IDENTIFICATION AND CHARACTERIZATION OF HOST MARKING PHEROMONES IN THREE *CERATITIS* SPECIES

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Identification and Characterization of Host Marking Pheromones in Three *Ceratitis S*pecies

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DECLARATION

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

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This thesis has been presented for examination with our approval as the supervisors.

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DEDICATION

To my family, who accorded me encouragement, support and patience during this study.

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LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
BCEU	Behavioral and Chemical Ecology unit
CRD	Completely Randomized Design
ESI	Electron Spray Ionization
FAO	Food and Agricultural Organization
FSC	Female Specific Compound
GA	Glutamic acid
GC-MS	Gas Chromatography-Mass Spectrometry
GSH	Glutathione
HCl	Hydrochloric acid
HMPs	Host Marking Pheromones
HPLC	High Performance Liquid Chromatography
icipe	International Centre of Insect Physiology and Ecology
IPM	Integrated Pest Management
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LC-MS	Liquid Chromatography – Mass Spectrometry
LC-MS/MS	Liquid Chromatography – Mass Spectrometry – Mass Spectrometry
LC-QTOF-MS	Liquid Chromatography – Quadruple time of flight – Mass Spectrometry
MDGs	Millennium Development Goals
MRLs	Maximum Residue Levels
NMR	Nuclear Magnetic Resonance

RPM	Revolution Per Minute
SNK	Student Newman Keuls
SSA	Sub-Saharan Africa
USDA	United States Department of Agriculture
VOCs	Volatile Organic Compounds

ABSTRACT

Fruit flies (Diptera: Tephritidae) are notorious pests of horticultural crops, causing significant economic losses especially in the tropics. The conventional approaches utilized in controlling these pests including biological and chemical methods are relatively inefficient and targets mostly males, making it imperative to identify new Integrated Pest Management (IPM) tools that are effective and economically sustainable. To boost current efforts in fruit fly control, females should also be controlled, for example through exploitation of their host marking behavior. Ovipositing females of certain fruit fly species have been established to mark their oviposition sites with a host marking pheromone (HMP), to deter other females from overexploiting the same fruit for egg laying. Previous work has identified HMPs for ovipositing females of the cherry fruit fly and the Mexican fruit fly. However, few are known for African indigenous fruit flies. In this study, the HMP of the African indigenous mango fruit fly *Ceratitis cosyra*,was identified as glutathione (GSH): - a ubiquitous animal and plant antioxidant tripeptide, while that of *C. rosa* and *C. fasciventris* is glutamic acid (GA). GSH was identified using bioassay-guided fractionation where aqueous extract of the fecal matter of adult females was analyzed by liquid chromatography coupled to quadrupole time of flight mass spectrometry (LC-QTOF-MS). A similar protocol was used to resolve the HMP of the two fruit fly species *C. rosa* and *C. fasciventris*. Dual choice oviposition assays showed that both the fecal matter extract and the isolated female specific compound significantly reduced oviposition responses in conspecific and heterospecific females of *C. cosyra*, *C. rosa* and *C. fasciventris*. GSH and GA levels in the respective female fecal matter extract increased with increasing age of the fruit fly, with optimal amounts detected in the fecal matter of approximately 2-week-old before slightly dropping. Additionally, GSH and GA levels were 5-10 and 10-20 times higher in female fecal matter than in the ovipositor or hemolymph extracts of the respective females fruit flies. Further analysis using synthetic GSH showed the molecule to reduce oviposition in conspecifics and the heterospecifics species (*C. rosa*, *C. fasciventris*, *C. capitata*, *Zeugodacus cucurbitae*) and to arrest the egg parasitoid *Fopius arisanus*. In contrast, GSH had no effect on oviposition responses of the invasive oriental fruit fly species *Bactrocera dorsalis* and *C. anonae*. Also, it did not alter the volatile profile of ripe mango fruit when topically applied. GA on the other hand, reduced oviposition in *C. rosa* and *C. fasciventris* but not in *C. cosyra*. Identification of GSH and GA as a host marking pheromone in females of *C. cosyra* and *C. rosa* and *C. fasciventris* improves our understanding of fruit fly chemical ecology, and that they could be used as a potential component in the IPM of these fruit fly species

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Tephritid fruit flies are among the most notorious pests of horticultural crops worldwide, with direct and indirect losses in Africa estimated at over $2 billion annually (Sunday Ekesi *et al*., 2017; Lux *et al*., 2002; Mwatawala *et al*., 2004). Direct losses reduce crop yield through damage from feeding larvae that emerge from eggs laid by female fruit flies (Sunday Ekesi *et al*., 2017; Kachigamba *et al*., 2012; Vaníčková *et al*., 2014). Indirect losses are a result of quarantine restrictions through introduction of uniform and strict maximum residue levels (MRLs) legislation for pesticides across Europe, causing a reduction in the export market from Africa (de Graaf, 2009).Management of fruit flies has focused on a number of techniques including the use of commercially available traps that can be combined with food baits, early harvesting, fruit bagging, biological control agents (parasitoids, predators and pathogens), chemical sprays and orchard sanitation (Rwomushana *et al*., 2009; Silva *et al*., 2012). While challenges to effective controlling of these pests abound, a combination of IPM packages is a viable option including the exploitation of semiochemicals such as host marking pheromones (HMPs)

After oviposition, many phytophagous insects especially those whose larvae develop in confined and limited food resource habitats, always leave behind HMPs (Nufio *et al*., 2004). This is achieved through the host marking behavior which involves dragging a protracted ovipositor on the surface of the fruit by females (Kachigamba *et al*., 2012). The HMP informs conspecifics and at times heterospecifics of already utilized oviposition substrate there by triggering a complex series of responses in ovipositing fruit flies that include oviposition deterrence, reduction in time spent by females in oviposition attempts, reduction of clutch size and the number of clutches per fruit and moving away from heavily infested oviposition resource patches. The ultimate aim is to minimize over exploitation of the brood resources resulting in increased survival rate and development of subsequent generation (Arredondo & Díaz-Fleischer, 2006; Kachigamba *et al*., 2012; Stelinski *et al*., 2009)

The existence and function of HMPs has been studied in many insects groups, among them Diptera, Coleoptera, Hymenoptera, Lepidoptera and Neuroptera (Barrera *et al*., 1994; Silva *et al*., 2012). Majority of these studies reported host marking behavior and not the chemical identity and characterization of the pheromone (Kachigamba *et al*., 2012). In the Order Diptera, host-marking behavior is prevalent in the family Tephritidae; most species in this family are of high economic importance worldwide (Copeland, Wharton, *et al*., 2006).

Semiochemicals mediate communication in many species of fruit flies and therefore can be exploited in integrated pest management strategies for these pests. For instance, HMPs can be used to manipulate the oviposition behavior of insects because they reduce/deter subsequent egg laying in conspecifics and heterospecific species (Aluja & Díaz-Fleischer, 2006; Kachigamba *et al*., 2012; Nufio & Papaj, 2001a). For example, the fatty acid glucoside derivative *N*-[15(*β*-glucopyranosyl)oxy-8-hydroxypalmitol]-taurine **[1]**, (Figure 1.1) identified as the HMP from the fecal matter of ovipositing females of the cherry fruit fly *Rhagoletis cerasi*, was found to significantly reduce conspecific infestation in cherry orchards (Nufio *et al*., 2004).

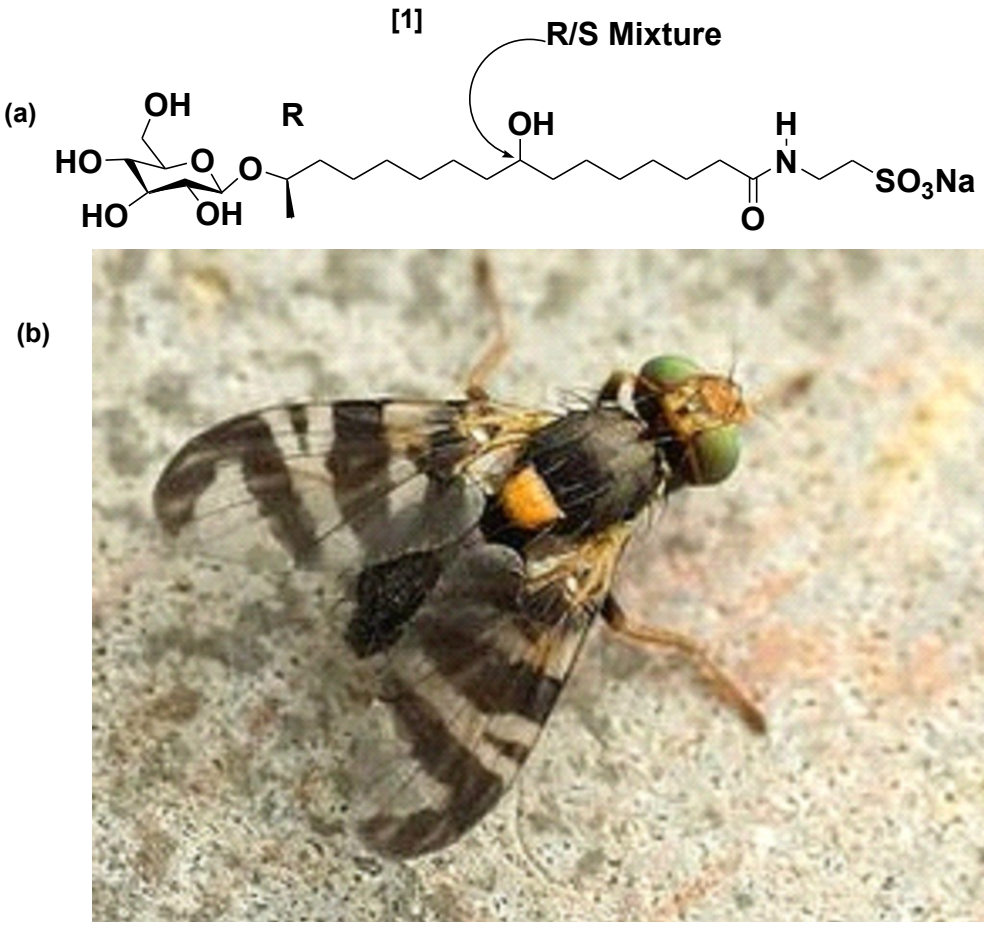

Figure 1.1: Host marking pheromone of *R.* ***cerasi*** (a) N-[15(β-glucopyranosyl)-oxy-8-hydroxypalmitol]-taurine (b) cherry fruit fly (Weems *et al*., 2015)

The HMP of the Mexican fruit fly *Anastrepha ludens* is the amino acid derivative *N*-(2,14-dimethyl-1-oxopentadecyl)-glutamic acid **[2]** (Figure 1.2). It was identified from fecal matter of the Mexican fruit fly and found to reduce conspecific infestation in the red mombin *Spondias purpurea* (Edmunds *et al*., 2010).

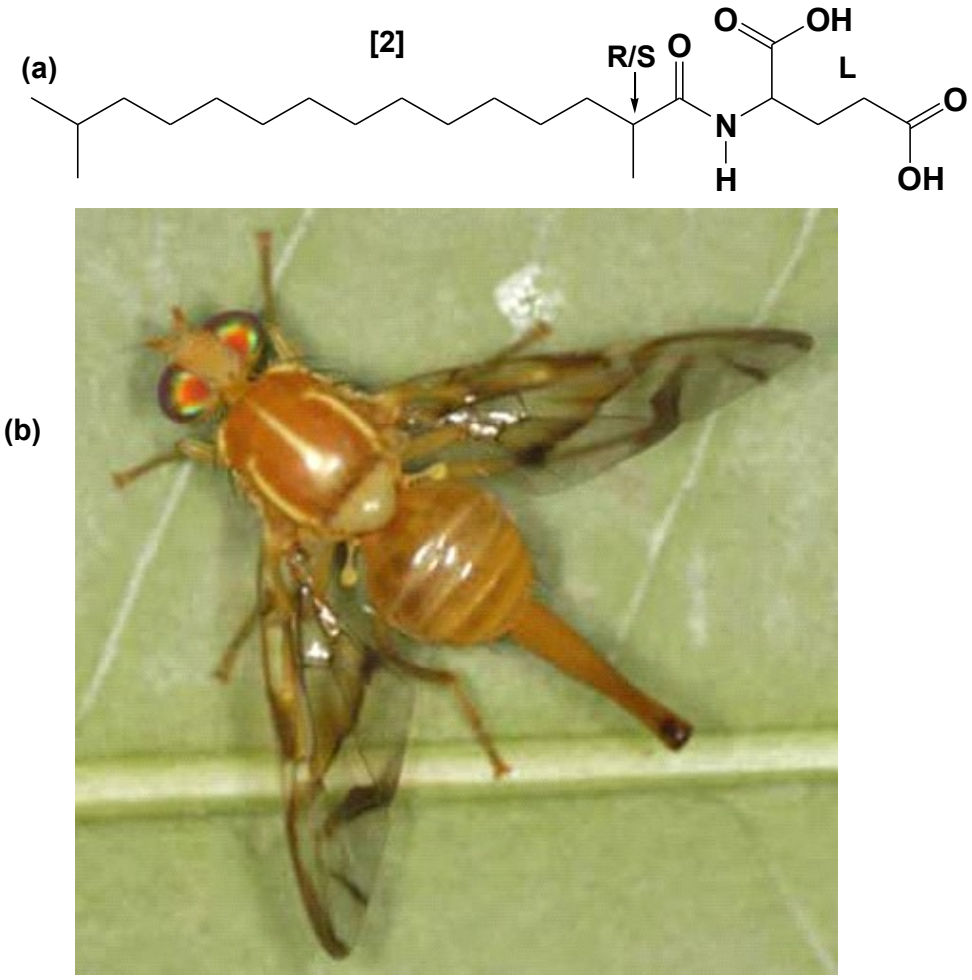

Figure 1.2: Host marking pheromone of *A. ludens* (a) *N*-[2,14-dimethyl-1-oxopentadecyl]-glutamic acid (b) Mexican fruit fly; L, levorotatory (Weems *et al*., 2015)

Additionally, the efficacy of externally applied fecal matter extracts from female Mediterranean fruit fly *C. capitata* on conspecifics have been demonstrated, (Arredondo *et al*., 2006), although the bioactive component in the fecal matter extract was not identified.

