuticular compound (vii)

The fragment ions occurring at CH_2 intervals with *m/z* 57 for base peak (figure 78) which was observed at 31.8 minutes on the GC chromatogram was matched to the compound 2-Methylheptacosane.

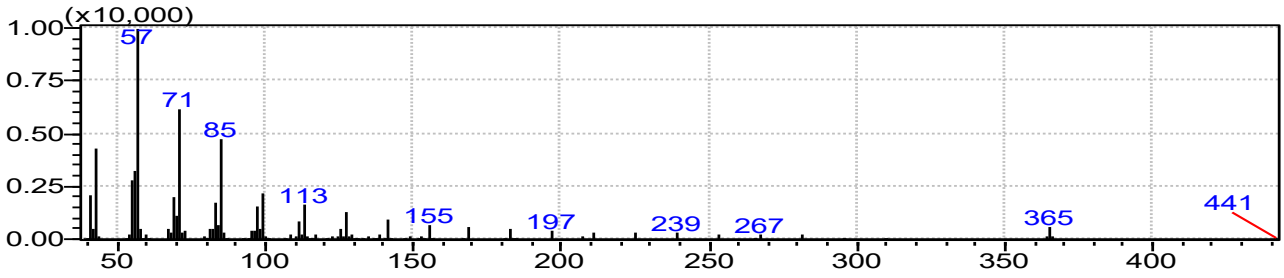

Figure 78: AnSp female cuticular compound (viii)

The analyte appearing at 32 minutes, produced fragment ions of the pattern 14n (figure 79), denoting a long chain alkane with base peak as *m/z* 57. This peak corresponds to an alkane compound, branched or straight, probably 2-methyl octacosane.

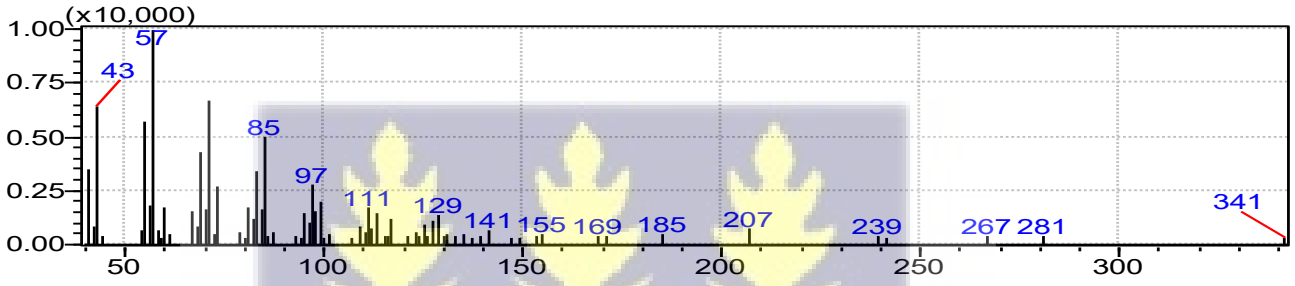

Figure 79: AnSp female cuticular compound (ix)

The compound detected at 32.8 minutes was nonacosane (figure 80), a longer chain methyl branched alkane.

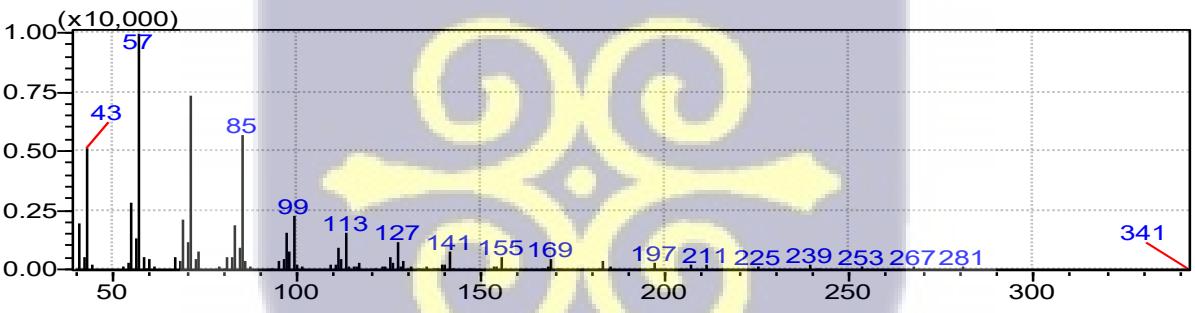

Figure 80: AnSp female cuticular compound (x)

The peak appearing at 33.1 and 33.3 minutes, producing fragment ions of *m/z* 57, 71, etc. with 14n masses (figure 81).

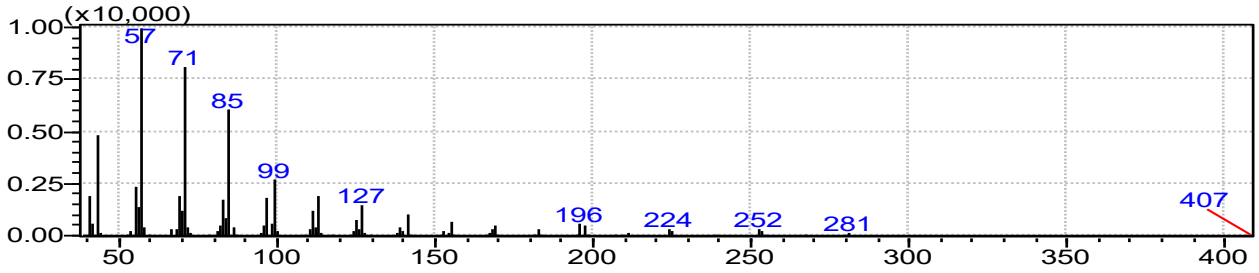

Figure 81: AnSp female cuticular compound (xi)

The remaining compounds had peak areas less than the appreciable level of detection and did not occur in the replicated samples.

Female whole-body compounds and diagnostics

At 12.81 minutes, the analyte with *m/z* 68 (figure 82) for base peak is characteristic of a terpenoid compound as a result of retro Diels-Alder. It was matched to the compound Limonene. The fragment ions were observed.

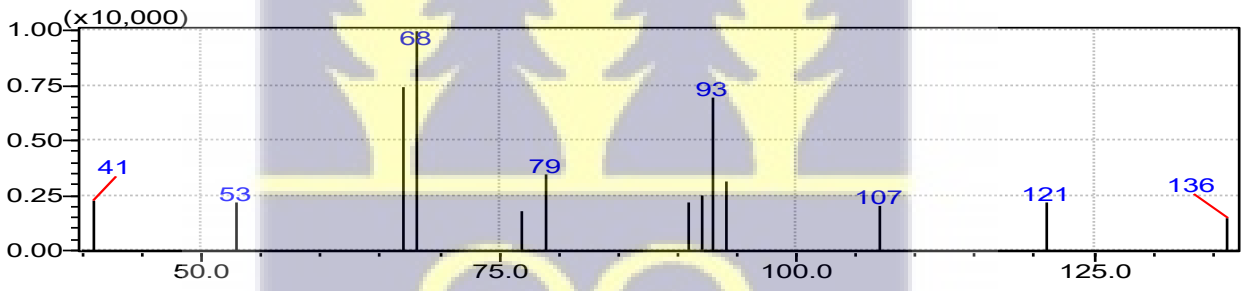

Figure 82: AnSp female whole-body compound (i)

The peak appearing at 12.9 and 12.4 minutes, producing base peak of *m/z* 57 (figure 83). The information corresponds to the compound 2-Ethyl-1-hexanol. Cleavage at the alkane side of the branch would afford the base peak.

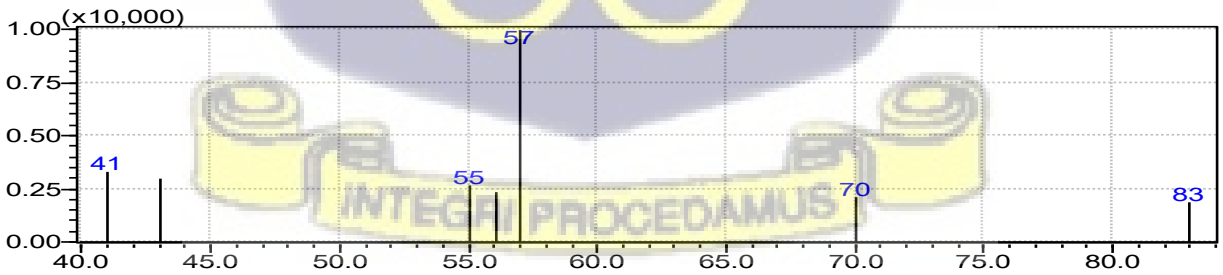

Figure 83: AnSp female whole-body compound (ii)

At 23 minutes, the peak occurring produced fragment ions at *m/z* 41, 55, 69, 71 and 85 with *m/z* 57 as base peak (figure 84). There is one degree of unsaturation.

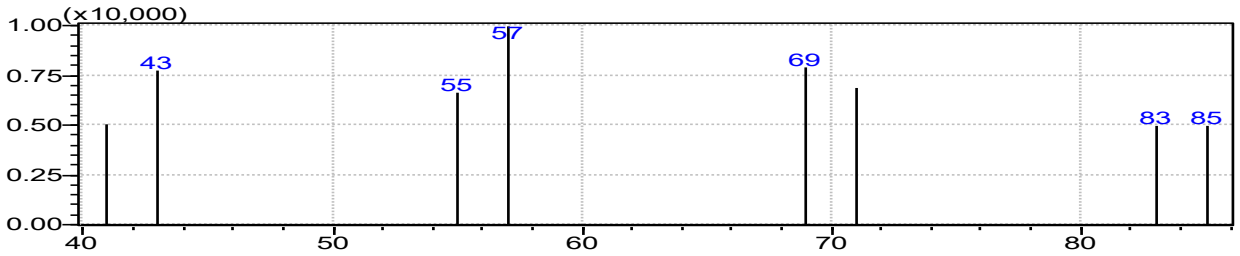

Figure 84: AnSp female whole-body compound (iii)

The compound occurring at 9.19 /9.46 minutes generated key fragment ions *m/z* 60, 73 (figure 85) pointing to carboxylic acid, *m/z* and 84, 98, 112, 116 predicting oxygenated functions. The base peak is at *m/z* 57. this compound was reported as Oxiran-2-ylmethyl palmitate.

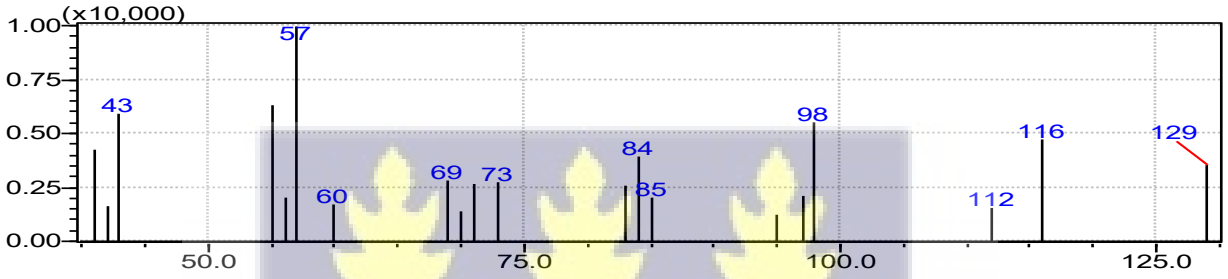

Figure 85: AnSp female whole-body compound (iv)

The analyte appearing at 26.5 minutes, produced fragment ions *m/z* 55, 41 (figure 86) indicating an alkene or oxygen functional group. Its base peak *m/z* 74 confirms the presence of a methyl ester, and *m/z* 60 the McLafferty rearrangement of a carboxylic group. This peak could correspond to compounds 17 octadecenoic acid methyl ester or an epoxy octadecanoic methyl ester.