The Mediterranean fruit fly *C. capitata* and the related species *C. cosyra*, *C. fasciventris C. anonae* and *C. rosa* are all indigenous to Africa and are economically important pests of a wide range of fruits (Copeland *et al*., 2006; Kachigamba *et al*., 2012). Because fruit growers in Africa export fresh fruits to various countries in the world, there is a potential risk of the introduction and establishment of these fruit flies

in some of these countries depending upon climatic conditions (Kriticos *et al*., 2007). For example, the larvae of *C. cosyra* in infested mangoes from Africa, is one of the most commonly intercepted fruit flies in Europe (Steck, 2012), where it is feared that it may establish, like the now cosmopolitan *C. capitata*. Therefore, robust, effective and eco-friendly IPM strategies including use of HMPs are necessary to control these pests wherever they occur.

1.2 Statement of the problem

Fruit flies continue to constrain production and utilization of mango and other horticultural produce in Kenya. Although several techniques are being used to mitigate the problem, the level of infestations and damages caused by the fruit flies continues to soar. Combination of IPM packages is the only viable option including the development of semiochemical based tools such as HMPs. This technique has been tested in Switzerland and USA to manage these devastating pests. However, the technique has not been exploited in Africa because not much is known about HMPs in the fruit fly species found in this region and their potential application in pest management.

Recent studies similar to those performed on HMP of *C. capitata,* (Arredondo *et al*., 2006) have demonstrated that related African indigenous fruit flies *C. cosyra, C. rosa* and *C. fasciventris* exhibit host marking behavior, (Kachigamba *et al*., 2012), suggesting their use of HMPs. This study traced the candidate HMP to the aqueous extract of the fecal matter of females of *C. cosyra*. However, like the study of the Mediterranean fruit fly (Roitberg *et al*., 1991), the chemical identity of the HMP responsible for bioactivity was not disclosed (Kachigamba *et al*., 2012). Therefore, identification of the pheromone responsible for oviposition deterrence would have a potential for its application in the management of several species of fruit flies

This study sought to isolate, identify and characterize HMPs in three *Ceratitis* species namely; i) *C. cosyra,* the most destructive of the four host-marking species of mango (Rwomushana *et al*., 2009) and the fact that no other fruit fly species deters it from ovipositing yet it deters others ii) *C. fasciventris* and *C. rosa* since both exhibit host-marking behavior and yet the identities of their HMPs are unknown. This was

achieved through bioassay-guided fractionation of the aqueous extract of the fecal matter and analysis by chromatography systems (LC and GC) that were coupled to mass spectrometry (MS, MS/MS and QTOF-MS).

1.3 Justification

The use of HMPs provides about 90% efficacy in curbing fruit fly infestation (Aluja & Boller, 1992, Nufio & Papaj, 2004a). Moreover the technique is target-specific, environmentally friendly and less laborious compared to the conventional methods. Aluja & Boller (1992), observed that the fruit fly problem could be better managed using behavior-based techniques rather than insecticides which is known to have delirious effect on the environment and human. In this regard, it is important that strategies such as the host marking technique be explored with the aim of addressing the fruit fly problem in Kenya and Africa at large.

Two host marking pheromones *N*-[15(*β*-glucopyranosyl)oxy-8-hydroxypalmitol]-taurine and *N*-(2,14-dimethyl-1-oxopentadecyl)-glutamic acid have so far been identified for *R. cerasi* and *A. ludens* respectively for fruit flies not found in Africa. However, in Africa, some indigenous fruit flies species (*C. capitate*, *C. cosyra*, *C. fasciventris* and *C. rosa*) have been shown to display host marking behavior although their HMP was not disclosed (Kachigamba *et al*., 2012), an avenue that can be exploited for their control.

1.4 Hypotheses

The following were the hypotheses of this study:

1. Fecal matter from *C. cosyra*, *C. fasciventris* and *C. rosa* do not contain host-marking pheromones
2. HMPs of the three *Ceratitis* species are not structurally similar to the known HMPs identified from *A. ludens* and *R. cerasi*
3. HMP of *C. cosyra* does not elicit arresting behavior in the egg parasitoid
4. HMP of *C. cosyra* does not change the volatile profile in ripe mango fruits following topical application

1.5 Objectives

1.5.1 General objective

To isolate, identify and characterize the host-marking pheromone (HMP) in three *Ceratitis* species (*C. cosyra*, *C. fasciventris* and *C. rosa*) and evaluate the most active HMP (*C. cosyra*) for oviposition deterrence in other fruit fly species; *C. capitata*, *Z. cucurbitae, B. dorsalis C. anonae*. Also, to establish the effect of *C. cosyra* HMP effect on the egg parasitoid *Fopius arisanus* and volatiles of oviposition substrate (mango) with the view of getting insight into its potential application in integrated pest management of fruit flies.

1.5.2 Specific objectives

1. To isolate and characterize the host-marking pheromone in three *Ceratitis* species (*C. cosyra*, *C. fasciventris* and *C. rosa*)
2. To determine the efficacy of the isolated compounds in oviposition deterring assays
3. To determine the efficacy of the synthetic analogue of compounds isolated in 2 above in oviposition deterring assays
4. To determine the effect of *C. cosyra* HMP on egg parasitoid *Fopius arisanus*
5. To determine the effect of *C. cosyra* HMP on volatile profile of ripe mango following topical application

CHAPTER TWO

LITERATURE REVIEW

2.1 World geographic distribution and economic importance of fruit flies

Fruit flies are considered one of the most economically important groups of insect pests worldwide (Vargas *et al*., 2015). They belong to the family Tephritidae, one of the largest, most diversified and fascinating acalypterate families of this order. They are commonly referred to as fruit flies due to their close association with fruits 0and vegetables and comprised of over 4500 species (Clarke *et al*., 2005). Sub-Saharan Africa (SSA) is the aboriginal home to 915 fruit fly species, out of which 299 species develop in either wild or cultivated fruit (Ekesi *et al*., 2016). The most economically important fruit fly pests belong to five genera: *Bactrocera* Macquart, *Ceratitis* MacLeay, *Anastrepha* Schiner, *Rhagoletis* Loew and *Dacus* Fabricius

The genus *Bactrocera* is the most economically important genus. Its members are native to the Old-World tropics and the major species include *B. invadens*, *B. dorsalis*, *Z. cucurbitae*, *B. oleae*, *B. tryoni* and *B. zonata* (Ekesi *et al*., 2016). *B. dorsalis* invaded Africa around 2003 and is currently the most destructive fruit fly pest of mango on the continent (Rwomushana *et al*., 2008).

The majority of *Ceratitis* species are found within the African continent except the Mediterranean fruit fly (*C. capitata*), which has spread to many tropical and subtropical parts of the world (Ekesi *et al*., 2016).

The genus *Anastrepha* is mainly found in the Neotropics and its most economically important pest species are the Mexican fruit fly (*A. ludens*), West Indian fruit fly (*A. obliqua*), and South American fruit fly (*A. fraterculus* complex (Ekesi *et al*., 2016).

The genus *Rhagoletis* is mainly found in the Holarctic and Neotropical regions and its most economically important fruit pest species are the apple fruit fly (*R. pomonella*), European and eastern cherry fruit flies (*R. cerasi* and *R. cingulata* respectively), blueberry fruit fly (*R. mendax*), walnut husk fly (*R. completa*), *R. striatella*, a pest of husk tomato, and *R. tomatis*, a pest of tomato (Ekesi *et al*., 2016).

The genus *Dacus* mainly occurs in the Afrotropical region and the most economically important pest species of this genus are *D. bivittatus* and *D. ciliates* (Ekesi *et al*., 2016) .

Fruit flies cause direct losses through the female laying their eggs under the skin of fruits and vegetables (Ekesi *et al*., 2016). The eggs hatch into larvae that feed in the decaying flesh of the crop. Infested fruits and vegetables quickly rot and become inedible or drop to the ground (Kachigamba *et al*., 2012; Vaníčková *et al*., 2014). Beside the direct damage to fruits, indirect losses are associated with quarantine restrictions because infestation and sometimes mere presence of the flies in a particular country could also restrict the free trade and export of fresh horticultural produce to large lucrative markets abroad. The introduction of uniform and strict maximum residue levels (MRL) across Europe compounds the problem and further jeopardizes export (de Graaf, 2009)

In order to ensure market standards are met, farmers have been forced to incur additional costs in terms of fruit fly management. As an example, the cost of eradicating *C. capitata* and several other major fruit fly pests in the State of California was estimated to range between $493-875 million, and an additional cost of $564 million following imposition of trade embargo from Asian countries and loss of more than 14,000 jobs (Siebert *et al*., 1995). In Africa, fruit fly loses is estimated at $2 billion annually on avocado alone (Ekesi *et al*., 2017; Lux *et al*., 2002; Mwatawala *et al*., 2004) while in Kenya, the average loss is KES 5.5 billion annually due to invasive fruit fly species (Mugo, 2017)

2.2 Taxonomic features of *C. cosyra, C. fasciventris*, *C. rosa*, *C. capitata*, *C. anonae*, *B. dorsalis* and *Z. cucurbitae*

2.2.1 *Ceratitis cosyra*

C. cosyra (Walker) also known as the mango fruit fly or marula fruit fly based on its common occurrence in these host plants is an indigenous species of Africa. The fruit fly is a serious pest to both smallholder and commercial mango farmers across Sub-

Saharan Africa. *C. cosyra*, also attacks a few other fruits such as avocados, citrus and peaches (Lux *et al*., 2003).

The presence of *C. cosyra* has been reported in several African countries such as Cameroon, Comoros, Kenya, Madagascar, Malawi, Mozambique, Seychelles, South Africa, Sudan, Tanzania, Togo, Zaire, Zambia and Zimbabwe, where it is more destructive than either the Mediterranean fruit fly, *C. capitata* or the Natal fruit fly *C. rosa* (Lux *et al*., 2003).

C. cosyra, like other *Ceratitis* species, has yellowish banded wings and body; sides and posterior of thorax prominently ringed with black spots, dorsum yellowish except for two tiny black spots centrally and two larger black spots near scutellum which has three wide, black stripes separated by narrow yellow stripes; wing length 4-6 mm, costal band and discal cross band joined. The pattern of grey flecks in the basal wing cells distinguishes *Ceratitis* spp. from most other genera of tephritids (De Meyer *et al*., 2008; Virgilio *et al*., 2008). Some taxonomic features of *C. cosyra are* shown in plate 2.1.

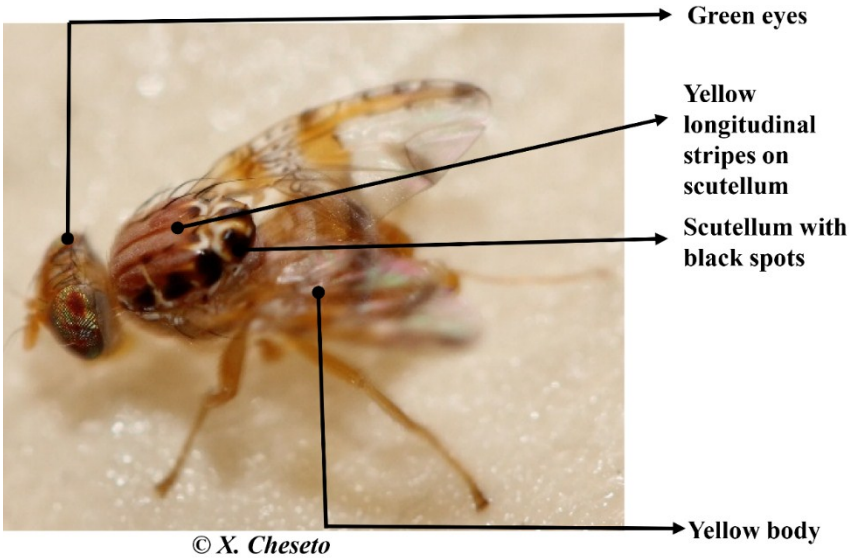

Plate 2.1: Main taxonomic features in *C. cosyra* female

2.2.2 *Ceratitis fasciventris*

C. fasciventris is an indigenous species of Africa. In Kenya, it is mostly found in the western region (Lux *et al*., 2002). It is also been reported in other African countries such as Angola, Benin, Congo-Kinshasa, Congo-DRC, Cote d' Ivoire, Ethiopia, Ghana, Guinea, Mali, Namibia, Nigeria, Sao Tome, Seirra Leone, Uganda and Tanzania (Ekesi *et al*., 2009, 2016). According to De Meyer and Freidberg, (2006), an adult *C. fasciventris* can be distinguished by the following characteristics: the body is 3.95 - 5.15 mm long; the wing is 4.45 - 5.75 mm long while the antennae are yellowish orange. The first flagellomere is 2-3 times longer than the pedicel. The arista has short to moderately long rays; ventral rays being shorter and sparser than the dorsal rays, especially basally. The frons is yellow; with short scattered setulae distinctly darker than the frons. Frontal setae are well developed. The face is yellowish white. Genal seta and setulae are dark and well developed.

The post frontal lobe is yellowish white, with no spot. The mesonotum is dark gray, sometimes with an orange tinge; with streaks and darker markings but without distinct spots, except white and separate prescutellar markings, usually with paler gray area in between, occasionally merged. Scapular setae are dark. The scutellum is yellowish white while the anepisternum on ventral half is yellowish brown and setulae pale. Legs are yellow except where otherwise noted. The foreleg is slightly yellow and its femur is without bushy feathering posteriorly, only a row of dispersed, long and usually black setulae. The midleg has its femur with dispersed pale setulae at the base. The femur has long setulae. Wing bands are brown or yellowish brown.

The cubital band is free, the medial band is absent; cross vein R-M is opposite the middle of the discal cell, sometimes just proximal to the middle. In female *C. fasciventris* however, the anepisternum on the ventral half is brown or yellowish brown, the cross vein is variable, the legs are not feathery and the oviscape is shorter than the pre-abdomen. Some taxonomic features of *C. fasciventris* are shown in plate 2.2

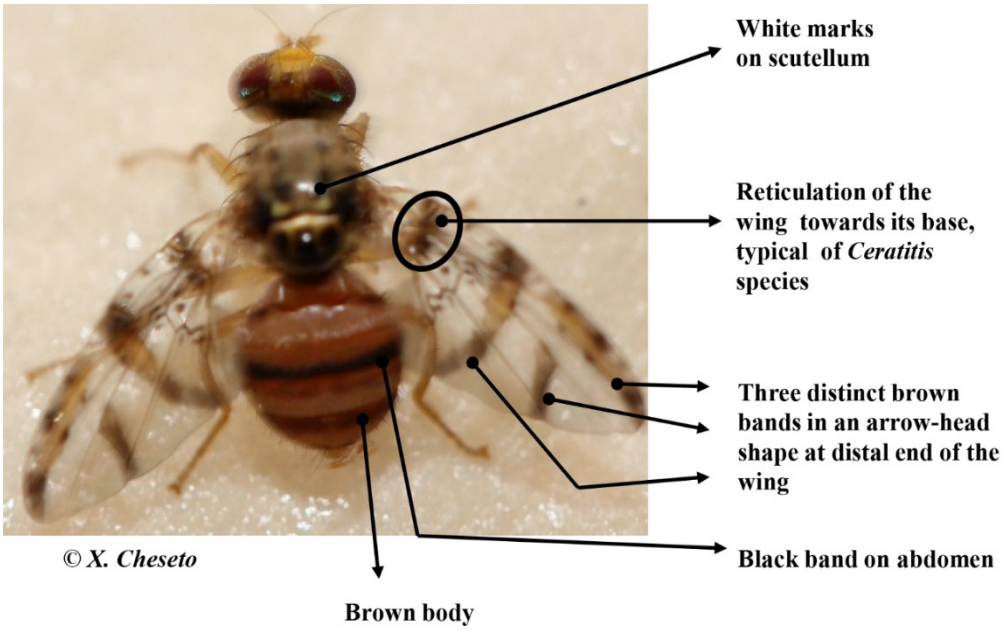

Plate 2.2: Main taxonomic features in *C. fasciventris*

2.2.3 *Ceratitis rosa*

C. rosa is also indigenous to Africa and has been reported in the following countries; Angola, Uganda, Tanzania, Malawi, Mozambique, Nigeria, Rwanda, Zambia South Africa and Swaziland. In Kenya, it is found mostly at the coastal region (Lux *et al*., 2002). *C. rosa* is a polyphagous species that attacks mangos, apples, apricots, avocados, citrus, guavas, figs, pawpaw's, peaches, pears, plums, quinces, tomatoes and grapes (White & Elson-Harris,1992; Ekesi *et al*., 2009).

C. rosa has the following characteristics: wing bands and general body are brown; the wing is 4-6 mm long; the body is 4-5 mm long. The scutellum is marked black and yellow, with yellow lines or areas meeting the margin, such that each apical scutellar seta is based in or adjacent to a yellow stripe. Male's mid-tibia has rows of stout setae along the distal half of both the anterior and posterior edges such that it looks feathery (Plant Health, Australia, 2011). Some taxonomic features of *C. rosa* are shown in plate 2.3.

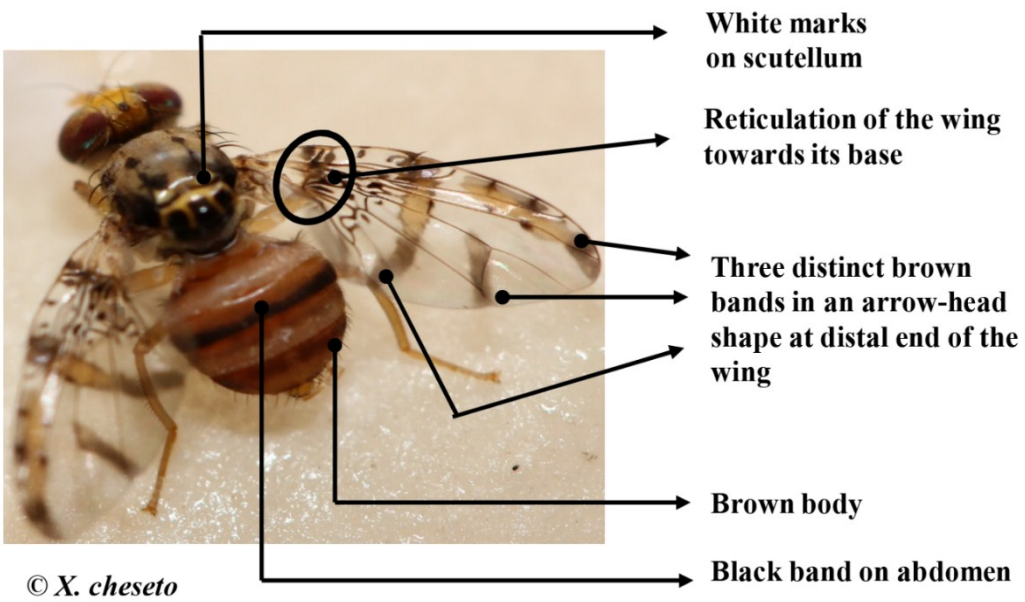

Plate 2.3: Main taxonomic features in *C. rosa*

2.2.4 *Bactrocera dorsalis*

B. dorsalis is an invasive species of Asian origin, first detected in Kenya in 2003 (Lux, *et al*., 2003). They are known to attack a wide range of fruits, vegetable and wild species examples include; mango, banana, guava, pepper and citrus (Rwomushana *et al*., 2008, Ekesi *et al*., 2006)

B. dorsalis can be identified by the following features: It is medium in size, the face is fulvous with a pair of medium to large oval black spots while the scutum is red-brown with variable dark fuscous to black patterns (in occasional specimens the scutum base color is black) (Drew and Romig, 2007). The post pronotal lobes and notopleura are yellow, the scutellum is yellow except for a narrow dark basal band and the femora of the legs are entirely fulvous. The abdominal terga III - V are orange brown with a 'T' pattern consisting of a narrow transverse black band across the anterior margin of tergum III. The fly has dark orange-brown shining spots on tergum V. Some taxonomic features of *B. dorsalis* are shown in Plate 2.4

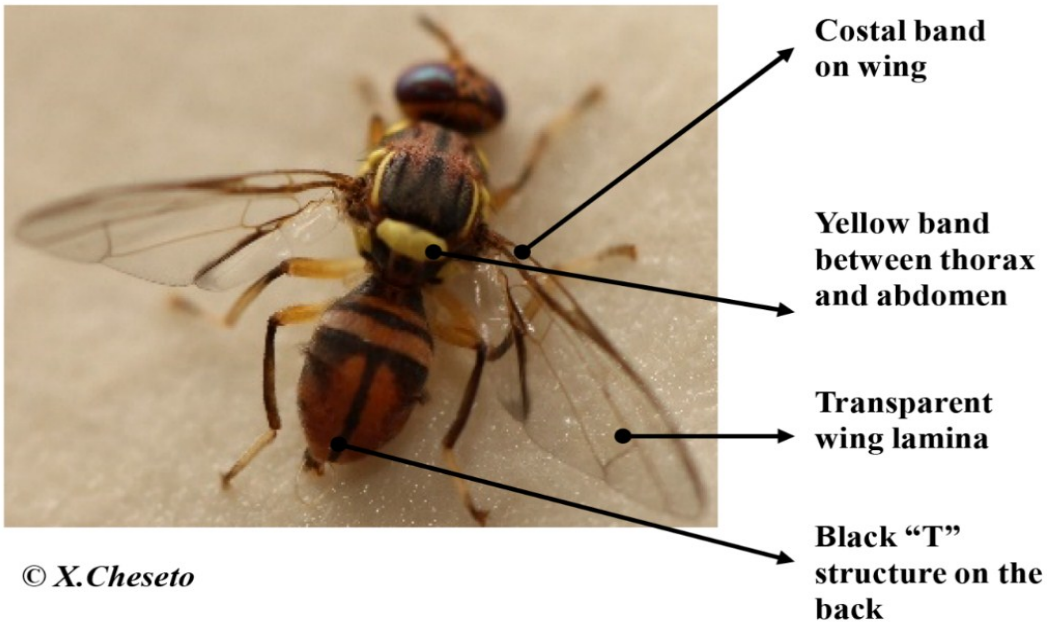

Plate 2.4: Main taxonomic features in *B. dorsalis*

2.2.5 *Ceratitis capitata*

C. capitata is indigenous to Africa, but has spread to the Mediterranean area and parts of Central and South America (Sunday Ekesi *et al*., 2009). In Kenya, *C. capitata* is found at the coastal region and in central and western highlands (Copeland & Wharton, 2006). It is a highly polyphagous species hosting on mango apples, avocados, citrus, figs pears and also from wild fruits ((White & Elson-Harris,1992); Ekesi *et al*., 2009).

C. capitata can be identified by the following features: the body is yellow; the eyes are reddish purple, the scutellum is entirely black in apical half, with a sinuate yellow line across it sub-basally; the costal band starts beyond the end of vein R1 and is separated from discal cross-band by a hyaline area at the end of R1 while the wing is 4 - 6 mm long (De Meyer and Freidberg, 2006). Males and females of *C. capitata* differ in a way that males have small black diamond-shaped nodules at the apex of their orbital setae, which is absent in females, while females have a characteristic yellow marking on their wings (De Meyer and Freidberg, 2006). Some of the taxonomic features of *C. capitata* are shown in Plate 2.5.

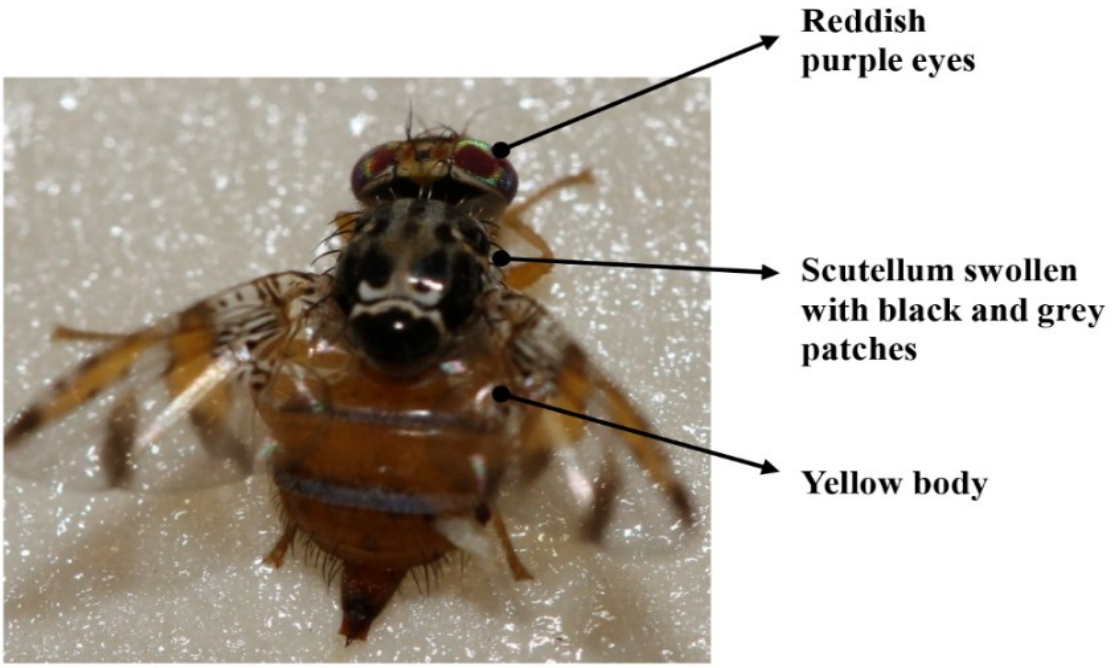

Plate 2.5: Main taxonomic features in *C. capitata*

2.2.6 *Ceratitis anonae*

C. anonae is indigenous to Africa. In Kenya, it is mostly found in the western part (Copeland, Wharton, *et al*., 2006). Other countries where it is found in Africa include: Benin, Cameroon, Central African Republic, Congo-Kinshasa, Congo-DRC, Equatorial Guinea, Gabon, Ghana, Guinea, Mali, Sao Tome, Senegal, Tanzania, Togo and Uganda (White & Elson-Harris,1992); Ekesi *et al*., 2009).

C. anonae is also a highly polyphagous species hosting on mango, *robusta* coffee, tropical almond, common guava and strawberry guava (Copeland, Wharton, *et al*., 2006). An adult *C. anonae* has the following descriptive morphological features; body length is 4.35-5.90 mm, wing length is 4.45 - 5.75 mm, antennae are yellow and the first flagellomere is three times as long as the pedicel. The arista has short to moderately long rays, the ventral rays being shorter and sparser than dorsal rays, especially basally. The frons is pale, sometimes completely yellow. The frontal setae are well developed, the face is white, sometimes yellowish white while the genal seta and setulae are dark and well developed. The post pronotal lobe is white, sometimes

yellowish white and has no spot. The scapular setae are dark, the scutellum is ash gray, sometimes yellowish white, the legs are yellow except where otherwise noted. Wing markings are yellowish brown, interruption between marginal and discal bands near vein R1 is clear and complete; the discal band is often partly or completely interrupted in the discal cell. The female of *C. anonae* is like the male except for the following characteristics: the legs are without feathering, the wing has a complete discal band and the oviscape is shorter than the preabdomen (De Meyer and Freidberg, 2006). Main taxonomic features of *C. anonae* are shown in Plate 2.6.