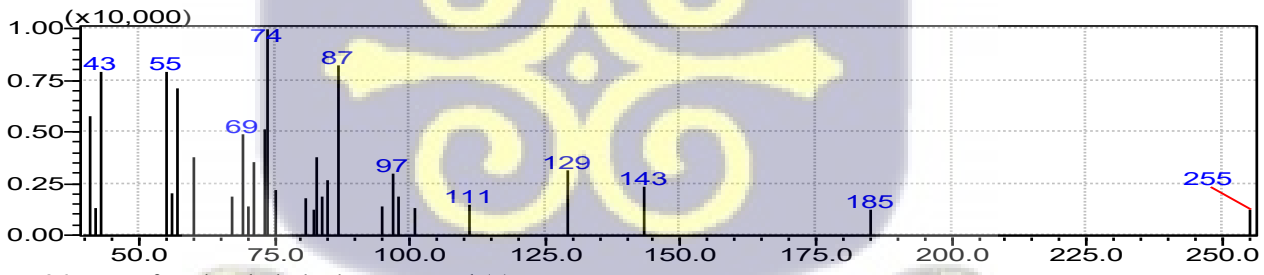

Figure 86: AnSp female whole-body compound (v)

At 29.1 minutes, the peak occurring produced fragment ions (figure 87) at *m/z* 43, 57, 69, 83, 97, 111, etc. (denoting long chain alkane) 41, 55, 60, 85, etc. (stemming from one level of unsaturation) and *m/z* 57 for base peak. This compound could be an alcohol or alkene.

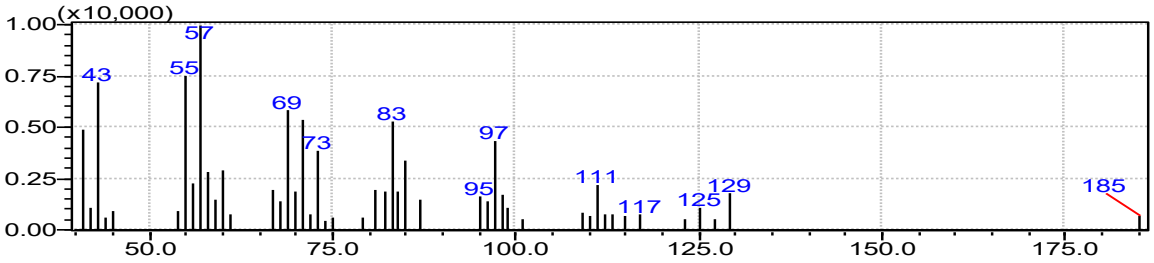

Figure 87: AnSp female whole-body compound (vi)

Figure 88 Fragment ions (figure 88) *m/z* 43, 57, 69, 83, 97, 111, etc. (denoting long chain alkane) 41, 55, 60, 85, etc. (stemming from one level of unsaturation) and *m/z* 57 for base peak was observed at 30.8 and 30.3. minutes.

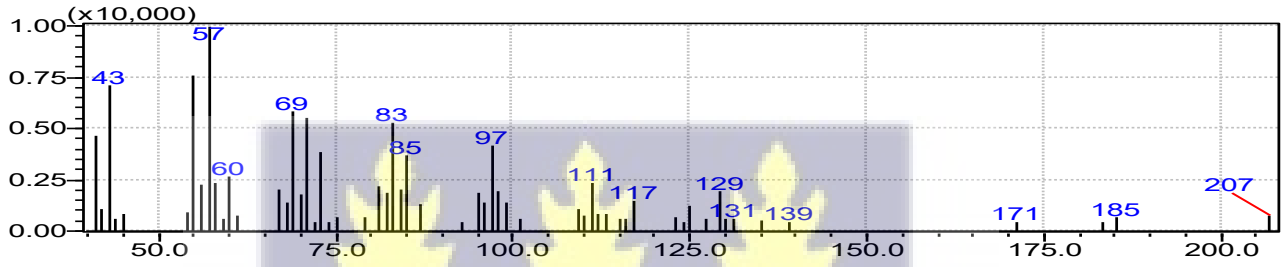

Figure 88: AnSp female whole-body compound (vii)

The compound occurring at 31.8 minutes generated key fragment ions corresponding to long chain alkanes (figure 89). The compound proposed is 2-Methylheptacosane

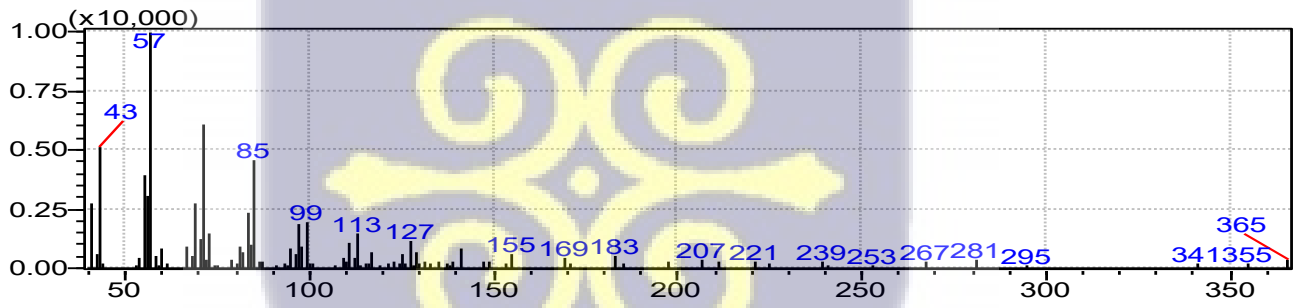

Figure 89: AnSp female whole-body compound (viii)

The fragment ions *m/z* 43, 57, 71, 85, 99, 113 etc. (figure 90) confer a long chain alkane status. With a base peak of *m/z* 57, proposed compound is triacontane.

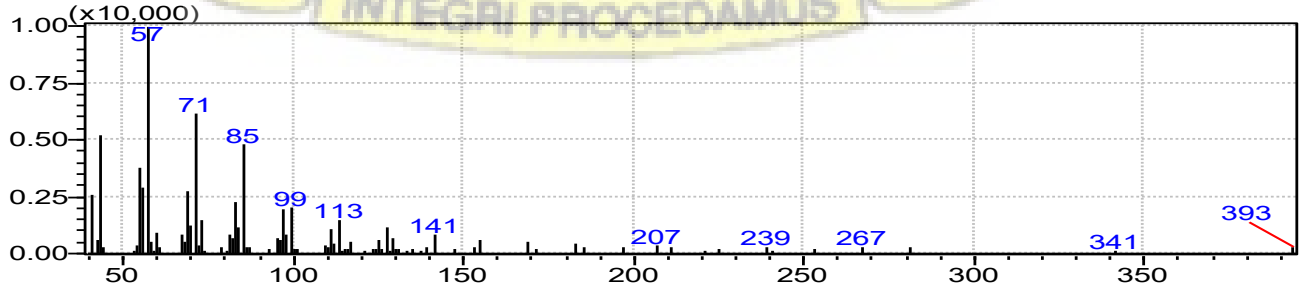

Figure 90: AnSp female whole-body compound (ix)

The remaining compounds did not occur in the replicated samples.

***Leptomastix* spp. [LeSp]**

Male cuticular compounds and diagnostics

The analyte appearing at 31.1 minutes, produced fragment ions consistent with a long chain alkane (figure 91). At *m/z* 55, 130 and 60 could also denote presence of oxygen-functionalities.

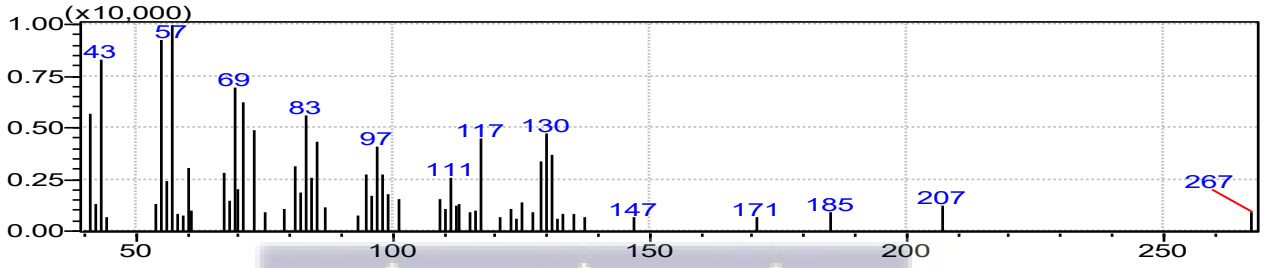

Figure 91: LeSp male cuticular compound (i)

The peak appearing at 33.1 minutes, producing fragment ions at 14amu intervals (figure 92), conferring long chain alkane.

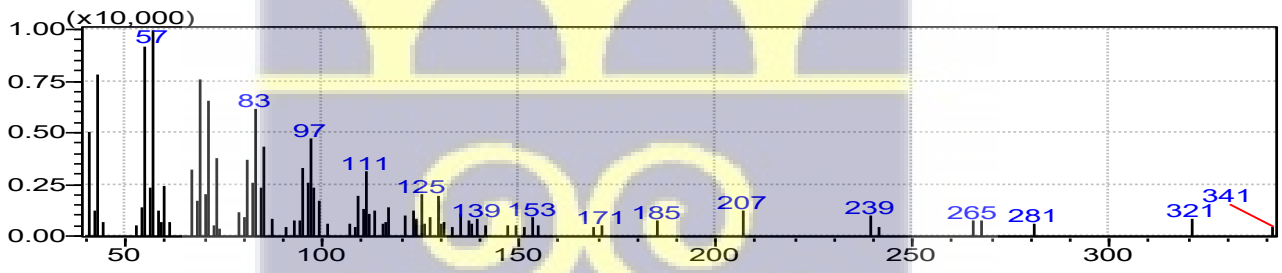

Figure 92: LeSp male cuticular compound (ii)

The remaining compounds did not occur in the replicated samples.

Male whole-body compounds and diagnostics

The fewer numbers of organisms account for an inability to sufficiently identify compounds which were detected. The NIST20 library matches were not highly comparable. The following are the spectra with their deductions;

The analyte appearing at 26.3 minute, produced a base peak of *m/z* 55 (figure 93). The peak at *m/z* 60, from a McLafferty rearrangement indicates a carboxylic group is present. A methyl ester could also be present with *m/z* 74 present. Thus, a carbonyl-containing compound.

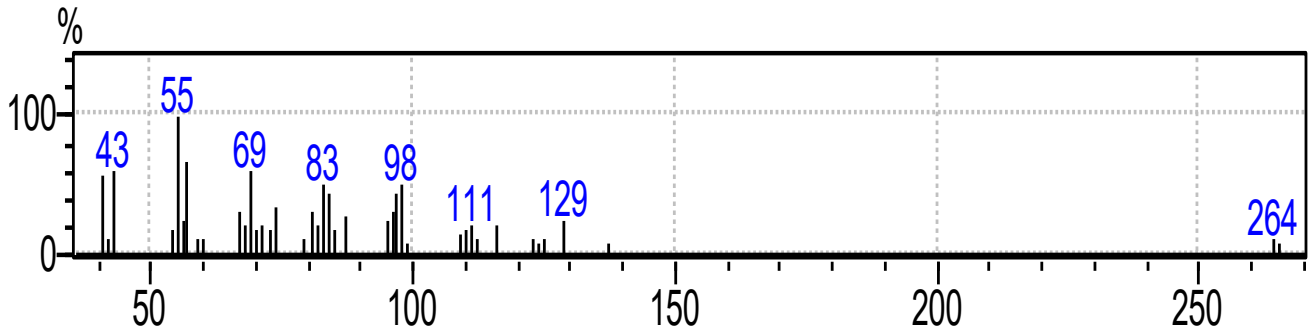

Figure 93: LeSp male whole-body compound (i)

At 31.8 minutes, the pattern (figure 94) appears similar to the previous.

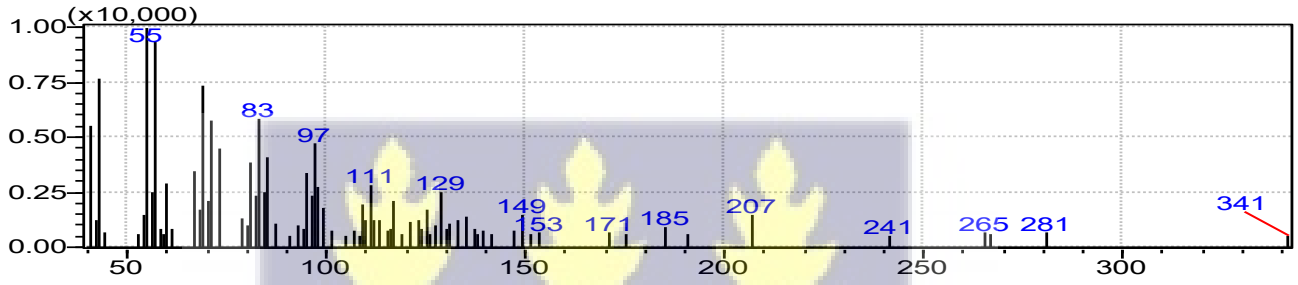

Figure 94: LeSp male whole-body compound (ii)

The peak appearing at 33.1 minutes, producing fragment ions at 14 amu interval (figure 95). This indicates the presence of a long chain hydrocarbon.