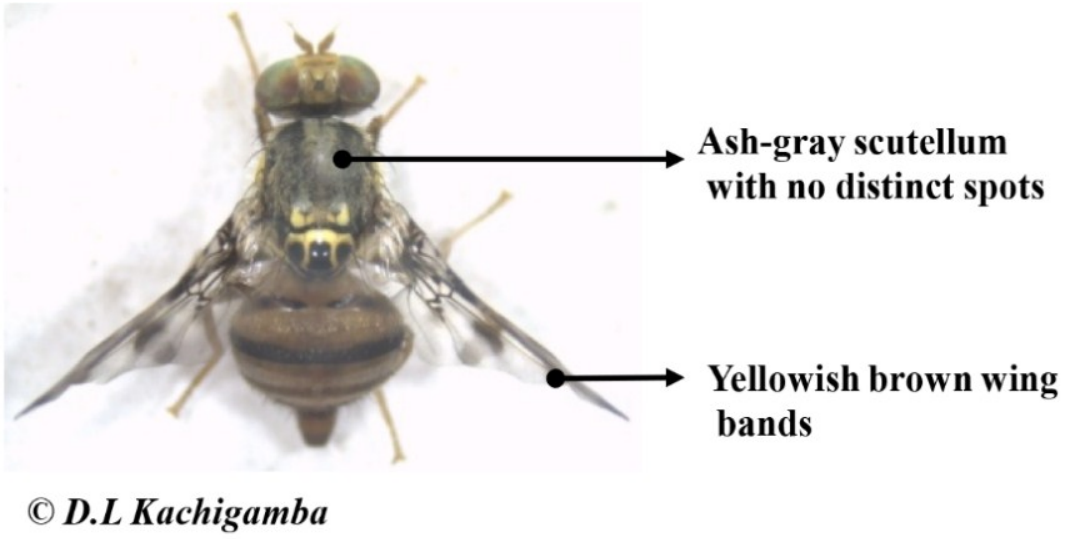

Plate 2.6: Main taxonomic features in *C. anonae*

2.2.7. *Zeugodacus cucurbitae*

The melon fly, *Z. cucurbitae* (Coquillett) is an invasive species of Asian origin. Its presence has been reported in the following continents, Africa, Asia, North America and Oceania. In African, it is present in the following countries; Cameroon, Cote d'Ivoire, Egypt, Gambia, Kenya, Mali, Mauritius, Reunion, Seychelles, Somalia, Tanzania (Jr H V Weems *et al*., 2015).

Z. cucurbitae is rather distinctive in adult morphology and can be differentiated from other related species by the following combination of characters: scutum red-brown, with medial and lateral yellow post sutural vittae; large apical spot on the wing with posterior margin reaching about halfway between vein R_{4+5} and vein M; infuscation

present over cross vein dm-cu and usually also cross vein r-m, wing cells bc and c hyaline, abdomen with a narrow transverse black band across basal margin of tergite 3 and a medial longitudinal black stripe over tergites 3-5 (White, 2006). Some taxonomic features of *Z. cucurbitae* are shown in Plate 2.7

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Plate 2.7: Main taxonomic features in *Z. cucurbitae*

2.3 Biology of fruit flies: Reproduction

Majority of fruit flies are holometabolous, i.e. they undergo complete metamorphosis with four stages; eggs, larvae (maggots), pupae and adults each briefly described below.

2.3.1 Eggs

The female adult fruit fly lays egg clutch (1-20) into the maturing and ripening fruit of the host plant. The eggs hatch into larvae inside the fruit after a few days (2-4 days).

2.3.2 Larvae

The hatching larvae (maggots) feed on the flesh of the fruit, gradually moving towards its center. The feeding activity of the larvae form galleries in the fruit which provide entry points for pathogens accelerating fruit decay and thus making the latter unsuitable for human consumption.

2.3.3 Pupae

Ripen fruits rot and fall to the ground. Fully mature larvae leave the fruit and burrow into the soil to pupate. They become oval, light to dark brown, hard pupae.

2.3.4 Adult

The adult flies may emerge from the pupae in as little as seven days during the summer, or after several months over winter (White & Elson-Harris,1992). The adult fruit fly looks for the nourishment it needs to reach maturity, breed, and lay eggs in new season crops. Young females need 1–2 weeks to become sexually mature while young males mature in one week or less. Adult fruit flies can live for 2–11 months depending on species and environmental conditions. The life cycle of a fruit fly is shown in figure 2.1.

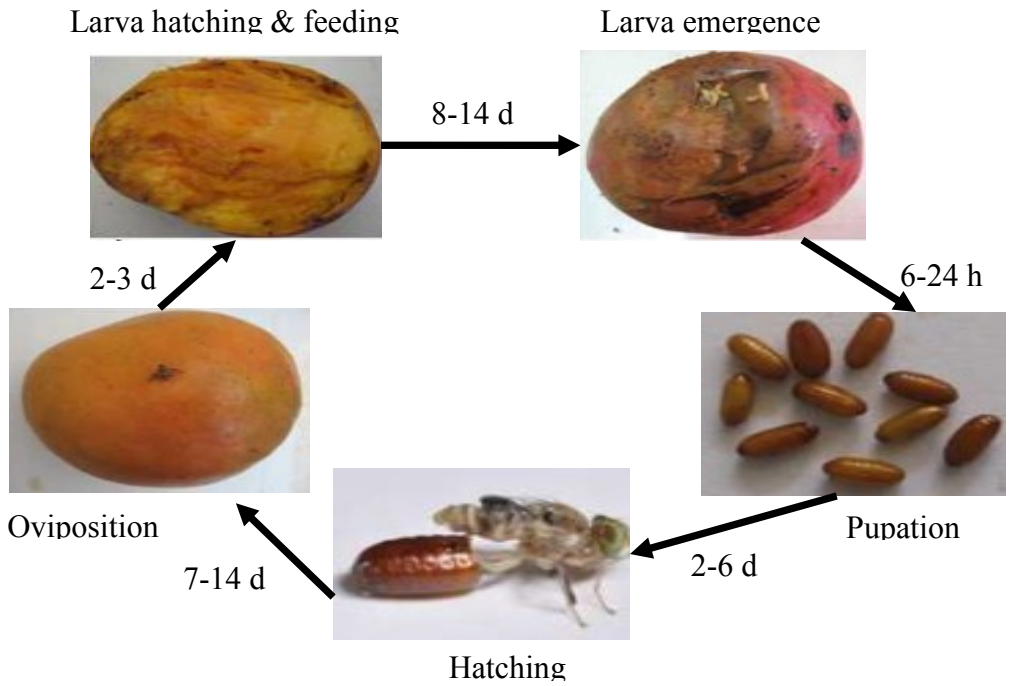


Figure 2.1: Life cycle of fruit fly; d, days

2.4. Pheromones in chemical communication

Communication is crucial to the survival and reproduction of animals. Animal communication is defined as the transmission of, reception of, and response to signals; a signal is a stimuli that causes a change in another animal's behavior (Campbell *et al*., 2005). Animals communicate through a variety of methods, including the use of visual, auditory, chemical, tactile, and electrical signals. The type of signal used depends on the environment and lifestyle of the animal, as well as the cost to benefit ratio of the signal.

The most ancient form of communication is believed to be chemically based (Bradbury *et al*., 1999). Chemicals that are utilized for animal communication are known as semiochemicals, broadly classified into pheromones and allelochemicals (Figure 2.2).

After the discovery of the first insect sex attractant bombykol **[3],** (Figure 2.3), (Butenandt *et al*., 1959), the term 'pheromone' derived from the Greek '*pherein'* (to carry or transfer) and '*horman'* (to stir up or excite) was defined by (Karlson *et al*., 1959) as substances secreted to the outside by an individual and received by a second individual of the same species, in which they stimulate a specific reaction, for example, a definite behavior or a developmental process.

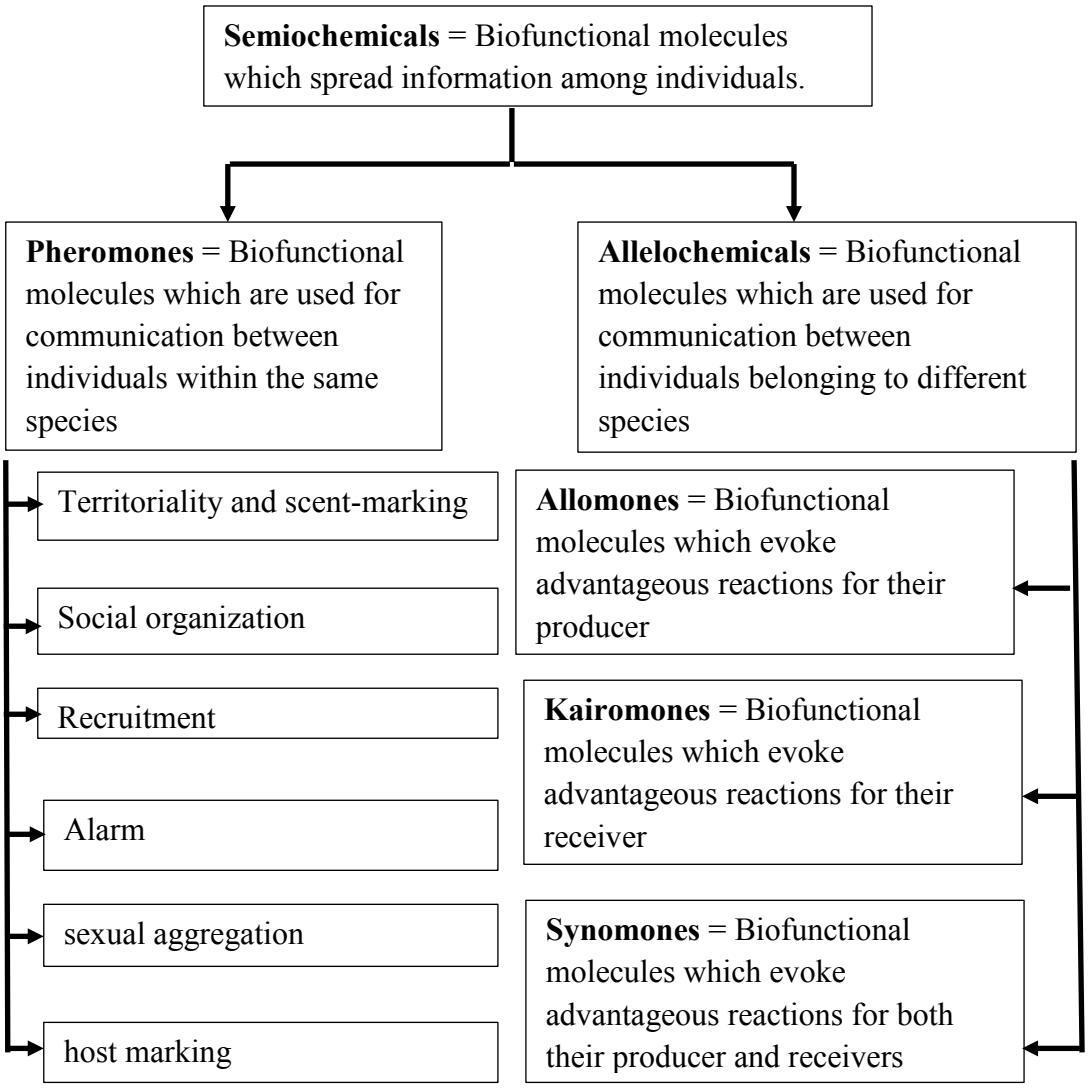


Figure 2.2: Classification and definition of semiochemicals, (Bradbury *et al*., 1999).

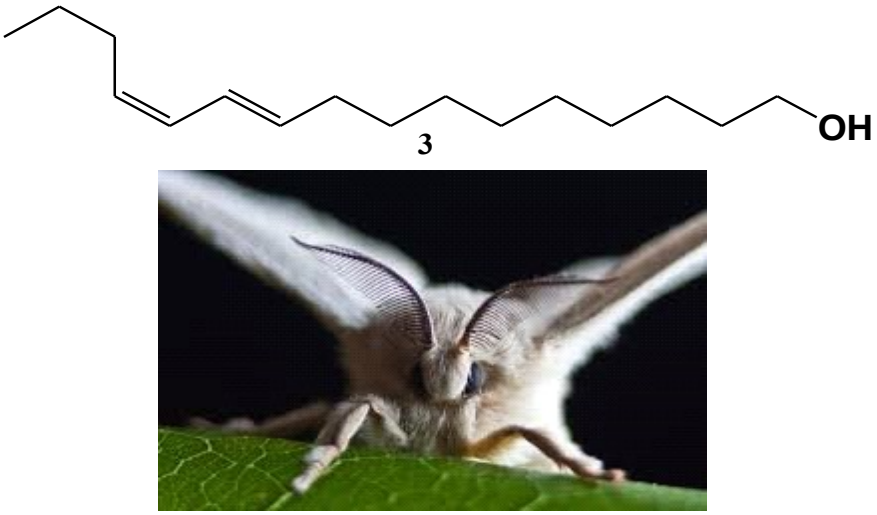


Figure 2.3: Sex pheromone of the silkworm moth *Bombyx mori* **bombykol**, **3**; the photo of silkworm moth (Gellez 2011)

Pheromones play an incredible role in mediating chemical communication of insects and are functionally classified as; sexual, territoriality and scent-marking, social organization recruitment, alarm, aggregation and host marking (Larsson, 2003). The family Tephritidae, has a wide range of pheromones that partly overlap in their functions. They have a complex mating systems incorporating host plants that influence male sexual behavior (Francisco Díaz-Fleischer *et al*., 2001). For species with narrow range of host plants e.g. *R. pomonella*, the plants majorly act as a dating site where males rest on the host fruit (oviposition site) waiting for the females, with which they mate upon arrival (Francisco Díaz-Fleischer *et al*., 2001). But for polyphagous fruit flies where female location is less predictable, the males usually aggregate and release sex pheromone in groups named 'leks' to attract females (Shelly & Kaneshiro, 1991).

In addition to oviposition site selection, the plants also supply key sex pheromone ingredients. Example, *B. dorsalis* males ingest methyl eugenol, a precursor in the synthesis of phenylpropanoids 2-allyl-4,5-dimethoxyphenol and (*E*)-coniferyl alcohol, that are sequestered, stored in the rectal gland and released during mating at dusk to attract the females (Haq *et al*., 2014). This phenomenon is also experienced when males of *B. tryoni* feed on plants containing cuelure and zingerone (Kumaran *et al*.,

2013). In *B. oleae* and *C. capitata* the exposure to α-copaene containing plant or orange peel volatiles increases mating performance index of these insects respectively (Papadopoulos *et al*., 2003; Shelly *et al*., 2007).