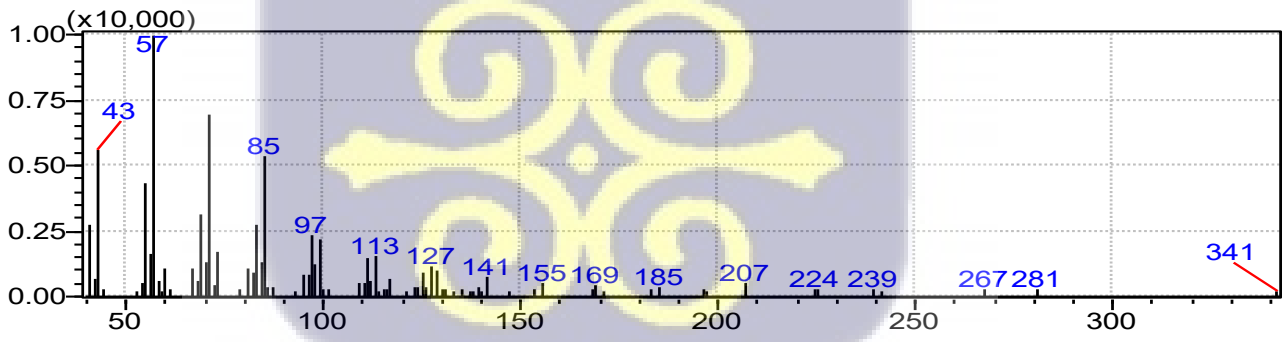

Figure 95: LeSp male whole-body compound (iii)

The remaining compounds did not occur in the replicated samples.

Female cuticular compounds and diagnostics

The only notable compound was inferred as a methylnonacosane or triacontane (figure 96), eluting after 33 minutes. Previous fragmentation pattern refers.

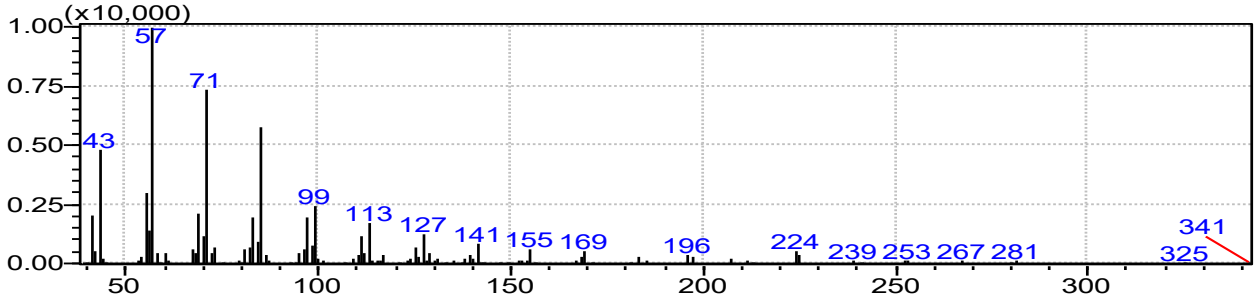

Figure 96: LeSp female cuticular compound (i)

Female whole-body compounds and diagnostics

At 12.9 minutes, the peak occurring produced its base peak on *m/z* 68 (figure 97). This is characteristic fragmentation of terpene compounds. The parent peak [M^+] was present, at *m/z* 136. This compound was identified to be limonene. Other noteworthy peaks occur at *m/z* 121 and 107.

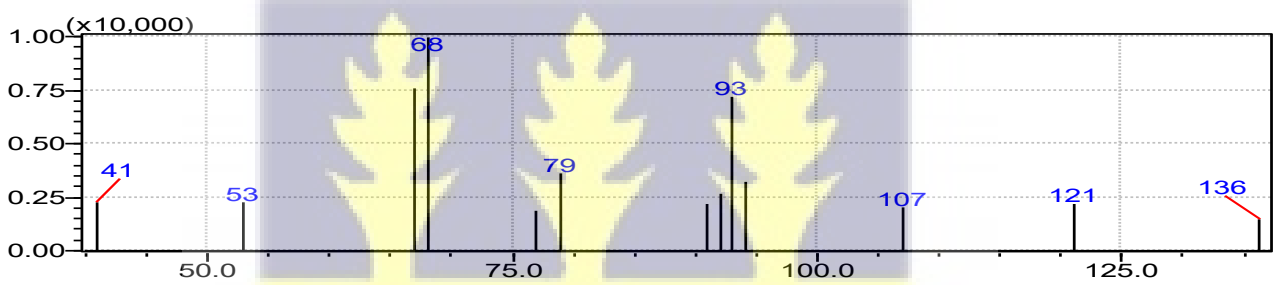

Figure 97: LeSp female whole-body compound (i)

The analyte appearing at 24.6 minutes, produced its base peak at *m/z* 74 (figure 98), indicative of a saturated methyl ester.

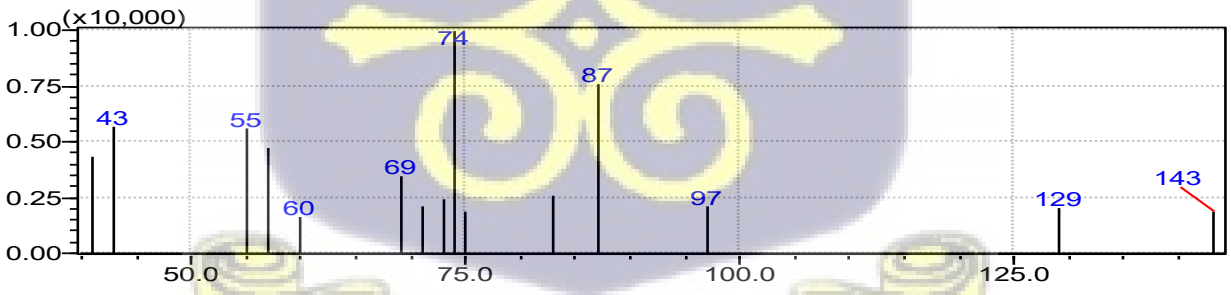

Figure 98: LeSp female whole-body compound (ii)

The peak appearing at 26.5 minutes, with base peak *m/z* 74 (McLafferty rearrangement of methyl ester) and *m/z* 55 (denoting an alkene), corresponded to the compound 13-Docosenoic acid, (Z)- methyl ester (figure 99).

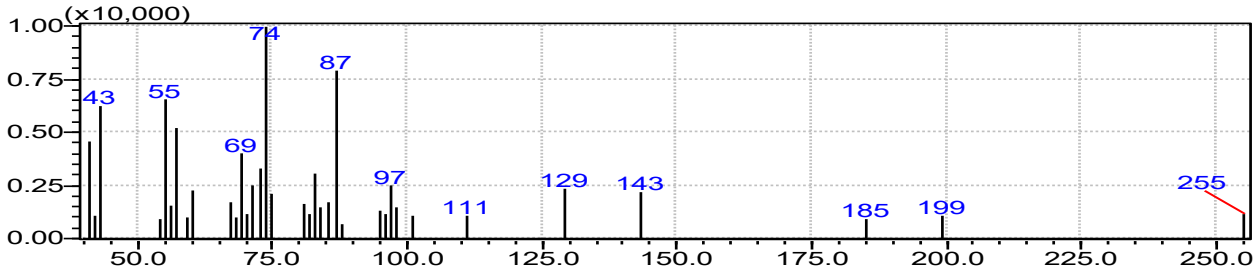

Figure 99: LeSp female whole-body compound (iii)

The fragment ions (figure 100) for the peak at 33.1/33.4 minutes contains a long chain alkane evident by 14amu intervals and base peak *m/z* 57. Methyl nonacosane is implicated.

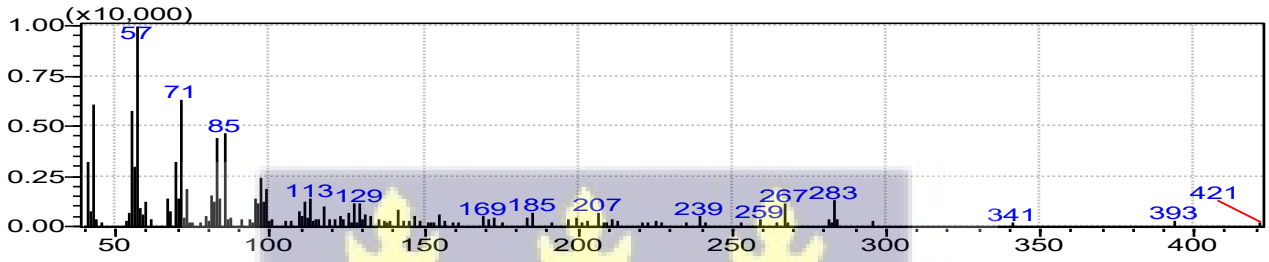

Figure 100: LeSp female whole-body compound (iv)

The remaining compounds did not occur in the replicated samples.

The pheromonal compounds detected for each insect species tested are summarized in Table 5 below.

Table 5. Summary of compounds of interest with pheromonal significance determined in this study

Compound	RT (min)	RI^L	RI^O		Class	Relative abundance ± S. E											
						CoSp male		CoSp female			AnSp male			AnSp female				LeSp male		LeSp female	
						Cuticular	Whole body	Headspace volatiles	Cuticular	Whole body	Headspace volatiles	Cuticular	Whole body	Headspace volatiles	Cuticular	Whole body	Cuticular	Whole body	Cuticular	Whole body
4-Hydroxy-4-methyl-2-pentanone	9.47/9.1, 9.28/9.32, 9.92/9.48	845	675.24 655.06, 599.69 605.92, 660.07/ 675.25	/	ketone	65.16 ± 14.96	46.91 ± 33.21				16.89 ± 10.03									
xylene o/m	9.78/9.92	907	699.51 692.27	/	aromatic hydrocarbon									23.09 ± 22.75						
Carbonic acid, 2-ethoxyethyl 2-methoxyethyl ester	12.5	N/A			ester						1.65 ± 1.05									
Limonene	12.8/93, 12.81	1018	1046.19 1053.47, 1046.75	/	terpene											1.36 ± 0.70				13.16 ± 12.05
Unknown carbonyl	12.93/4		1053.47 1023.80	/	carbonyl										5.25 ± 3.31					
2-Ethyl-1-hexanol	12.931/41, 12.94/12.96	995	1053.5 1024.4, 1054.03 1034.99	/	alcohol									15.90 ± 15.56		4.13 ± 3.64				

3,5,5-Trimethyl-1-hexene, or unknown carbonyl	14.11/13	757	1121.61 1122.85	/	-					4.04 ± 2.69	
2,2-Dimethyl-3-hexanone	16.98/7	868	1307.94 1287.91	/	ketone					0.50 ± 0.29	
6-ethyl-2-methyl-decane	18.36/8	1390	1407.14 1440.94	/	branched alkane					1.10 ± 0.66	
2 6 10 trimethyl tridecane/ 3 ethyl 2 6 10 trimethyl undecane	18.95, 21.07/21.29	1419 , 1355	1452.46 1452.61, 1642.7 1623.11	/ /	branched alkane	1.005 ± 0.005	46.44 ± 45.43				
4, 8 Dimethyl dodecane	18.95	1494	1452.46, 1452.46 1410.22	/	branched alkane			0.67 ± 0.33	0.67 ± 0.33		
Unknown alkene	18.95		1452.46 /		hydrocarbon		2.25 ± 1.25				
Unknown ketone	19.67/9	868	1508.22 1526.95	/	ketone					1.33 ± 0.80	
Unknown hydrocarbon	20.93		1598.86 1623.11	/	hydrocarbon	1.51 ± 0.51					
Unknown -diol	20.94		1612.03		alcohol			1.09 ± 0.59	1.09 ± 0.59		
Unknown hydrocarbon	23.361/22.79		1827.7 1774.9	/	hydrocarbon						2.62 ± 2.14
Oxiran-2-ylmethyl palmitate	24.28	2168	1915.81		ester					4.05 ± 2.12	4.53 ± 3.52