The territorial and scent-marking pheromone is well studied for some fruit fly species example *Drosophila grimshawi, C. capitata* and *A. suspensa* where males deposit a pheromone by dragging their abdomen along the perching substrate to attract fruit flies of both sexes and also to discriminate among potential mates based on quantity, an indicator of male genetic superiority (Droney, 1994; Shelly, 2004; Sivinski *et al*., 1994).

The social organization, recruitment and aggregation pheromones are the least studied in Tephritidae; example *D. melanogaster* the larval and adult fruit flies have shown high preference to odors emanating from food substrates occupied by larvae (Sarin *et al*., 2009). Additionally, aggregation pheromone such as *cis*-vaccenyl acetate has been shown to induce grouping behavior in both sexes of *D. melanogaster* and is usually produced by the males and transferred to females during mating (Sarin *et al*., 2009; Venu *et al*., 2014).

The alarm pheromone, also the least important pheromone in nonsocial insects is a semiochemical used by many species to alert conspecifics or other related species of impending danger. In *D. melanogaster* stressed flies have been shown to emit an odorant which elicits avoidance behavior in naïve flies (Enjin *et al*., 2013).

The last group of pheromones which is the focus of this project is the host marking pheromones (HMPs). HMPs are employed by many insects to mark their egg laying sites which serve to inform other gravid females of already occupied host thus preventing over-exploitation. The HMPs in relation to fruit fly is discussed in section 2.5.

2.5. Fruit fly host-marking pheromones

2.5.1 Host-marking behaviour

The behavior of marking the host is a well-studied aspect of the oviposition in many Tephritid. Host-marking behaviour does not cut across all fruit flies species, but is prevalent in some (Arredondo *et al*., 2006), occasional in some (Duyck *et al*., 2006; Nufio *et al*., 2004) and absent in others (Arredondo *et al*., 2006).

After mating on the host plant, fruit flies females exhibit a sequence of behaviors that assist it assess information about the potential host (Edmunds *et al*., 2010). The oviposition behavior has been shown to occur in four steps: arriving at the fruit, searching, puncture and drawing (Arredondo *et al*., 2006). When the female arrives on the fruit, she uses, at short distance, visual stimuli and appraises the fruit about its size, color and shape (Edmunds *et al*., 2010).

The female surveys all the surface of the fruit during the searching, touching it with the anterior part of the head, the labelo and the ovipositor. In this step, she analyses the physical (size and shape) and chemical properties of the fruit (Fletcher, 1991). The female then inserts its aculeus into the fruit pulp, keeping her ovipositor in a perpendicular position to the surface (Díaz-Fleischer *et al*., 2000). The female does not lay eggs obligatorily but, in some cases, she removes the aculeus making only the puncture. At last, in the drawing step, the female surveys again the fruit surface, but with the aculeus protract. At this point, she leaves a pheromone, the host marking pheromone (HMP) (Edmunds *et al*., 2010).

2.5.2 Production of host-marking pheromones in fruit flies

Fruit flies produce and store their HMP in the posterior half of the midgut and as such, fecal matter of host-marking fruit flies contains large quantities of their pheromones (Edmunds *et al*., 2010).

2.5.3 Chemical properties of fruit fly host marking pheromones

Fruit fly HMPs have low volatility, highly polar and have molecular weight of ≤ 10,000 Daltons (Aluja, 2003). Majority are soluble in water and methanol (Aluja *et*

al., 2003). Fruit fly HMP are also persistent on surfaces where they have been deposited regardless of whether they have been deposited directly by the fruit flies or as extracts (Aluja *et al*., 2003). For example, the half-life of the HMP of *R. pomonella* is 10.7 days with activity persisting for three weeks (Aluja *et al*., 2003). Persistence of host-marking pheromones of other fruit fly species has been reported as follows: 4 days for *R. indifferens*, (Aluja *et al*., 2003) , 6 days for *A. suspensa* (Aluja *et al*., 2003), 6 days for *C. capitata*, 9 days for *R. fausta* and 12 days for *R. cerasi* (Aluja *et al*., 2003). Aluja *et al*., (2009) observed that the deterrent efficacy of fecal matter extract of *A. ludens* on *A. obliqua* in an orchard of tropical plum dropped by just 10% after 27 days despite heavy rainfall.

2.5.4 Perception of host-marking pheromones in fruit flies

Fruit flies perceive HMPs using sensilla found on the ventral side of the second, third and fourth tarsomeres of prothoracic tarsi (Stadler *et al*., 1992) and the short hairs on the labellum and meso- and meta- thoracic legs (Loy *et al*., 2016; Städler *et al*., 1994). These sensilla contain contact-chemoreceptor cells which are sensitive to HMPs and are influenced by several factors such as concentration of the pheromone, physiological state of the fly, nature of the fly (e.g., whether wild or laboratory reared), and the type of host.(Faraone *et al*., 2016)

Although HMPs of fruit flies are generally perceived to be effective for conspecifics only (Nufio *et al*., 2001, 2004), some studies have shown that interspecific recognition of HMPs also occurs in fruit flies. Nufio *et al*., (2001) observed cross-recognition of host-marking pheromones among species of the genus *Rhagoletis*. Aluja and Díaz-Fleischer, (2006) observed cross-recognition of HMPs among *A. ludens*, *A. obliqua* and *A. serpentina*. It has also been observed that host-marking is one important mechanism by which fruit fly species displace one another from ecologies (Aluja and Díaz-Fleischer, 2006).

Further, several interspecific ecological displacements based on heterospecifics chemical interference have been reported worldwide (Duyck *et al*., 2004). In situations where polyphagous tephritid species have been introduced or in areas already occupied by a polyphagous tephritid, interspecific chemical interference has

resulted in a displacement status. For example, *C. capitata* displaced *C. catoirii* in the Reunion Island and Mauritius between 1939 and 1942. Several other interspecific and even intergeneric displacements through chemical interference involving *Ceratitis, Bactrocera* and *Anastrepha* species have been reported in various countries from 1950's to 2003 (Duyck *et al*., 2004).

2.6 Fruit fly control measures

Methods currently being used to control fruit fly include; the use of fumigants, insecticidal bait sprays, early harvesting, chemical sprays and use of the sterile insect technique (SIT) (Aluja & Boller, 1992). Despite their effectiveness, large-scale use of insecticides has environmental implications that have to be considered. These include pollution of the soil and water sources, potential danger posed to farmers who apply the chemicals and the harm caused to other non-targets (Asquith *et al*., 1992). Malathion was initially used as the main insecticides but has been replaced by gibberelic acid which enhances the innate resistance of fruit to fruit fly attack (Birke *et al*., 2006).

Farmers have also employed insecticide growth regulators such as cyromazine (Moreno *et al*., 1994), pathogens such as *Bacillus thuringiensis* and *Metarhizium anisopliae* (Ojeda-Chi *et al*., 2010), bacterial toxins such as spinosad (Wang *et al*., 2005), photoactivated dyes (Broillet *et al*., 2001), and mass releases of parasitoids (Montoya *et al*., 2000). Although these seem to be viable alternatives to toxic bait sprays, some of these methods could still prove unacceptable because of the potential deleterious effect on humans, non-target insects and reluctance of farmers to accept them for socio-economic reasons (Mack *et al*., 2005). While challenges to effective controlling these pests abound, a combination of IPM packages is a viable option including the exploitation of semiochemicals such as host marking pheromones (HMPs) as they are known to mediate communication in many species of fruit flies.

2.6.1 The host-marking technique in fruit fly management

HMPs are deposited by flies on the surface of a fruit after oviposition which deters conspecifics and at times heterospecifics from ovipositing in the same fruit at

biologically significant concentration (Faraone *et al*., 2016). As exemplified in a field scenario, work done by Aluja and Boller, (1992) demonstrated significant reductions in fruit infestation by *R. cerasi* in commercial cherry orchards in Switzerland.

Their findings indicated that application of synthetic HMP to the entire tree crown reduced the number of larvae per kilogram of fruit by a factor of 10 when compared with an untreated tree (Aluja & Boller, 1992). The efficacy of the same synthetic HMP was later confirmed by Boller *et al*., (1994) in different parts of Switzerland where reductions in *R. cerasi* infestation of up to 100% were achieved in sweet cherries. Effectiveness of feces extracts and two simplified synthetic analogues of the *A. ludens* HMP (Figure 2.4) have also been shown to reduce the infestation of *A. obliqua* in tropical plum and mango trees under natural conditions.

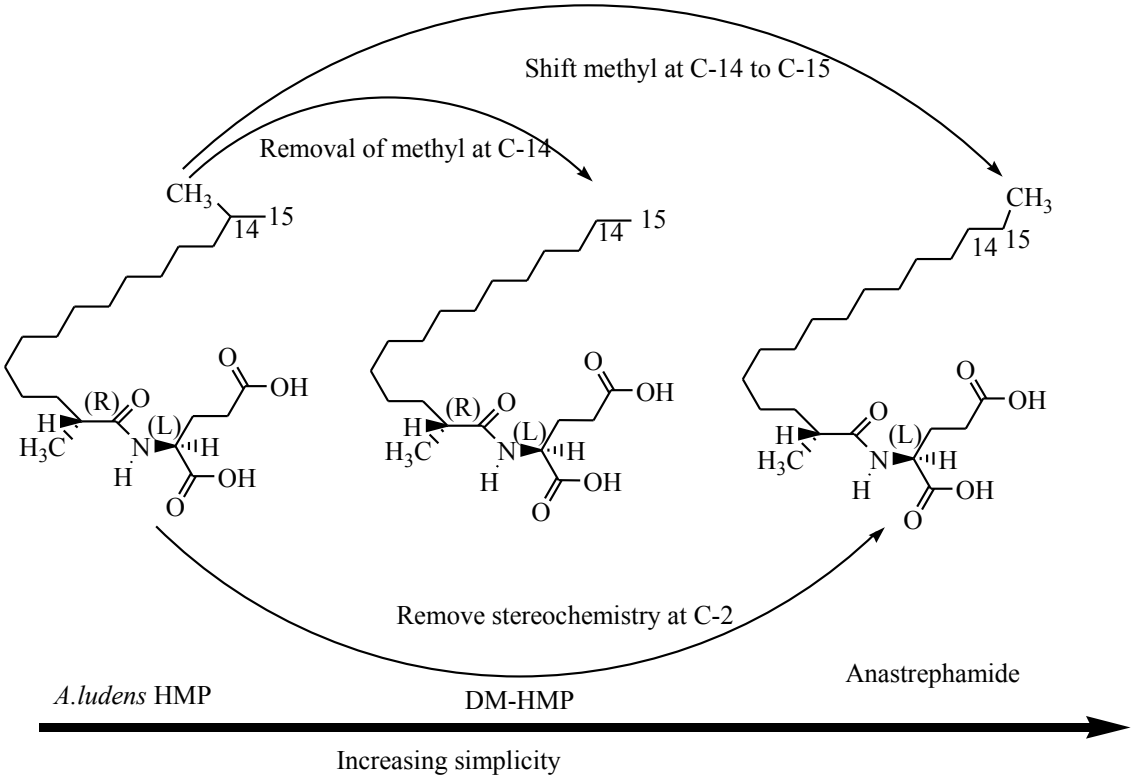


Figure 2.4: *A. ludens* HMP extracted from feces and the two synthetic analogues DM-HMP and Anastrephamide (Aluja *et al*., 2009).

The HMPs technique is advantageous because it can be used as conventional insecticides, and even applied with conventional spray equipment. It is also target

specific because only the pests are eliminated and not any other beneficial insects. In their first experiment on the potential of host-marking pheromones for field management of tephritid*s*, Aluja *et al*., (1992) observed a low concentration of aqueous solution of partially purified pheromone gathered from artificial oviposition substrate reduced *R. cerasi* infestation in cherries by 63%. In their second experiment they observed that higher concentration resulted in 90% infestation reduction. They also observed that spraying vertical halves of the cherry plants was more effective than spraying of whole plants. They recommended the technique for tephritid management, particularly in Integrated Pest Management (IPM) programs. Nufio and Papaj, (2004a) tested the technique in cherry fields and observed that it reduced *R. cerasi* infestation by up to 90%.

To date, there are few documented cases where attempt has been made to exploit the host marking behavior to manage fruit fly. Examples include; control of the fruit flies, *R. cerasi* in cherries in Switzerland (Aluja *et al*., 1992); *R. juglandis* in walnuts in the United States (Aluja *et al*., 1992); *C. capitata* in coffee in Mexico (Arredondo *et al*., 2006), and *A. obliqua* (Macquart) in mango and plums in Mexico (Martñ Aluja *et al*., 2009). There is therefore, a huge potential for its adoption in Africa if well understood and developed.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Study site and conditions

All experiments were laboratory-based and carried out at the Duduville Campus of the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi, Kenya (1.2219° S, 36.8967° E; 1600 m ASL). The experiments were run during day, at peak of fruit fly oviposition activity, between 9:00 a.m. and 4:00 p.m. (Kachigamba *et al*., 2012) at 24 ± 2˚C and 50 ±10% relative humidity with LD 12:12 h cycle.

3.2 Camera

All pictures were taken from *icipe* by Cheseto using canon powershot A530 digital camera. USA unless otherwise stated.

3.3 Research design

The research approach involved six major steps as shown (Figure 3.1):

1. Collection and extraction of fecal matter from both females and males of different fruit fly species and carryout oviposition deterrence experiments.
2. Purification of the fecal matter extract using HPLC in bioassay-guided experiments and characterization of the active fraction using HPLC, LC-MS and LC-QTOF-MS.
3. Testing for oviposition deterrence activity of the synthetic HMPs identified in 2.
4. Testing for the effect of *C. cosyra* HMP on egg parasitoid behavior
5. Testing for the effect of *C. cosyra* HMP on mango surface volatiles
6. Evaluating the effect of larvae development on mango volatiles

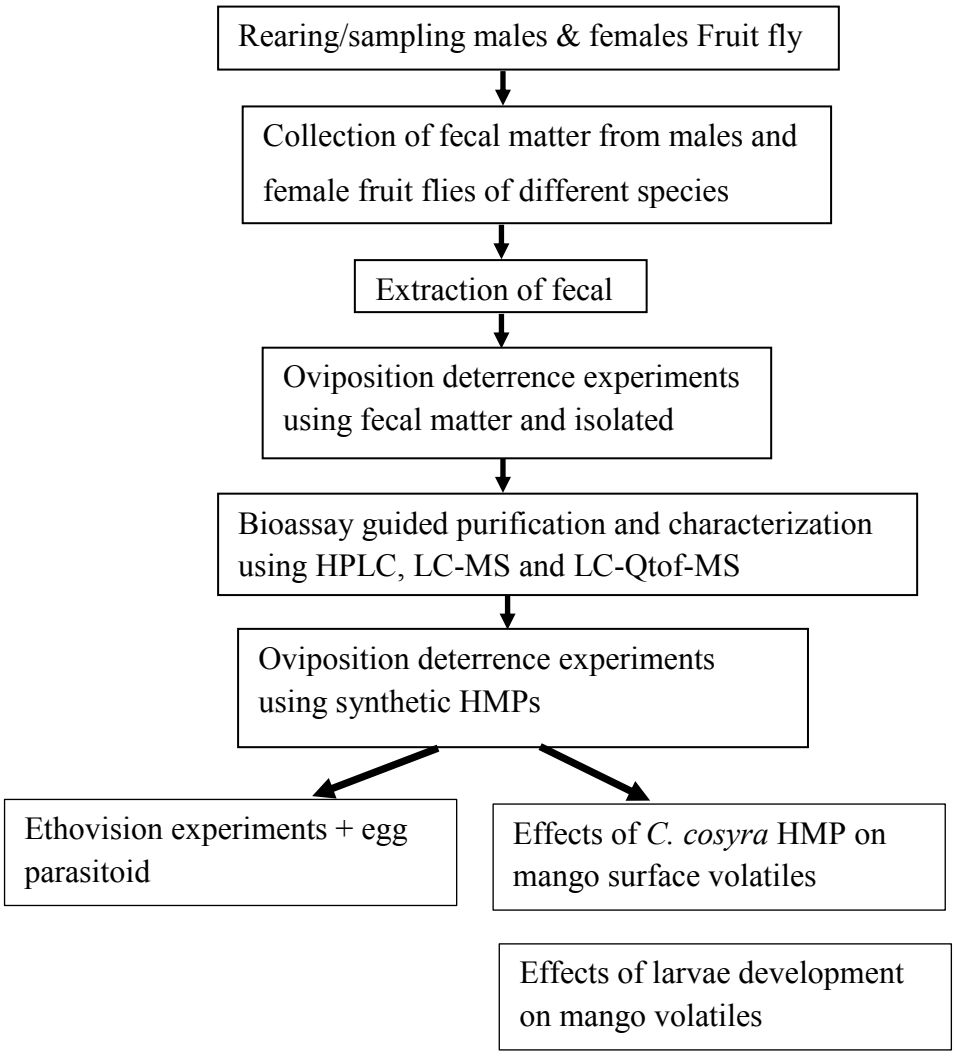


Figure 3.1: Flow diagram showing research design

3.4 Sampling

The sample size for oviposition deterrence bioassay experiments was 100 adult female fruit flies from all the different species used per experiment and were aged between 8-21days (Kachigamba *et al*., 2012). More details for the sampling are elaborated in specific experiments.

3.5 Experimental techniques

3.5.1 Insects

The colonies of the major three fruit fly species for this study, *C. cosyra, C. fasciventris* and *C. rosa* and the supplementary fruit flies *B. dorsalis*, *C. capitata* and *C. anonae* used in this project were obtained from International Centre of Insect Physiology and Ecology *icipe,* Kenya, Arthropod Rearing and Quarantine Unit where they have been maintained since 1997 (over 300 generations) with yearly infusion with wild-caught species to reduce inbreeding depression and loss of genetic variability.

Original colonies of *C. cosyra* were derived from collections obtained from mango, *Mangifera indica L.,* and marula, *Sclerocarya birrea* (A. Rich.) Hochst. at Nguruman, Kenya (1˚ 47′S; 36˚ 05′E; 700 m ASL). *C. fasciventris* and *C. capitata* originated from coffee, *Coffee arabica L*. collected from farms in the Central highlands of Kenya at Ruiru (1° 5.72′ S; 36° 54.22′E; 1609 m above sea level) while *C. rosa* was collected from wild plant *Lettowianthus stellatus* in coastal region of Kenya, Mrima Hill (4° 29.32′ S; 39° 15.27′E; 290 m above sea level). For *B. dorsalis, C. anonae* and *Z. cucurbitae* the flies were raised and supplied by John Kiilu a senior technician at fruit fly program *icipe*. The flies were reared at *icipe*, Kenya (01˚ 13’ 25.3” S, 36˚ 53’ 49.2” E, 1,609 m ASL) using the methodology described by Ekesi *et al*., (2006) and Kachigamba *et al.*, (2012).

Forty (40) adult flies (10 d old) of each sex and species were obtained from the stock culture for raising an experimental colony. They were transferred into 30 × 30 × 30 cm clear Perspex rearing cages made locally in *icipe* with fine netting ventilation on one side and maintained on a 4:1 volumetric mixture of sugar (Mumias Sugar Co., Nairobi, Kenya) and an enzymatic yeast hydrolysate (USB Corporation, Cleveland, OH). The fruit flies were provided with water *ad libitum* in 9 cm Petri dishes filled with pumice granules to prevent drowning. The fruit flies were also provided with three whole ripe mangoes, apple variety for 2 d as an oviposition substrate. The mangoes were spiked several times using colored push pins sterilized with an ethanol wipe (1 mm hole, ~150 holes/mango). Thereafter, the egg-infested fruits were

removed and incubated in 20 × 12.5 × 8 cm plastic containers covered with a perforated plastic lid (Kenpoly, Nairobi, Kenya). The inner bottom of these containers was cushioned with a thin layer of paper towel to absorb sap produced by the rotting fruits. The paper towels were replaced every 2 d with clean, dry towels.

On day 10, the time it takes for the three species to reach 4th instar, the paper towel was removed and the larvae-infested, rotten mango washed under running tap water into an incubation container. The fiber, peels, intact seed and other dirt were removed leaving only the fourth instars, which sank to the bottom of the containers, and recovered through sieving. The brood was then transferred into clean incubation vessels containing 40-60 mm of sterilized sand for pupation. On day 3 the sand was soaked in water allowing the puparia to float and were subsequently collected by sieving. The puparia were placed in Petri dishes with moistened filter paper and kept in rearing cages until eclosion. They were subsequently supplied with food and water as described above and at day 6, were continuously provided with ripe mangoes for oviposition. The rearing room was maintained at 23-25 °C and 40-60% RH with a photoperiod of 12:12 h (L: D) cycle. The fruit fly species were used at 6-21 d old, when female fruit flies are usually at their peak of behavioral and biological activity (Faraone *et al*., 2016).

3.5.2 Oviposition substrates

Ripe mango apple variety was selected as an oviposition substrate owing to its high economic value and susceptibility to the fruit flies (Griesbach, 2003). It was sliced lengthwise into two equal halves and the endocarp and mesocarp carefully scooped out. The remaining exocarp was thoroughly washed with distilled water, dried using paper towel and fitted in covers of 50 mm-diameter Petri dishes with the rinds on the top surface. The size of each oviposition substrate was ca. 20.4 cm^2 (surface area) and 5 mm thick.

3.5.3 Collection of fecal matter

The collection and extraction of fecal matter was performed as described previously, (Aluja *et al*., 2003; Kachigamba *et al.*, 2012) but with the following modifications.

Fecal matter of three fruit fly species, was collected by placing 150 fruit flies (optimized carrying capacity of the bottle) of a given species, sex, either males or females of a known age starting from day 1 after eclosion up to day 30, in a clean glass bottle (200 mL) fitted with a net lid tied to the rim for five consecutive photoperiods. The collection was initiated in the late afternoon and run until the next morning (4:00 pm – 8:00 am) to allow for deposition of their fecal matter in the vessel. Each morning, the fruit flies were released into their respective cages containing food, water and ripe mangoes until 4:00 pm when they were taken back into their respective bottles. The fecal matter was washed from the jars using 5 mL distilled water, freeze dried on a laboratory scale freeze-dryer (Virtis Advantage Plus freeze-dryer; SP scientific, Warminster, USA) and pooled after 5 d based on species and sex. For each day 10-15 mg of the fecal matter was obtained from each bottle. The feces were stored at -80 °C until sufficient quantities for bioassay and extraction were obtained.

3.6 Data collection

The data collection process involved a dual-choice oviposition assays, HPLC, LC-MS, LC-QTOF-MS analysis of aqueous fecal matter extract of fruit flies. For dual-choice oviposition assays, sample size of the fruit flies used were based on Kachigamba *et al*., (2012).Fruit flies used in all the investigations were randomly picked from designated cages based on species, sex, age and species. The types of data collected in the dual-choice oviposition experiments included percentages of fruit flies, counts and duration (time). HPLC, LC-MS, LC-QTOF-MS data were collected as chemical component profiles comprising of retention time, peak area and absorbance.

In all experiments involving oviposition, the total oviposition time was measured beginning 30 s after fruit fly introduction into the observation cage and the session ended only if the female inserted its aculeus into the fruit pulp, keeping her ovipositor in a perpendicular position to the surface, eggs deposited, and dragged the protracted ovipositor on the surface of the oviposition substrate before flying away from the host. All fruit flies were only allowed to oviposit once. After an observation session was terminated for a female, she was returned to the rearing cage. The maximum total

oviposition time allowed per observation was 30 min. The fruit flies which failed to make a choice between the treated substrate and control after 30 min were deemed to be non-responsive and were replaced by fresh fruit flies.

3.7 Data analysis

Analysis of the data collected in behavioral observation and dual-choice oviposition assays involved analysis of variance (ANOVA) with SNK mean separation, Chi-square test or regression analysis, with due transformation of the data where necessary. R-statistical program 2.11.0 was used for data analyses (R Development Core Team, 2012). Analysis of HPLC, LC-MS and LC-QTOF-MS profiles involved comparison of retention times of chemical components, mass fragmentation pattern to those of authentic sample where available or literature searches. More detailed on data analysis is shown in the specific Chapters

CHAPTER FOUR

HOST MARKING PHEROMONE IN CERATITIS COSYRA

4.1 Introduction

This chapter reports the identity of the host marking pheromone of *C. cosyra*. The specific objectives were to: (i) confirm the bioactivity of aqueous extracts of the fecal matter of *C. cosyra* against conspecifics; (ii) isolate female specific component(s) (FSC) from the fecal matter and determine its bioactivity (iii) identify the HMP and test its synthetic equivalent for bioactivity against conspecifics and the if it has any effect on heterospecific indigenous species *C. rosa*, *C. fasciventris*, *C. capitata* and invasive species *Z. cucurbitae*; and, (iv) assess the distribution of the HMP in *C. cosyra* specific body tissue (ovipositor and hemolymph) and determine the relationship, if any, between HMP concentration and fruit fly age.

4.2 Materials and Methods

4.2.1 Rearing of insects and collection of fecal matter

The stock colonies of *C. cosyra, C. rosa*, *C. fasciventris*, *C. capitata* and *Z. cucurbitae* previously identified (Marc *et al*., 2002) were obtained and raised using the methods described in detail in section 3.5

4.2.2 Bioassays

For all the bioassays, sexually mature (10-21 day(d) old) female fruit flies were used (Kachigamba *et al*., 2012) and a ripe mango of apple variety selected as an oviposition substrate (Griesbach, 2003). Female fruit flies (100) of each species were observed to choose between ovipositing on:

a) a marked mango slice treated with 1 mL of aqueous fecal matter solution (10 mg/mL; 100 insect equivalent) or a control (treated with 1 mL distilled water). The tests were conducted for conspecifics (*C. cosyra* female aqueous fecal matter extract using *C. cosyra* females).

b) a marked mango slice treated with 1 mL known concentration of *C. cosyra* female specific compound (FSC) (1 mg/mL, 5 mg/mL or 10 mg/mL) versus a control (treated with 1 mL distilled water) using *C. cosyra* females.

c) a marked mango slice treated with 1 mL known concentration of GSH (1 mg/mL, 5 mg/mL or 10 mg/mL) and a control (treated with 1 mL distilled water) using *C. cosyra*, *C. rosa*, *C. fasciventris*, *C. capitata* and *Z. cucurbitae* females.

Test samples and controls soaked in cotton swabs were applied to the mango substrate. The 100 fruit flies used for each experiment were grouped into 10 batches of 10 fruit flies with each batch representing a replicate. Each replicate used ten pairs of oviposition substrates placed at the center of the observation cage, side by side and in contact with each other to allow fruit fly movement to allow for searching for oviposition sites. For each observation, one new pair of mango slices prepared from one mango was used in order to minimize differences in their chemistry, which could influence choice of the fruit flies, and their relative positions randomly varied before introducing the next fruit fly.

In all experiments involving oviposition, the total oviposition time was measured beginning 30 s after fruit fly introduction into the observation cage and the session ended only if the female inserted its aculeus into the fruit pulp, keeping her ovipositor in a perpendicular position to the surface, eggs deposited, and dragged the protracted ovipositor on the surface of the oviposition substrate before flying away from the host as explained in section 3.6.