6, 10 dimethylundecan-2-one	24.3/28	1321	1917.19 1915.81	/	ketone	0.67 ± 0.33						
Methyl 12-methyltetradecanoate	24.61/57, 24.78	1715	1948.61 1944.63, 1965.51	/	ester		20.29 ± 16.67	20.29 ± 16.67		1.23 ± 0.90		
Unknown methyl ester	24.642		1951.79		ester							1.00 ± 0.00
Unknown alkene	25.42/4		2030.52 2028.44	/	hydrocarbon					1.53 ± 0.80		
-enoic acid	25.88		2078.13 2079.27 2079.58	/ /	carboxylic acid		6.79 ± 5.16					
Unknown hydrocarbon	26.04/25.91		2095 2081.56	/	hydrocarbon				1.25 ± 0.80			
Unknown carboxylic acid	26.05		2096.15		carboxylic acid					0.67 ± 0.33		
Unknown carbonyl	26.3		2123		carbonyl						4.53 ± 2.44	
9-Octadecenoic acid (Z)-, methyl ester	26.31/2	2077	2124.27 2125.35	/	ester				17.12 ± 5.02			
C18 carbonyl	26.32		2125.35		carbonyl			26.08 ± 21.16				
13-Docosenoic acid, (Z)-methyl ester	26.5	2483	2144.94		ester							10.35 ± 7.45

9,12-Octadecadienoic acid (Z, Z)-	26.51	2183	2146.03		carboxylic acid		168.71 ± 151.627	168.71 ± 151.63				
Octadecanoic acid, methyl ester	26.54	2077	2149.29		ester					16.38 ± 6.75		
Epoxy Octadecanoic acid, methyl ester or 17 octadecenoic acid methyl ester	26.54/492	2105 or 2165	2149.29 or 2144.07	/	ester						11.40 ± 6.69	
Unknown alkene/carbonyl	26.55/26.76		2151.03 2173.34	/	-	225.92 ± 219.98						
Methyl octadecenoate	26.57/5	2077	2152.56 2144.94	/	ester				19.37 ± 13.35			
Eicosanoic acid,	27.88/93	2366	2299.17 2305.09	/	carboxylic acid	0.88 ± 0.44						
18-eicosenoic acid	28.94	2374	2425.62		carboxylic acid	13.33 ± 9.08						
Unknown carbonyl	28.95		2426.85		carbonyl					11.11 ± 8.51		
Unknown carbonyl	28.95/31.83		2801		carbonyl							2.94 ± 1.94
Unknown hydrocarbon	29.1		2445.32		hydrocarbon						4.12 ± 1.88	

Unknown carbonyl	29.9/30.08		2545.6 / 2568.6		carbonyl					24.61 ± 5.60		
9-Eicosenoic acid, (Z)-	29.93	2549.3	2318, 2213		carboxylic acid	66.90 ± 56.74	67.45 ± 56.42					
Methyl ketone	30.08		2568.63		ketone			1.78 ± 0.91				
2-Methylpentacosane	30.27	2562	2592.96		branched alkane			4.28 ± 1.99				
Unknown carbonyl	30.27/32		2592.72 / 2822.8		carbonyl					7.66 ± 2.08		
Methylhexacosane	30.35/40	2641	2603.31 / 2611.24		branched alkane			0.67 ± 0.33				
Unknown carbonyl	30.69/73		2648.28 / 2653.71		carbonyl			1.33 ± 0.76				
Unknown hydrocarbon	30.75/3		2656.22 / 2596.7		hydrocarbon						1.59 ± 0.79	
Unknown carbonyl	31.05/10		2695.90 / 2702.51		carbonyl							1.00 ± 0.00
Unknown carbonyl	31.2		2758.65		carbonyl	2.2 ± 1.72						
2-Methylheptacosane	31.24/31.29, 31.71, 31.97, 32.03/32.1	2763	2751.5 / 2759.65, 2797.69, 2798.91 / 2818.45,		branched alkane	130.7 ± 125.53		1.02 ± 0.24	27.17 ± 9.01		29.76 ± 17.83	

			2826.56 2835.29	/						
2-Methylhexacosane	31.24, 31.25	2641	2721.6, 2722.96		branched alkane		0.50 ± 0.22		14.87 ± 11.40	
Methyloctacosane	31.46/52	2840	2784.10 2811.28	/	branched alkane			12.80 ± 5.03		
Methylheptacosane	31.71	2763	2796.33 2797.7	/	branched alkane					3.70 ± 1.16
Unknown aldehyde	31.75/31.97		2818.83 2790.90	/	carbonyl	88.71 ± 87.65				
Triacontane	31.79/80	3000	3000 2963.57	/	alkane					14.33 ± 8.66
Unknown hydrocarbon	31.8		2822.82 2829.05	/	hydrocarbon				47.65 ± 35.21	
Nonacosane	32/.05	2900	2925.27 2872.69	/	alkane				22.08 ± 8.91	
2 methyl octacosane.	32.21/2, 32.80/32.84	2840	2849 2847.76, 2924.18 2919.8	/	branched alkane		1.17 ± 0.49	3.58 ± 3.09		
unknown alcohol or alkene	32.27		2856.48		-	11.29 ± 9.74				
Unknown alkene	32.27		2856.48		hydrocarbon	11.29 ± 9.74				

Unknown alkane	32.60/32.81		2920.57 2898.63	/	alkane	50.13 ± 1.97						
Unknown alkane	32.85/4		2958.1 2974.51	/	alkane				73.72 ± 55.77			
2 methyl nonacosane	32.9/33.08, 33.16, 33.17	2960	2930.74 2950.44, 2959.63 / 2959.41, 2960.72 / 2963.57	/	branched alkane	237.9 4 ± 141.6 2	195.1 3 ± 175.5 6		56.32 ± 38.65			
methylnonacosane or triacontane	33.1, 33.4,33.9	2960 or 3000	2952.63 2985.45 / 3000+	/	alkane						1.00 ± 0.00	32.02 ± 4.28
Unknown alkane	33.16/33.10		2952/2959		alkane					15.42 ± 12.07		
Unknown long chain alkane	33.9		2952.63		alkane				11.92 ± 7.20			

In CoSp, the volatiles were sufficient to yield two compounds. While the second one may be an alkane, with the first compound, the presence of a methyl ketone is suggestive of an oxygen function at position 2. Midges of Cecidomyiidae family use pheromones with at least one oxygen functional group in the form of an acetoxy- or ketone- functional group. A few have hydroxy- or aldehyde functionalities though. This compound is worth exploring. On the other hand, several long-chain hydrocarbons have been useful as attractants of certain Cecidomyiidae. (Young & Severson, 1994) determined that certain Cecidomyiids in cocoa ecosystems responded better to floral volatiles of higher molecular weights than to oils of lower molecular weights. This was however for kairomonal rather than pheromonal interaction. The females tested in this study were attracted to all pheromone extracts. A wide range of hydrocarbons, alcohols, esters and fatty acids are detected in these extracts. These could be aggregators or biological metabolites. 2-methyltetracosane for instance has pheromonal use in various insects such as *Drosophila pseudoobscura* (Blomquist et al., 1985).2 6 10 trimethyl tridecane is found in many plant sources as a metabolite while 3 ethyl 2 6 10 trimethyl undecane is not much found naturally. 9,12-Octadecadienoic acid (Z, Z)- would also serve metabolic functions. 9-Eicosenoic acid, (Z)- is also utilized as pheromone in a wide range of sea creatures and a few land mammals, however, in insects, it may serve as a metabolite. 2-Methylnonacosane is also used in many insects, including Diptera such as *Glossina austeni* (Nelson & Carlson, 1986). Thus, these cuticular hydrocarbons alongside the methyl ketone can be explored as an attractant for both males and females CoSp.

The Encyrtidae family which includes AnSp and LeSp are known for using plant cues to identify their host. As shown in the bioassays here, one sex of each seems to be much interested in plant cues provided; females for LeSp and males for AnSp. However, all sexes are attracted to the opposite's extracts used in this study. Thus, a wide range of compounds may be available for exploration for an attractant. The long chain alkanes such as 2 methyl hexacosane, 2 methylheptacosane, 2-methyloctacosane, etc. are commonly found in other insect organisms, being intraspecific communicative cues. Limonene, a terpenoid compound has been identified in plants in the D-isomer and animals in L-isomer. Terpene compounds come into good play in many Isopteran and Hymenopteran species (Wheeler & Duffield, 1985). Both D- and L- limonene were found to be pheromonal attractants of the bed bug *Cimex lectularius* Linnaeus (Siljander et al., 2008). 2-Ethyl-1-hexanol also has pheromonal use in other insects, such as Panamian leafcutter ant *Acromyrmex echinatior* Forel (Norman et al., 2017). A wide range of methyl esters and carboxylic acids, apart from being metabolites, are also in use in various taxa for pheromones.

Kairomones mixed with pheromones usually make for very potent and reliable field attractants. Out of the total of five categories (of 3 insect and 2 sexes each, minus one) used in this study, at least 3 are attracted to host mealybug volatiles. These are male AnSp, female AnSp and male LeSp. Three also are attracted to their induced HIPVs, the female CoSp, male AnSp and LeSp female. also, two are attracted to just the plant constitutive volatiles; the female CoSp, female AnSp and female LeSp. The account by (K. M. Harris, 1967) on CoSp may explain their behaviour towards host mealybug volatiles. Apparently, they are predatory, developing on the outside of the bodies of mealybugs. They pupate on the host plant on the underside of the leaves; however, their pupae stage requires parasitic nutrition from mealybugs. The parasitoid wasps however are endoparasitic and both larval and pupal stages are completed inside the body of the mealybug.

Mealybug Female volatile compounds and diagnostics

The following volatile compounds were identified from mealybug hosts;

The analyte appearing at 9.3 minutes, had *m/z* 43 for its base peak, a methyl ketone present (figure 101). The peak corresponded to compound 4-Hydroxy-4-methylpentan-2-one. *m/z* 101 occurs as a result of alpha-cleavage at the methyl ketone area.

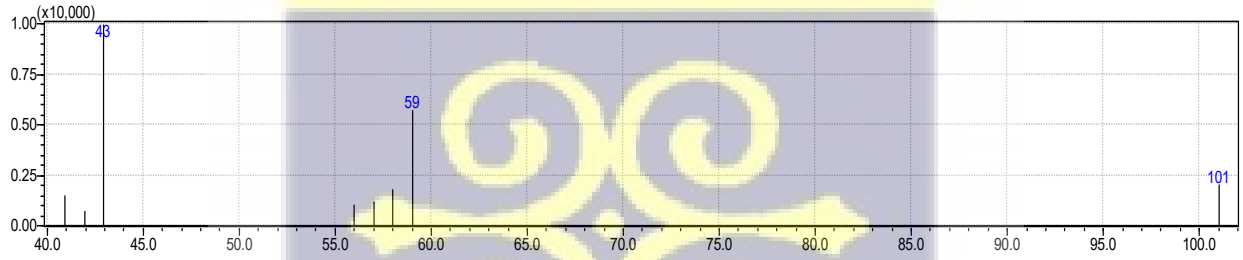

Figure 101: Mealybug headspace volatile compound (i)

At 26.3 minutes, the peak occurring produced fragment ions at *m/z* 55 as base peak (figure 102). This compound would contain a long chain alkane and a ketone, from the abundance of *m/z* 43 and 55. The abundant *m/z* 57 could also be derived from an acylium cation of a ketone. The retention index was identified to be 2119.9.