4.2.3 Chemical analyses

4.2.3.1 Fecal matter

The fecal matter of *C. cosyra* (1 mg) previously collected from both males and females of specific age was separately dissolved in 1 mL 0.01% formic acid/acetonitrile (95: 5, LC-MS grade Chromasolv, Sigma-Aldrich, St. Luis, MO), vortexed for 30 s, sonicated for 30 min and centrifuged at 14,000 rpm for 5 min to remove any insoluble material after which 5 µL of the supernatant was analyzed on a VP series HPLC system (Shimadzu, Kyoto, Japan) equipped with a prominence SPD-

M20 diode array detector (wavelength set at 190-360 nm for UV and 360- 700 nm for visible range). The column oven was set at 30 °C with the following column parameters, 250 mm × 10 mm i.d., 5µm, ACE 5 C-18 column (Advance Chromatography Technologies, Aberdeen, Scotland). The mobile phases consisted of water (A) and acetonitrile (B), each containing 0.01% formic acid. The following gradient program was used 0 min, 5% B; 0-5 min, 5-50% B; 5-10 min, 50-80% B; 10-15 min,80-100% B; 15-25 min 100% B; 25-30 min 5% B; 30-35 min 5% B. The flow rate was held constant at 1 mL/min (Ferreres *et al*., 2003)

In order to obtain sufficient quantities of *C. cosyra* FSC for bioassay, 100 g of *C. cosyra* female fecal matter collected from sexually mature females (10-21 d old) was dissolved in 200 mL of the same solvent, vortexed for 30 s, sonicated for 30 min and centrifuged at 14,000 rpm for 5 min, after which 100 µL of the supernatant was injected into semi-preparative column (250 mm × 10 mm i.d., 5µm, ACE 5 C-18 column (Advance Chromatography Technologies, Aberdeen, Scotland) installed on an HPLC-PDA and the peak at retention time 4.5 min, monitored at 220 nm wavelength collected from ~1200 runs over a period of 3 months, pooled and freeze dried to yield 2 g of FSC. Each day's collection was stored at -80 °C until sufficient quantities (2 g) for bioassay were obtained.

The HPLC fractionated pure FSC from *C. cosyra* (1 mg) was dissolved in 1 mL 0.01% formic acid/acetonitrile (95;5, v/v) LC-MS grade solvent, vortexed for 30 s, and centrifuged at 14,000 rpm for 5 min after which 0.2 µL of the supernatant was analyzed by LC-QTOF-MS. Chromatographic separation was achieved on an Acquity UPLC I-class system (Waters Corp., Milford, MA). The column used was a 250 mm × 4.6 mm i.d., 5 µm, ACE C-18 column (Advance Chromatography Technologies, Aberdeen, Scotland) with a heater turned off and an autosampler tray cooled to 5 °C. Mobile phases of water (A) and acetonitrile (B), each with 0.01% formic acid were employed. The following gradient was used 0 min, 5% B; 0−3 min, 5-30% B; 3−6 min, 30% B; 6−7.5 min, 30−80% B; 7.5-10.5 min, 80% B; 10.5-13.0 min, 80-100% B, 13-18 min, 100% B; 18-20 min, 100-5% B; 20-22 min, 5% B. The flow rate was held constant at 0.7 mL/min. The injection volume was 0.2 µL.

The UPLC system was interfaced by electrospray ionization to a Synapt G2-*Si* QTOF-MS (Waters) operated in full scan MS^E in positive mode. Data were acquired in resolution mode over the range *m/z* 100–700 with a scan time of 1 s using a capillary voltage of 0.5 kV, sampling cone voltage of 40 V, source temperature of 100 °C and desolvation temperature of 350 °C. The nitrogen desolvation flow rate was 500 L/h. For the high-energy scan function, a collision energy ramp of 25−45 eV was applied in the T-wave collision cell using ultrahigh purity argon (≥99.999%) as the collision gas. A continuous lock spray reference compound (leucine enkephalin; $[M+H]^+$ = 556.2766) was sampled at 10 s intervals for centroid data mass correction. The mass spectrometer was calibrated across the mass range 50- 1,200-Da mass range using a 0.5 mM sodium formate solution prepared in 90:10 propan-2-ol: water (90;10, v/v). MassLynx version 4.1 SCN 712 (Waters) was used for data acquisition and processing. The elemental composition was generated for every analyte. Potential assignments were calculated using the monoisotopic masses with specifications of a tolerance of 10 ppm deviation and both odd- and even-electron states possible. The number and types of expected atoms were set as follows: carbons ≤ 50; hydrogens ≤ 100; oxygens ≤ 50; nitrogens ≤ 10; chlorines ≤ 10; sulfurs ≤ 10. Data acquisition and analysis by LC-QTOF-MS were based on the following defined factors: mass accuracy (ppm) = 1,000,000 × (calculated mass - accurate mass)/calculated mass; fit conf% refers the confidence with which the measured mass (accurate mass) matches the theoretical isotope models of the elemental composition in the list. The empirical formula generated was used to predict structures that were proposed based on the online database (METLIN, ChemSpider and ChemCalc, fragment ion calculator), fragmentation pattern, literature and authentic standard (L-glutathione reduced, 98 % purity), (Sigma-Aldrich, St. Louis, MO). The application manager ChromaLynx, also a module of MassLynx software, was used to investigate the presence of non-target compounds in samples. Library searching was performed using the commercial NIST -MS/MS library.

4.2.3.2 Ovipositor

Ten sexually mature females (10 d old) of *C. cosyra* were dissected and the ovipositor tips excised into a vial containing 500 µL 0.01% formic acid/acetonitrile (95:5);

vortexed for 1 min, extracted by ultrasonication in a sonication bath for 30 min, centrifuged at 13,000 rpm for 5 min at 5 °C and the supernatant analyzed using LC-QTOF-MS. This was repeated three times from different batches of 10 females and the samples similarly analyzed.

4.2.3.3 Hemolymph

Hemolymph collection and extraction was performed as previously described (Teal *et al*., 2002). Sexually mature males and females (10-15 of each sex) of *C. cosyra* (10-12 d old) were separately held in a waxed bottom dish with pins pierced through the wings and head. A small slit was made down the center line of the thorax after which the hemolymph was allowed to flow into a calibrated 5 µL disposable micropipettes (by gravity). The hemolymph was pooled separately based on sex to obtain sufficient quantities for extraction (3 µL); approximately 0.3 µL of hemolymph was obtained per insect. The hemolymph was then transferred into a vial containing 500 µL 0.01% formic acid/methanol (30:70); vortexed for 1 min, sonicated for 30 min, centrifuged at 13,000 rpm for 5 min at 5 °C to remove any precipitated protein, and the supernatant analyzed using LC-QTOF-MS. This was repeated three times with a different batch of males and females.

4.2.3.4 Acid hydrolysis of *C. cosyra* female-specific compound

The FSC from *C. cosyra* (10 mg) was transferred into a 5 mL micro-reaction vial into which 2 mL of 6N HCl was added and closed after careful introduction of nitrogen gas. The sample was hydrolyzed for 24 h at 110 °C. After the hydrolysis, the mixtures were evaporated to dryness under vacuum. The hydrolysates were reconstituted in 1 mL 0.01% formic acid/acetonitrile (95: 5), vortexed for 30 s, sonicated for 30 min, and then centrifuged at 14,000 rpm and the supernatant analyzed by LC-QTOF-MS. This was repeated three times using different samples.

4.2.3.5 Relationship between glutathione amount in fecal matter and *C. cosyra* age.

The fecal matter (10 mg) of *C. cosyra* was obtained as earlier described starting from day one after eclosion and stopped at day 30 when majority of the flies were dead. On

a daily basis, the fecal matter for both males and females were separately washed off the jar with 5 mL distilled water, freeze dried, re-dissolved in 1 mL 0.01% formic acid/acetonitrile (95: 5), vortexed for 30 s, sonicated for 30 min and centrifuged at 14,000 rpm for 5 min after which 10 µL of the supernatant were analyzed by LC-MS. The same procedure was used to analyze the extracting solvents and three different samples (10 mg each) obtained from the rearing diet.

The LC-MS operating conditions were as follows: a quaternary LC pump (Model 1200) coupled to Agilent MSD 6120-Single quadruple MS with electrospray source (Palo Alto, CA) was used. The system was controlled using ChemStation software (Hewlett-Packard). Reversed-phase liquid chromatography was performed on an Agilent technologies 1200 infinite series, Zorbax SB C-18 column, 2.1 x 50 mm, 1.8 µm (Phenomenex, Torrance, CA) using a gradient program and mobile solvents similar to the LC method described above. The injection volume was 10 µL and data was acquired in a full-scan positive-ion mode using a 100 to 800 *m/z* scan range. The dwell time for each ion was 50 ms. Other parameters of the mass spectrometer were as follows: capillary voltage, 3.0 kV; cone voltage, 70 V; extract voltage, 5 V; RF voltage, 0.5 V; source temperature, 110 ºC; nitrogen gas temperature for desolvation, 380 ºC; and nitrogen gas flow for desolvation, 400 L/h. Serial dilutions of glutathione standard (1-100 ng/µL) were analyzed by LC-MS in full scan mode to generate linear calibration curve (peak area vs. concentration) with the following equation; [$y=6008.9x - 5250.3$ ($R^2 = 0.9987$)] which served as a basis for external quantification.

4.3 Statistical analyses

The number insects responding to the treatments and controls in the dual choice assays was analyzed by Chi-square goodness of fit to assess (a) *C. cosyra* female's discrimination to aqueous *C. cosyra* females fecal matter compared to control (b) *C. cosyra* females discrimination to different doses of aqueous *C. cosyra* FSC compared to control (c) *C. cosyra, C. rosa*, *C. fasciventris*, *C. capitata* and *Z. cucurbitae* females discrimination to different doses of GSH against control. Non-respondents were not included in the analysis. The two-sample Wilcoxon test was used to test for

differences in the median oviposition time between the control and various treatments. Analysis of HPLC profiles involved comparison of retention times of peaks of individual chemical components. The peak area from the total ion chromatogram corresponding to GSH obtained from LC-MS analysis was used to compute concentrations from the calibration curve. Concentration of *C. cosyra* FSC determined at different insect ages was expressed as mean ± standard error. Analysis of variance was carried for all the concentrations for the various ages and means were separated using Tukey's studentized HSD. R-statistical program version 2.11.0 software (R Development Core Team, 2012) was used to perform the statistical analyses and all tests were performed at 5% significance level.

4.4 Results and Discussion

4.4.1 Bioactivity of *C. cosyra* fecal matter extract confirmed.

First tested was whether the HMP modulated *C. cosyra* oviposition behavior. As previously found, mango slices treated with the aqueous extract of the fecal matter of *C. cosyra* significantly reduced oviposition responses in conspecific females (Kachigamba *et al*., 2012) (Figure 4.1A). Ovipositing females on average required three times longer to assess the suitability of the treated oviposition substrate than controls (two sample Wilcoxon test, W= 258, P <0.001), (Table 4.1). This suggests that they recognized that the treated hosts were marked with a compound whose volatility was relatively low and acted at close range, as well as present at a concentration within the levels to influence their oviposition decision. *C. cosyra* ovipositing female performance on the treated and control hosts is a survival strategy to minimize the over-exploitation of the same resource for egg laying by conspecifics to ensure success and fitness of their progeny in terms of access to food resources and maximum use of nutrients for development. Most studies focusing on insect host marking pheromones have made similar observations. For example, in scarce large blue butterfly, *Phengaris (Maculinea) teleius*,(Sielezniew *et al*., 2013) pepper weevils, *Anthonomus eugenii*,(Addesso *et al*., 2007) egg parasitoid *Trissolcus basalis*,(Rosi *et al*., 2001) *Pieris brassicae* and *Pieris rapae*.(Schoonhoven, 1990)

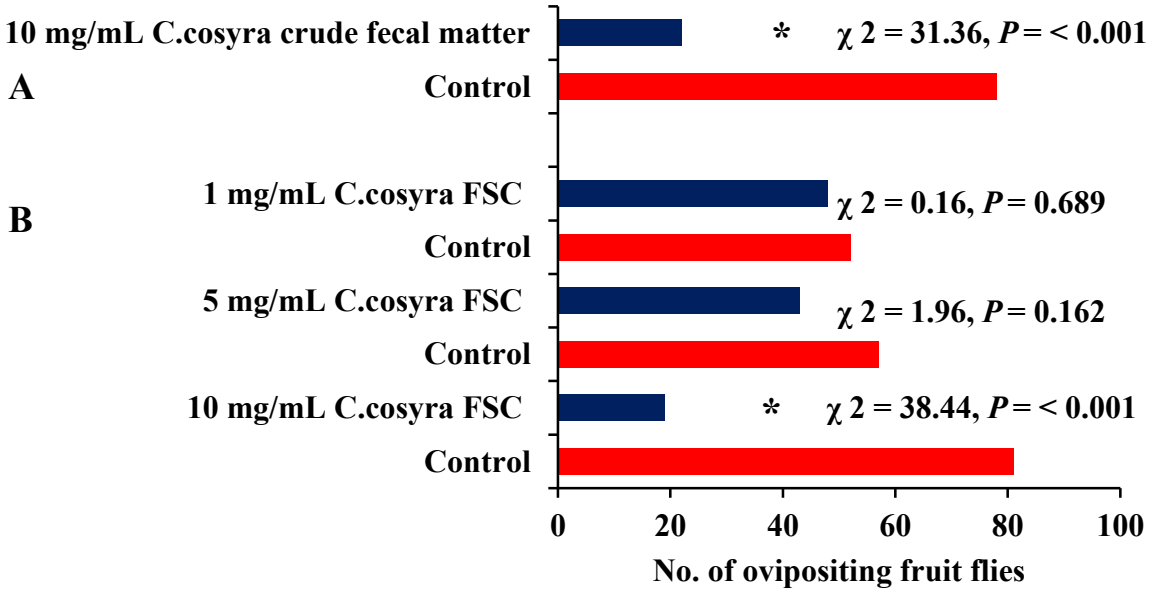


Figure 4.1: Discrimination of oviposition substrates treated with (A) 10 mg/mL aqueous solutions of fecal matter by conspecific females (B) aqueous solution of FSC isolated from fecal matter of *C. cosyra* by conspecific females. *Denotes significantly different at 0.05.

Table 4.1: Oviposition time of *C. cosyra* females on mango slices treated with a) aqueous solution of *C. cosyra* female fecal matter and b) aqueous solution of *C. cosyra* female specific compound and a control (n=100 females/ treatment)

Sample concentration	treatment median (range) (minutes)	control median (range) (minutes)	*P* value
a) Fecal matter (10 mg/mL)	18.7 (10.0-27.0)	5.3 1.6-13.5	< 0.001
FSC (1 mg/mL)	7.2 (3.9-13.3)	6.5 (3.6-15.6)	0.412
FSC (5 mg/mL)	13.1 (8.3-17.0)	3.9 (2.0-7.0)	0.287
FSC (10 mg/mL)	10.1 (7.1-25.1)	5.2 (2.7-8.4)	< 0.001

4.4.2 Bioassay-guided fractionation of *C. cosyra* female-specific component

Chemical analysis of the aqueous extract of the fecal matter collected from both males and females of *C. cosyra* at different ages by HPLC revealed the presence of a female-specific peak at retention time 4.5 min with UV absorption at λ_{max} 220 nm (Figure 4.2).