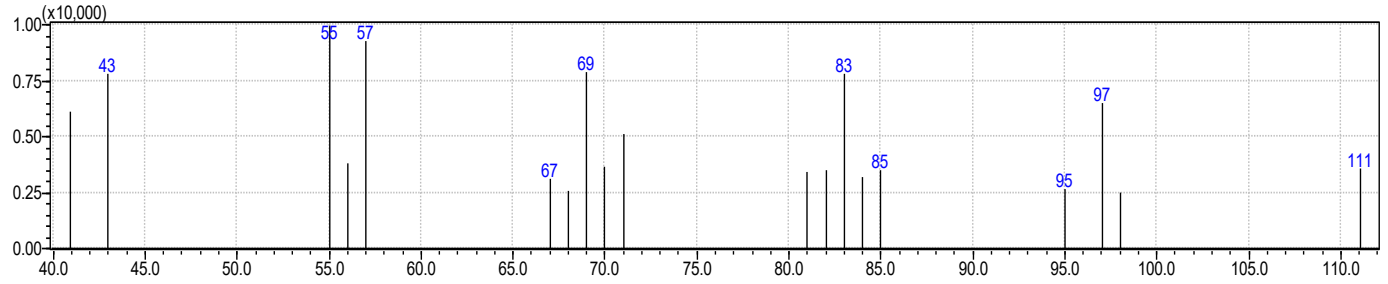

Figure 102: Mealybug headspace volatile compound (ii)

The fragment ions *m/z* 43, 57 71, 85, 97, etc. (figure 103) denote a long chain alkane. Retention index for this compound was calculated as 2785.46 across profiles 2729.76. ester

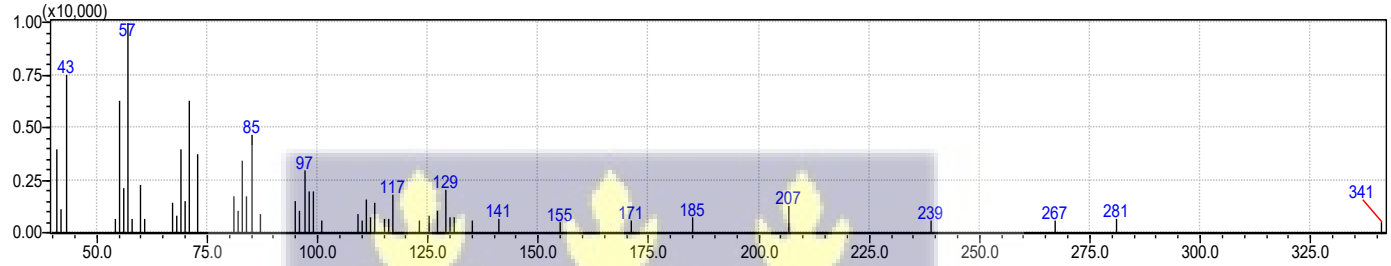

Figure 103: Mealybug headspace volatile compound (iii)

The remaining compounds did not occur in the replicated samples.

The compound 4-Hydroxy-4-methylpentan-2-one is a pheromone for several other insect species such as the Neotropical brown stink bug *Euschistus heros* (Lopes, 2015). This compound was also detected in female volatiles of AnSp, male cuticular and whole-body extracts of CoSp. Out of these three only the female AnSp are highly active towards mealybug volatiles in the bioassays yet. Thus, this compound may be interesting for further studies with the AnSp.

Further investigation into the two remaining compounds in the mealybug volatiles to ascertain their identities would be beneficial as well.

Plant compounds and diagnostics

The following compounds were detected in detached plant constitutive volatiles; Ethylbenzene, xylenes, heptane, limonene, naphthalene, p-xylene, azulene and benzene 1,3-dimethyl.

In the attached pods, the following were picked up; Nonanal, o-xylene, alpha-pinene and methyl hexadecanoate

It was noticed that nonanal and the xylenes are consistent for both. However, it should be noted that only the detached were used for the data set in this study.

The volatile profile seemed to change slightly when mealybugs were introduced and allowed to feed. The following compounds were detected in the HIPVs; Iso-Longifolene, 1,6-Dioxacyclododecane-7,12-dione, Furan, tetrahydro-2,5-dimethyl-, Limonene, Naphthalene, Nonanal.

Nonanal is consistent across all three categories. However, limonene and iso-longifolene are two interesting compounds that emerged under insect stress. Iso-longifolene, a terpenoid compound commonly found in pine plants, may play a synomonal role in the interaction between the mealybugs and their natural enemies. This compound can be further explored for attractant property. It was observed that several other components could be determined from these extracts had they been more concentrated. Future work should combine at least 3 days collection, similar to pheromones as in Amarawardana (2009), for enrichment of low-level content in analytes. The full profile of the plant volatiles would be better elucidated. This can also be extended to the mealybug volatiles, so as to unearth compounds that are active but at low concentrations. It is possible that a blend of compounds rather than single molecules would be responsible for attraction.

GC-MS is primarily designed for volatile and semi-volatile compounds, making it ineffective for non-volatile metabolites such as larger polar molecules (Deshoju Srinu, 2025). This is optimum for our sample type and target molecules. The methods of extraction simplified sample preparation, and there was no need for extra processing, preserving target compounds. The wide range of temperature programming is expected to avoid decomposition of some compounds which may have incurred loss of critical information (AL-Bukhaiti et al., 2017). Due to the small sample sizes, preliminary runs informed a decision to further concentrate the extracts, thus ensuring they fall within the optimal detection range of the instrument. Therefore, samples were also sufficiently diluted to minimise co-elution.

Although GC-MS is highly sensitive and can detect low concentrations of compounds in extracts, some limitations in detecting all active compounds are acknowledged. GC-MS broadly lacks selectivity. The technique can struggle to distinguish between compounds with similar mass-to-charge ratios, potentially leading to false positives or misidentifications (AL-Bukhaiti et al., 2017). Also, there could be matrix effects as extract samples obtained were of biological nature. These are known to have complex matrices. The result is the suppression or enhancement of target compound ionisation, complicating determination

(Zhang et al., 2024). Careful study under varying conditions, solvent types and even column types is required as precaution.

Moreover, GC-MS as a stand-alone method has a limited dynamic range, making it challenging to quantify compounds present at both high and low concentrations simultaneously (Kyle, 2017; Watson & Sparkman, 2007). Compounds that were present at lower concentrations were significantly obscured by detector saturation of those present at high concentrations. Conversely, low-abundance compounds might fall below the limit of detection (LOD) (Zhang et al., 2024) when the instrument is optimized for higher concentrations. Moreover, different compounds exhibit varying ionization efficiencies, meaning that one compound might generate a stronger signal than another at the same concentration. This variation can lead to skewed results, where stronger signals mask weaker signals—sensitivity becomes a critical factor in quantification (AL-Bukhaiti et al., 2017; Zhang et al., 2024).

It is recommended that in quantification studies, this should be considered, to avoid under-quantification and overestimation. Multi-point Calibration could be used to improve accuracy (Jensen et al., 2023). Derivatization could also be utilised prior to analysis to increase compound volatility and detection sensitivity (Watson & Sparkman, 2007). Advanced Instrumentation such as high-resolution mass spectrometry, may offer broader dynamic ranges compared to traditional GC-MS systems. Specifically, the use of GC coupled to electroantennography (EAG) could also be beneficial to target compounds of semiochemical value to the organisms under study

GC-MS systems is expensive to purchase and maintain, limiting accessibility for some laboratories (Kyle, 2017). Privileged access was required to undertake this study, limiting the possibility for additional runs under different conditions. This could have afforded extra useful information from the samples.

CHAPTER FIVE

5.0. CONCLUSION

This study examined the behavioural and chemical interactions between cocoa mealybug vectors and their natural enemies within the cocoa agro-ecosystem, with a view to improving biological control strategies. Despite the year-round presence of parasitoids such as *Leptomastix* spp., *Anagyrus* spp., *Aenasius abengouroui*, and *Coccodiplosis coffeae*, parasitism rates remained notably low, rarely exceeding 5.2%. This limited natural control, attributed to pesticide use, absence of shade, and availability of alternative hosts, reflects a significant ecological imbalance that threatens the sustainability and productivity of cocoa farming systems, particularly in the face of Cocoa Swollen Shoot Virus Disease (CSSVD).

Attempts to control mealybug vectors using insecticides have proven largely ineffective, constrained by the protective interactions between mealybugs and ants and the mealybugs' morphological adaptations. Consequently, the prospects for alternative control measures under Integrated Pest Management (IPM) remain critical. This research provided important insights into the chemical ecology underpinning parasitoid behaviour, particularly the use of pheromones and herbivore-induced plant volatiles (HIPVs) in host location.

The identification of specific semiochemicals offers a promising avenue for enhancing the recruitment and efficacy of natural enemies against cryptic pests such as mealybugs. Artificial amplification of these chemical signals could significantly improve parasitoid foraging success, thereby strengthening biological control efforts within the cocoa ecosystem. These findings reinforce earlier studies demonstrating the functional specificity of plant volatiles and their practical relevance to pest management.

Overall, the study highlights the need for further exploration and field validation of identified semiochemicals to optimize their application across diverse cocoa-growing regions. Integrating chemical ecology into pest management frameworks offers a sustainable, environmentally friendly alternative to conventional pesticide use. Adoption of such strategies could not only improve pest suppression but also promote biodiversity conservation, reduce environmental contamination, and contribute to the long-term resilience and sustainability of cocoa production systems.

RECOMMENDATIONS

Based on the findings of this study, it is recommended that the identified semiochemicals be subjected to extensive field validation across diverse cocoa-growing regions to confirm their practical efficacy. Integrated Pest Management (IPM) strategies should incorporate these chemical cues to enhance natural enemy recruitment and reduce reliance on chemical pesticides. Promoting agroforestry practices, such as maintaining shade trees, would further support natural enemy populations and improve ecological balance.

Population intensification of natural enemies by rearing or breeding programs along with pre-training with confirmed semiochemical compounds can be undertaken over a period of time. This can enhance their recruitment and effectiveness in suppressing mealybug populations in the field.

Future research should also address the disruption of ant–mealybug mutualisms to increase the vulnerability of mealybugs to parasitoids. Additionally, the development of cost-effective semiochemical-based monitoring tools and stronger policy support for sustainable cocoa farming practices are critical steps towards improving pest control while preserving biodiversity and environmental health.

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APPENDICES

APPENDIX A; Alkane standards table

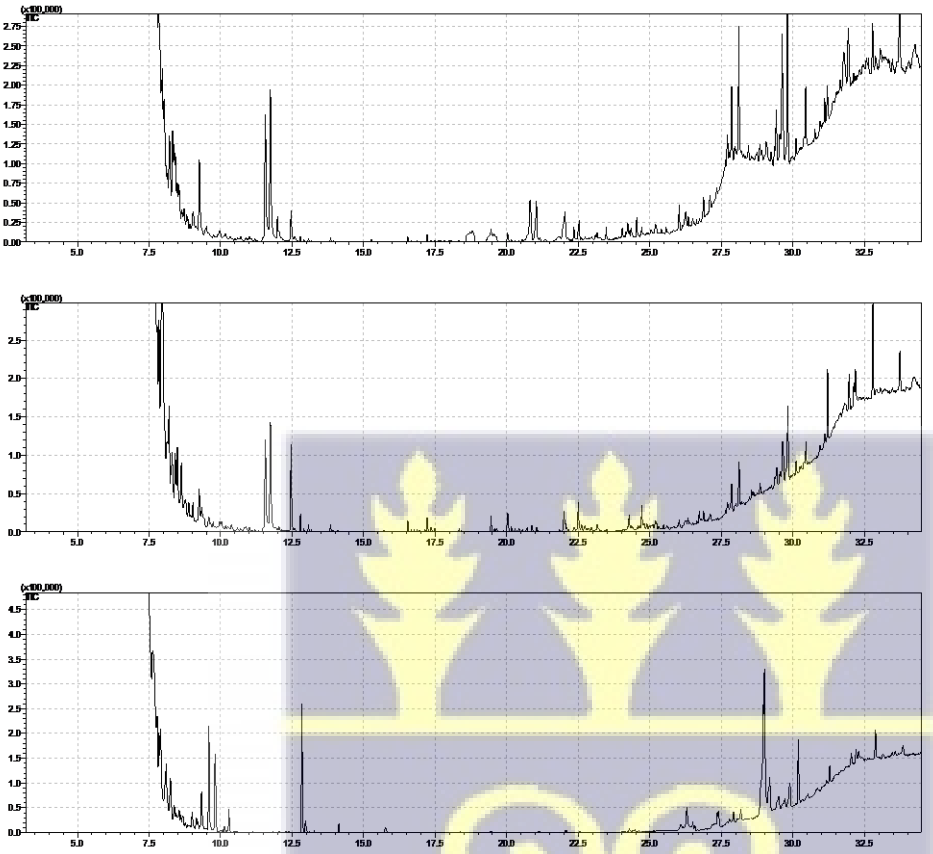
S/N	Compound	RT (min)	RI
1	Solvent front (hexane)	8.086	600
2	Octane	10.566	800
3	Nonane	10.935	900
4	Decane	11.975	1000
5	Undecane	13.761	1100
6	Dodecane	15.376	1200
7	Tridecane	16.869	1300
8	Tetradecane	18.267	1400
9	Pentadecane	19.569	1500
10	Hexadecane	20.797	1600
11	Heptadecane	21.961	1700
12	Octadecane	23.068	1800
13	Nonadecane	24.121	1900
14	Eicosane	25.127	2000

15	Heneicosane	26.087	2100
16	Docosane	27.006	2200
17	Tricosane	27.887	2300
18	Tetracosane	28.732	2400
19	Pentacosane	29.544	2500
20	Hexacosane	30.325	2600
21	Heptacosane	31.081	2700
22	Octacosane	31.817	2800
23	Nonacosane	32.619	2900
24	Triacontane	33.533	3000

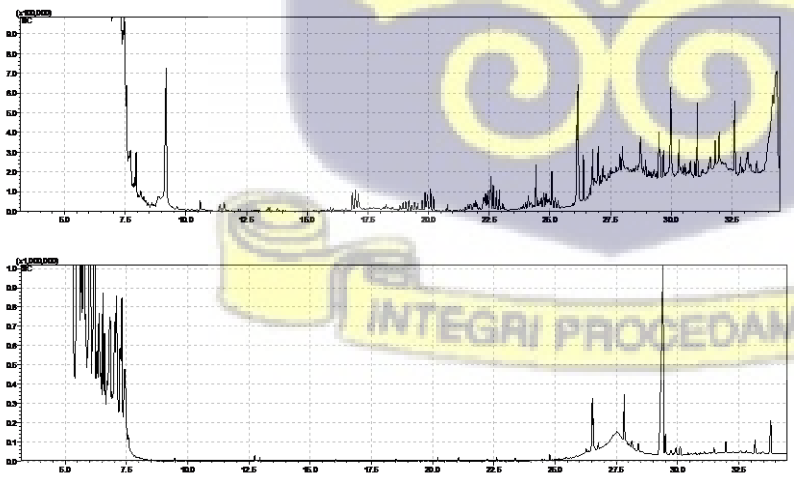
APPENDIX B; Raw Chromatograms

Coccodiplosis coffeae chromatograms

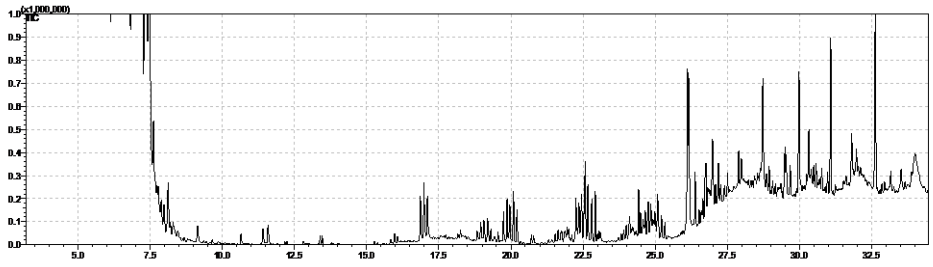
Female headspace volatiles

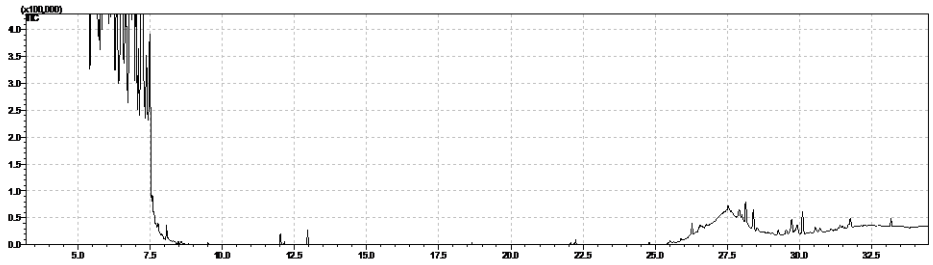


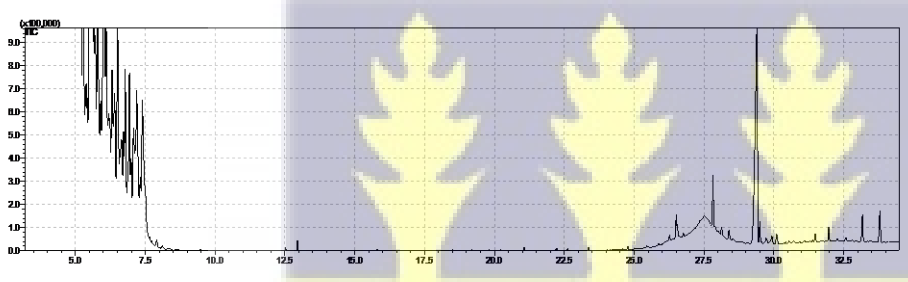
Male cuticular extracts



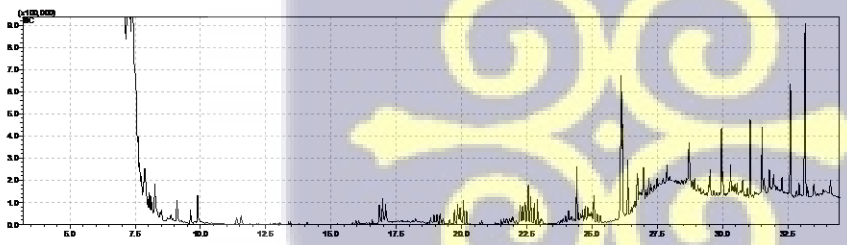
Female cuticular extracts

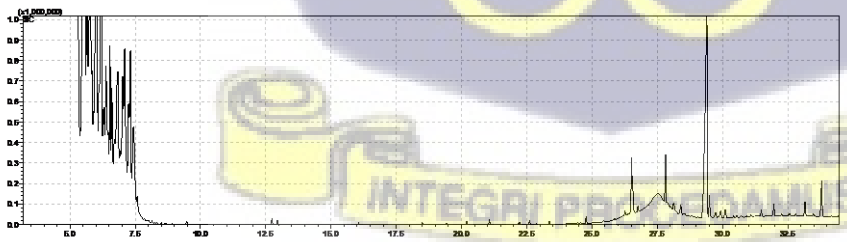




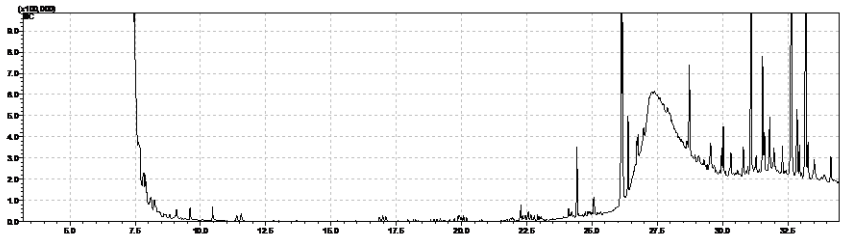


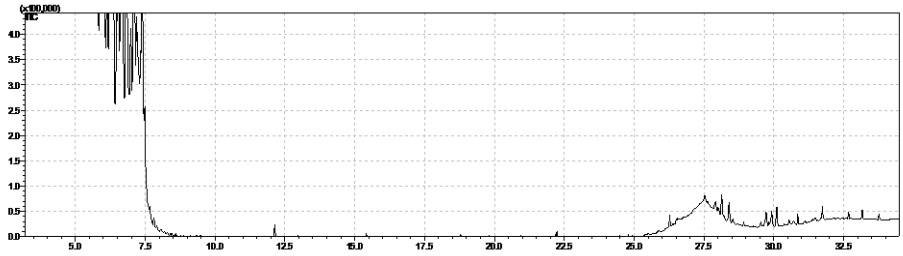
Male Glandular extracts (whole body)

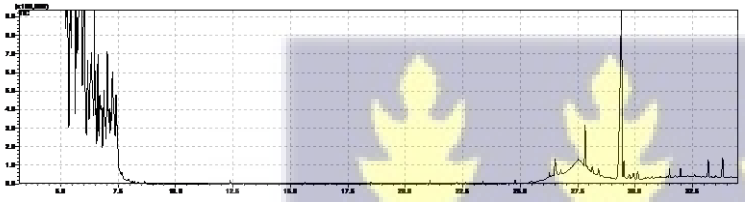




Female Glandular extracts (whole body)

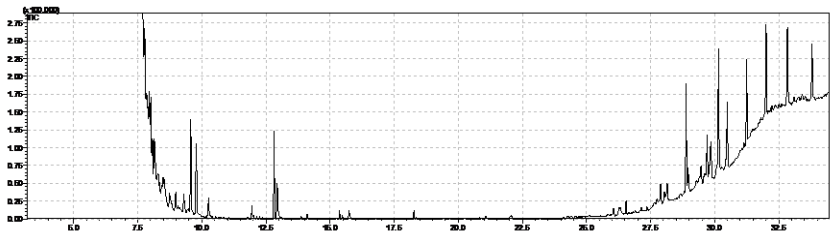


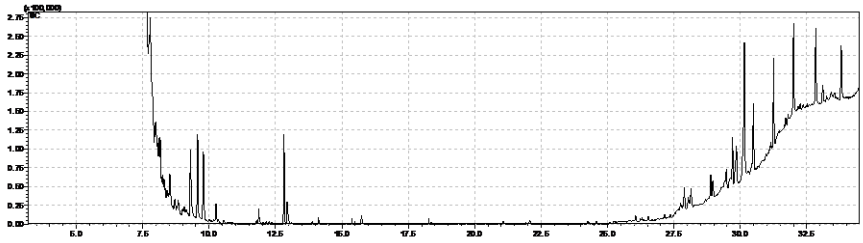


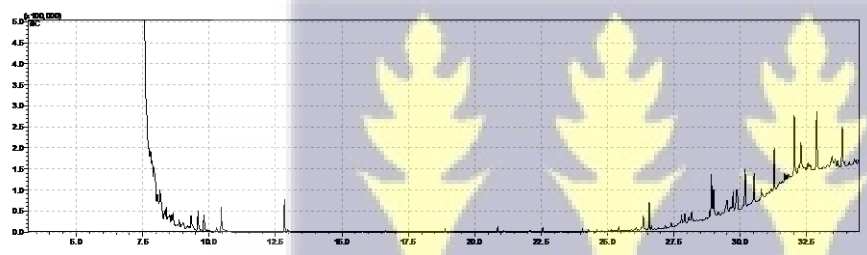


Anagyrus beneficans chromatograms

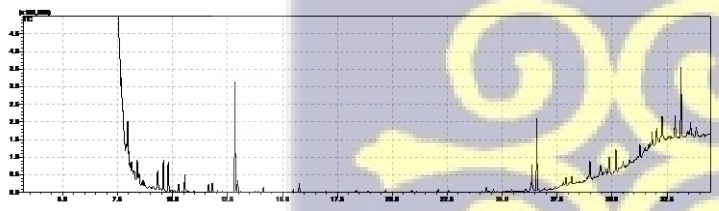
Male headspace volatiles

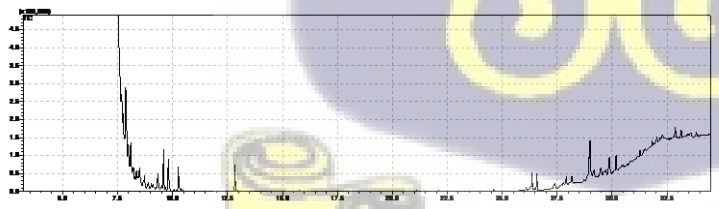


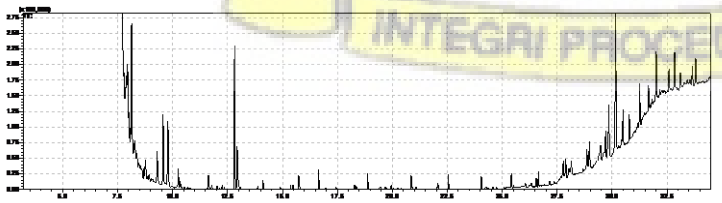




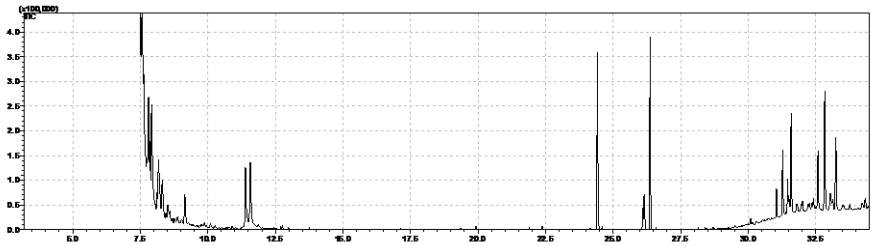
Female headspace volatiles

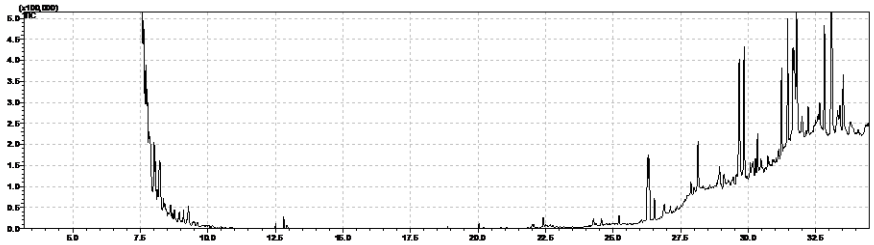


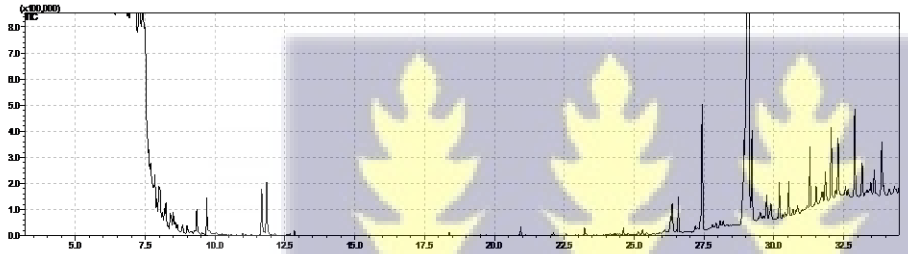




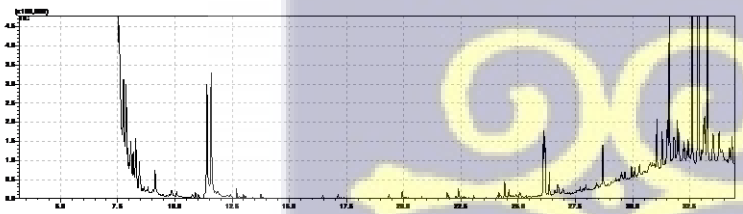
Male cuticular extracts

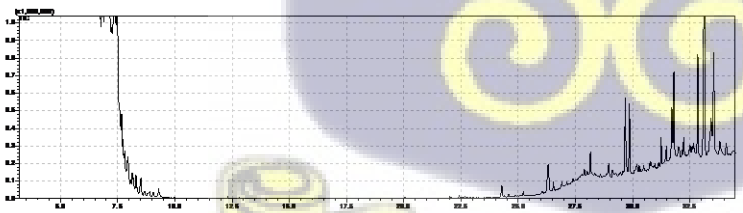


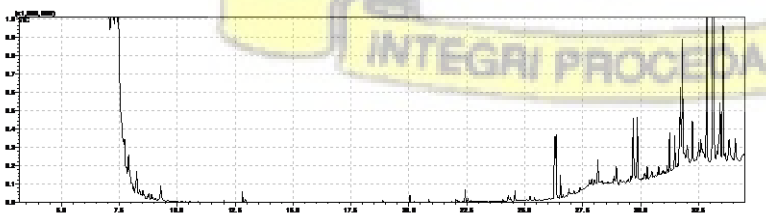




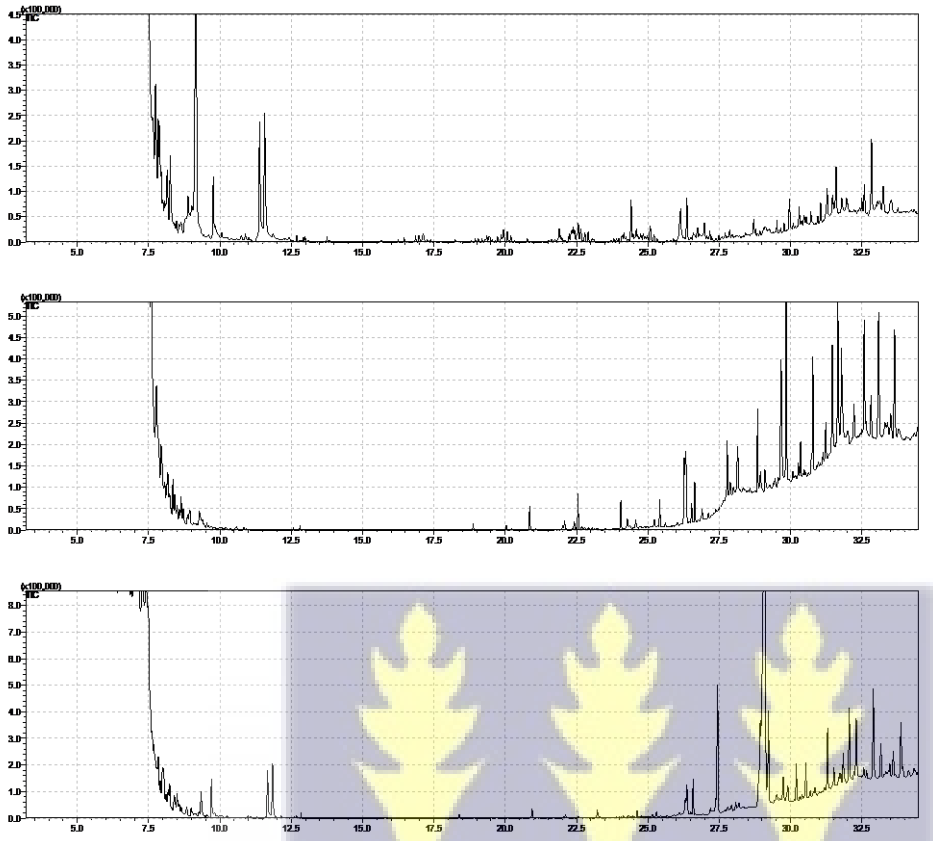
Female cuticular extracts



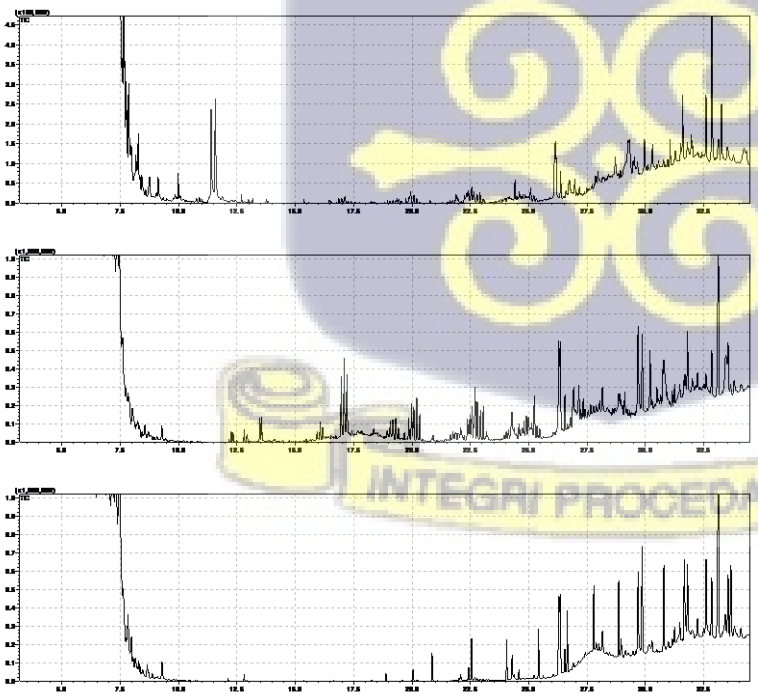




Male Glandular extracts (whole body)

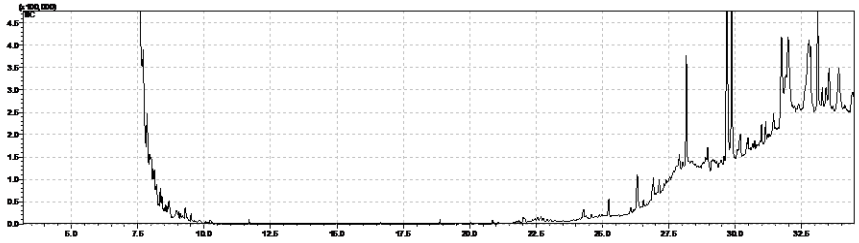


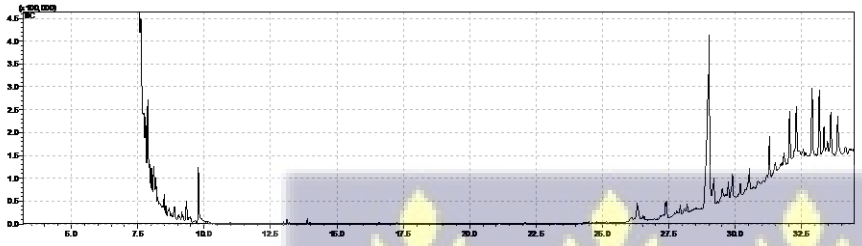
Female Glandular extracts (whole body)



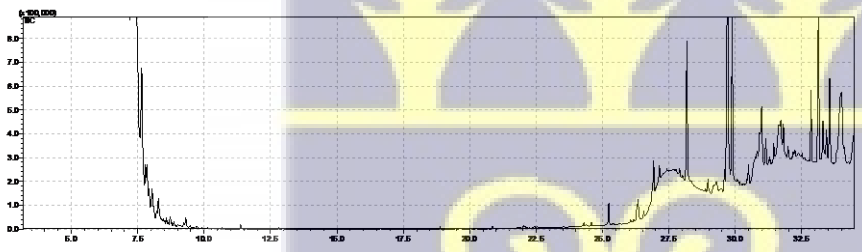
Leptomastix dactylopii chromatograms

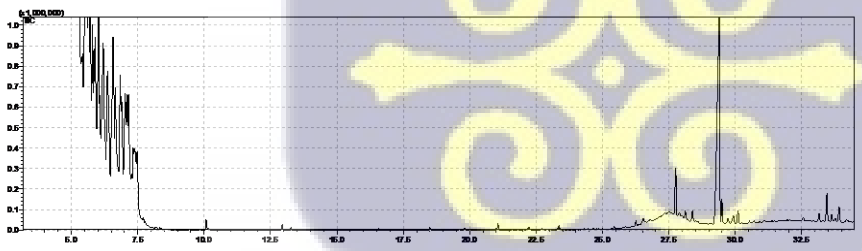
Male cuticular extracts



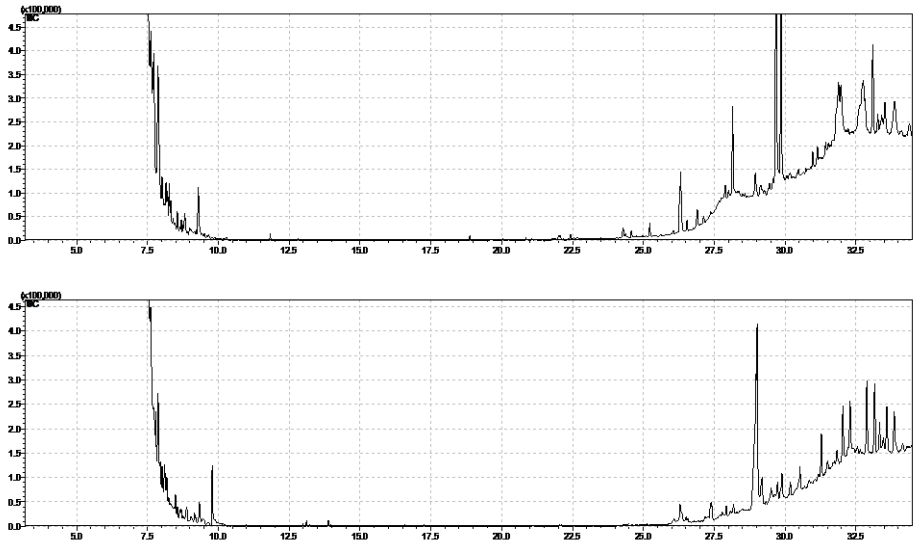


Female cuticular extracts

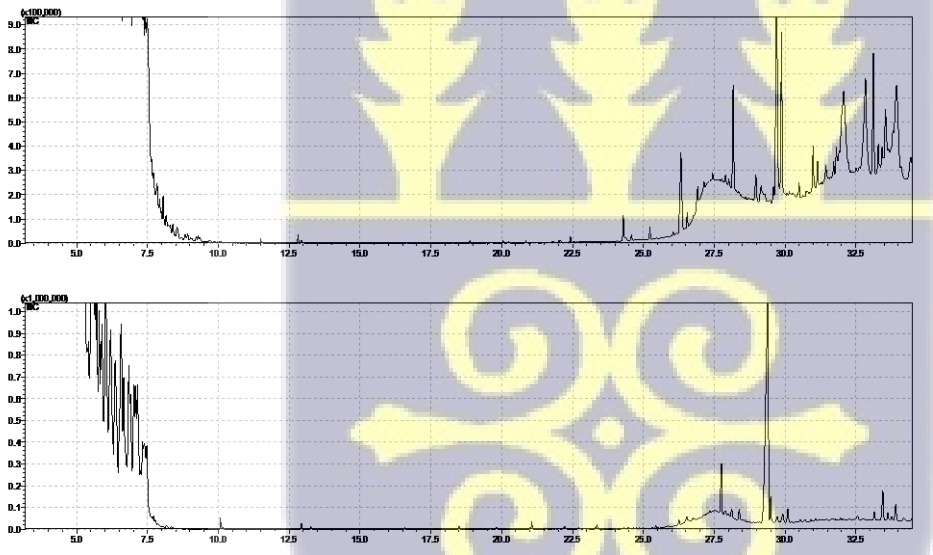




Male Glandular extracts (whole body)

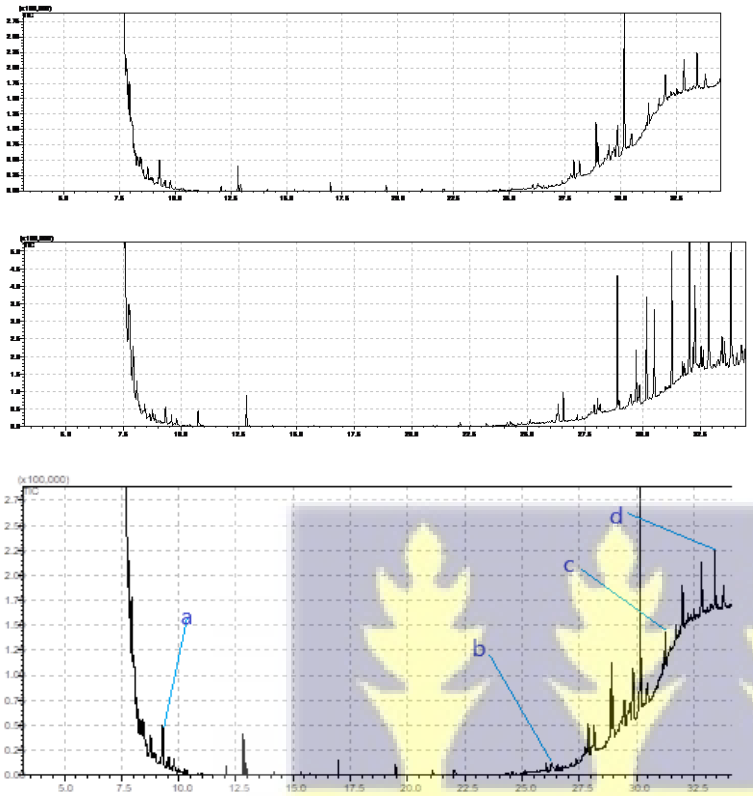


Female Glandular extracts (whole body)



Formicococcus njalensis chromatograms

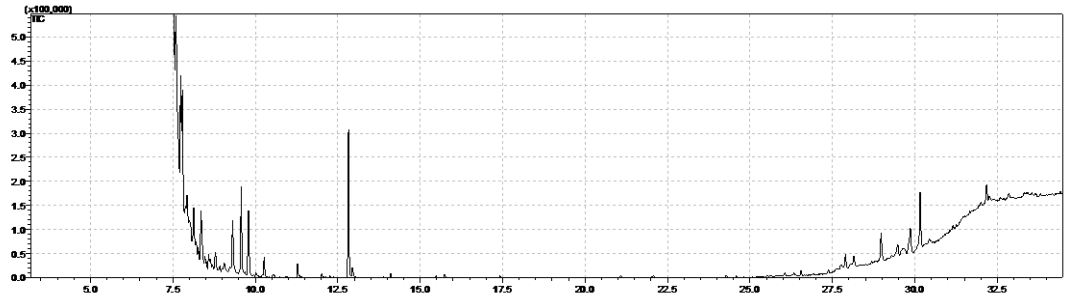
Female headspace volatiles

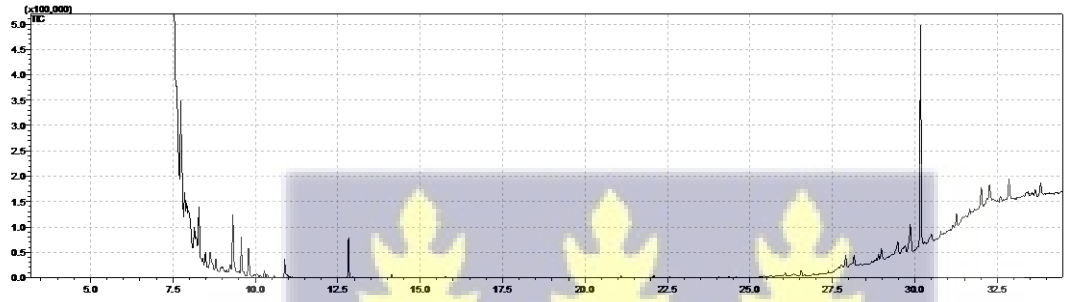


. 4-Hydroxy-4-methylpentan-2-one, b. 1-Heptadecanol, c. Hexadecanoic acid, 2-(octadecyloxy)ethyl ester*, d. Unknown ester

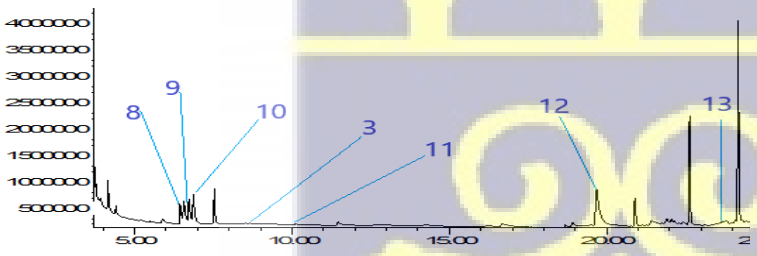
Theobroma cacao chromatograms

Detached cocoa pod headspace volatiles



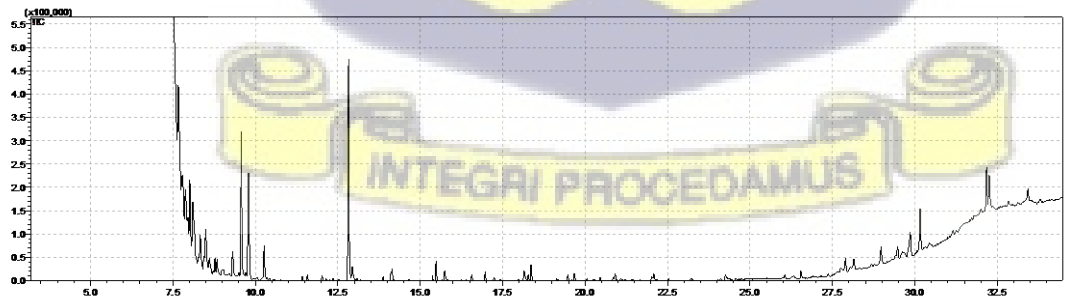


Attached cocoa pod headspace volatiles

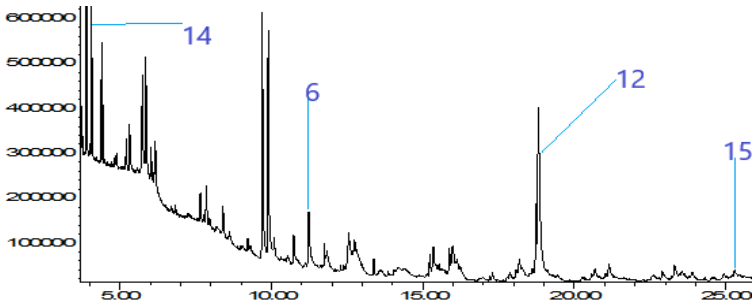


3-hexanone[8], 3-hexanol[9], 2-hexanol[10], 1,3-dimethyl benzene[3], α-pinene[11] ,1,6-dioxacyclododecane-7,12-dione[12], methyl hexadecanoate[13]

Detached cocoa pod with mealybugs (HIPV) headspace volatiles

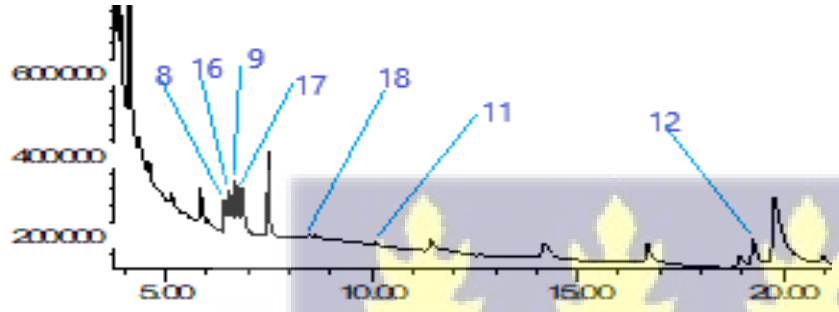


Attached cocoa pod with mealybugs (HIPV) headspace volatiles



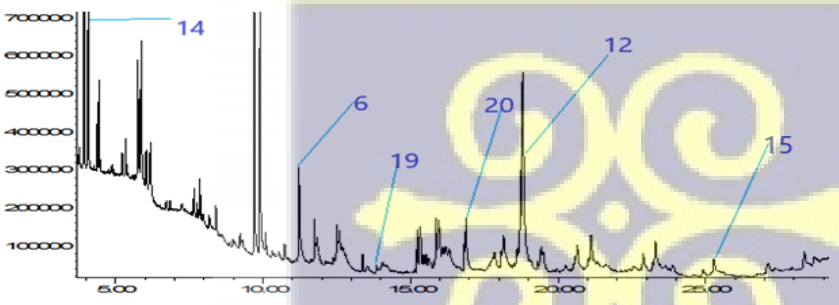
2,5-dimethyl-tetrahydrofuran[14], limonene[6], 1,6-dioxacyclododecane-7,12-dione[12], eicosane[15]

Cocoa seedlings headspace volatiles



3-hexanone[8], 2-hexanone[16], 3-hexanol[9], 4-methyl pentanol[17], o-xylene[18], α-pinene[11], 1,6-dioxacyclododecane-7,12-dione[12]

Cocoa seedlings with mealybugs (HIPV) headspace volatiles



2,5-dimethyl-tetrahydrofuran[14], limonene[6], naphthalene[19], isolongifolene[20], 1,6-dioxacyclododecane-7,12-dione[12], eicosane[15]