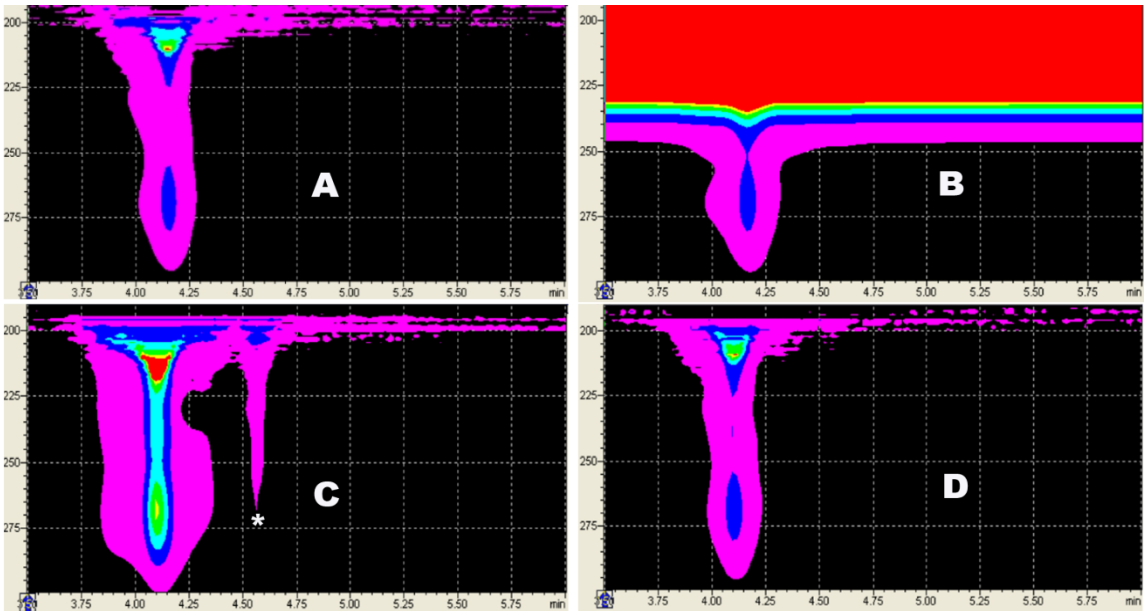


Figure 4.2: HPLC-PDA contour view of aqueous extract of the fecal matter of *C. cosyra* (A) 3-day old females, (B) 3-day old males, (C) 6-day old females, and (D) 6-day old males. *Denotes peak of female specific component (FSC) of *C. cosyra*

To aid in identifying the component that appeared to be a biomarker for gravid female *C. cosyra* FSC, the LC-MS profiles of the aqueous extracts of the fecal matter of females were compared with a similar extract obtained from the rearing diet. FSC was present only in the aqueous extracts of the fecal matter of 6- to 30-d old gravid females (figure 4.3)

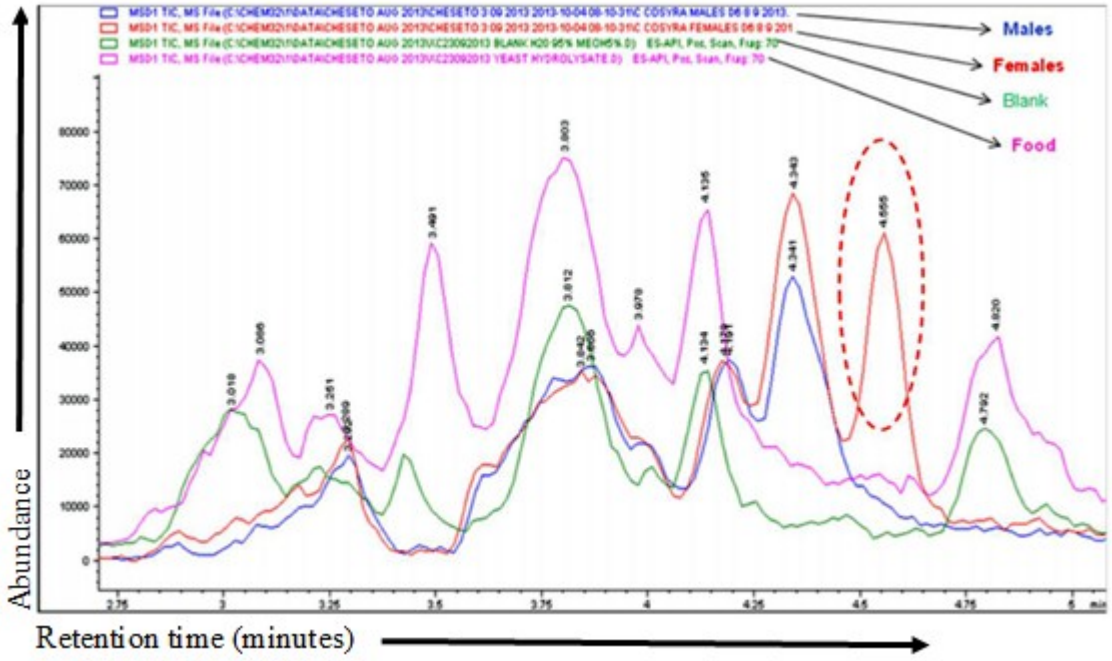


Figure 4.3: LC-MS Representative overlaid total ion chromatography showing fecal matter extract, diet and blank; *C. cosyra* males (blue) *C. cosyra* females (red), Solvent blank (green) and yeast hydrolysate (pink)] showing *C. cosyra* FSC (circled at rt 4.5 min)

Fractionation of the aqueous extracts of fecal matter obtained from 3 day (d) and 6 d old females and males by liquid chromatography gave a FSC in 6 d but not 3 d old female fecal matter. This component was also absent in the fecal matter of males at both ages. These results suggest that the production and release of the FSC is dependent on the developmental state of the female. The physiological basis for this was not investigated but these findings suggest that in females the biochemical pathway for synthesis of the FSC is switched on as the female reaches a specific age of maturity. As such, it is possible that the onset of the production and release of the FSC may occur earlier than 6 d. Age-dependent pheromone production and release has important implications in the biology and ecology of insects because they influence certain physiological processes such as reproduction, feeding, oviposition, and development (Alfaro *et al*., 2011; Diamantidis *et al*., 2008; Ferguson *et al*., 1999; Flath *et al*., 1993; Quilici *et al*., 2013; Teal *et al*., 2000). An investigation of the physiological basis for production the FSC would enhance our understanding of the behavioral ecology of females.

To obtain enough FSC for bioassays and chemical analyses, 100 g of crude female fecal matter was fractionated by semi-preparative HPLC to obtain 2 g of the FSC. In dose-response tests at concentrations of 1, 5 and 10 mg/mL (1 mg of FSC = 95 insect fecal matter equivalents), host discrimination in conspecifics increased with increasing dose with 10 mg/mL of FSC eliciting the highest oviposition reducing response in females which almost mirrored the bioactivity of the crude fecal matter extract tested at the same dose ($\chi^2 = 38.44$; df = 1; $P < 0.001$) (Figure 4.1 B). Furthermore, ovipositing fruit flies required longer times to assess the suitability of the treated oviposition substrate compared to the control (two sample Wilcoxon test, $W = 255$, $P < 0.001$).

These results confirm the sensitivity and suitability of our extraction method, avoiding possible degradation of the bioactive component. They are also consistent with previous studies which found that fecal matter and their aqueous extracts obtained from the related fruit flies *C. capitata*, *C. rosa* and *C. fasciventris* (Kachigamba *et al*., 2012) and those of the cherry fruit fly *R. cerasi* (Aluja & Boller, 1992) and the Mexican fruit fly *A. ludens* contained chemicals that reduced oviposition responses in conspecifics (Edmunds *et al*., 2010).

4.4.3 LC-QTOF MS identification of *C. cosyra* HMP.

Similar to the analysis of crude fecal matter aqueous extract, analysis of this bioactive fraction by LC-QTOF-MS showed a major peak at retention time 2.38 min (Figure 4.4) which gave a molecular ion peak $[M+H]^+$ at *m/z* 308.0928 having a molecular formula of $C_{10}H_{18}N_3O_6S$. library search of *m/z* 308.0928 returned the tripeptide glutathione as a compound which fitted the predicted empirical formula (Figure 4.5). Acid digestion of the bioactive fraction followed by LC-MS analysis identified the digestion products as the amino acids glycine (G), cysteine (C) and glutamic acid (E) (Figure 4.6). A fragment ion calculator search of the six possible arrangements, ECG, GCE, EGC, GEC, CEG or CGE of the tripeptide predicted a N-C terminal structure of the tripeptide as glutamyl-cysteinyl-glycine (ECG). The tripeptide structure was supported by the presence of major expected fragments of monoisotopic masses for z_2, y_2 and b_2 ions *m/z* 162, 179, and 233 (Figure 4.7a and b) (Ramanathan *et al*., 1998)

and confirmed the identity of the HMP of *C. cosyra* as glutathione based on comparison of mass spectra data, retention time and co-injection of the natural product with an authentic standard (Figure 4.5). To determine whether glutathione was present in other body tissues of females of *C. cosyra*, the aqueous extracts of both the ovipositor and hemolymph were similarly analyzed by LC-QTOF-MS. Glutathione was detected in the two tissues of 10 d old gravid females.

Chemical analysis identified unambiguously the FSC as the tripeptide glutathione consisting of glycine, cysteine and glutamic acid. This suggests that GSH is likely synthesized from these three amino acids ingested from the rearing diet of the fruit fly. Future research will evaluate whether GSH would be detected in *C. cosyra* fecal matter when reared on varying diets, or other combinations of these amino acids. Interestingly, the HMP of *C. cosyra* is highly distinct when compared to the other HMPs identified from the fecal matter of other fruit flies; *N*-[15(*β*-glucopyranosyl)oxy-8-hydroxypalmitol]-taurine, identified from the cherry fruit fly *R. cerasi* (Aluja & Boller, 1992), and *N*-[2,14-dimethyl-1-oxopentadecyl)-glutamic acid, identified from the Mexican fruit fly *A. ludens*. However, the HMP of *C. cosyra* appears to be more closely related to the HMP of the Mexican fruit fly, which contains glutamic acid. In contrast, the HMP of *R. cerasi* is a fatty acid glucoside nonetheless, it appears that in general and irrespective of the fruit fly species and complexity of the structure of the HMP, binding of the components involves at least an amino acid or a carbohydrate to enhance solubility in water.

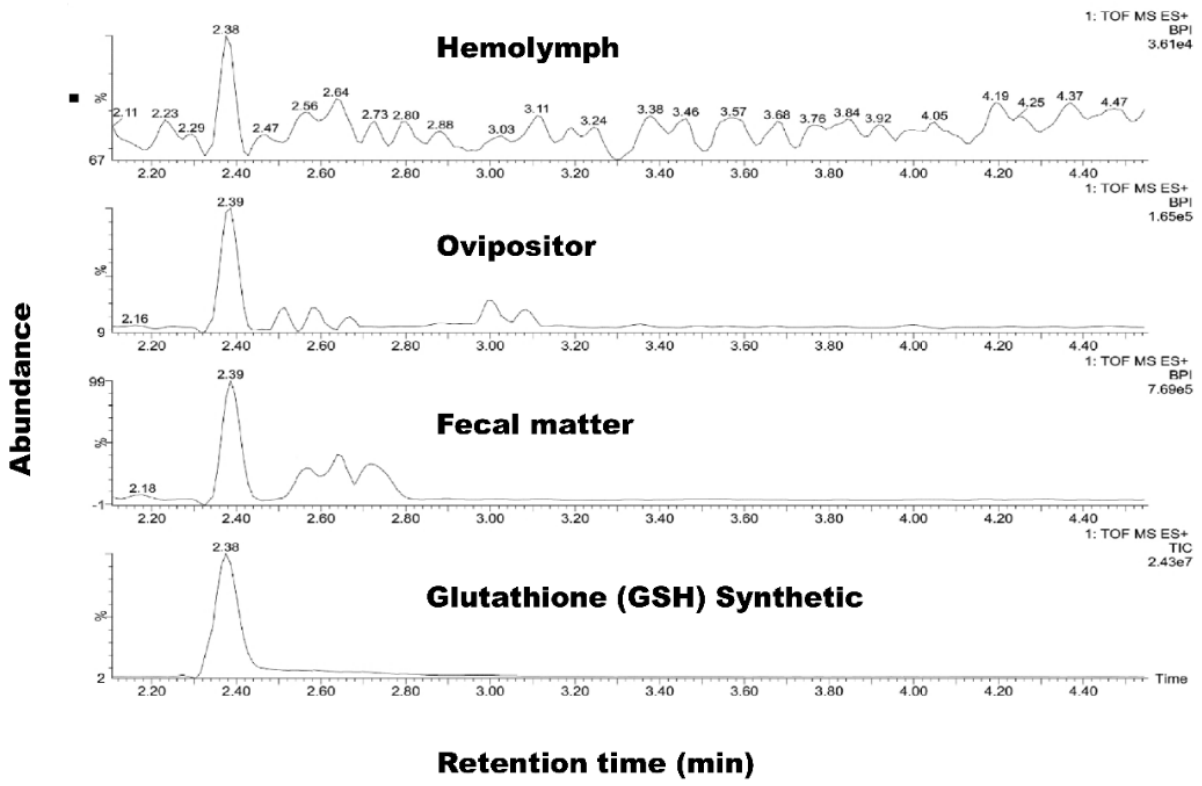


Figure 4.4: LC-QTOF-MS representative total ion chromatogram showing detection of GSH in gravid female *C. cosyra* hemolymph, ovipositor and fecal matter compared to the authentic compound. The retention time of GSH is 2.38 min

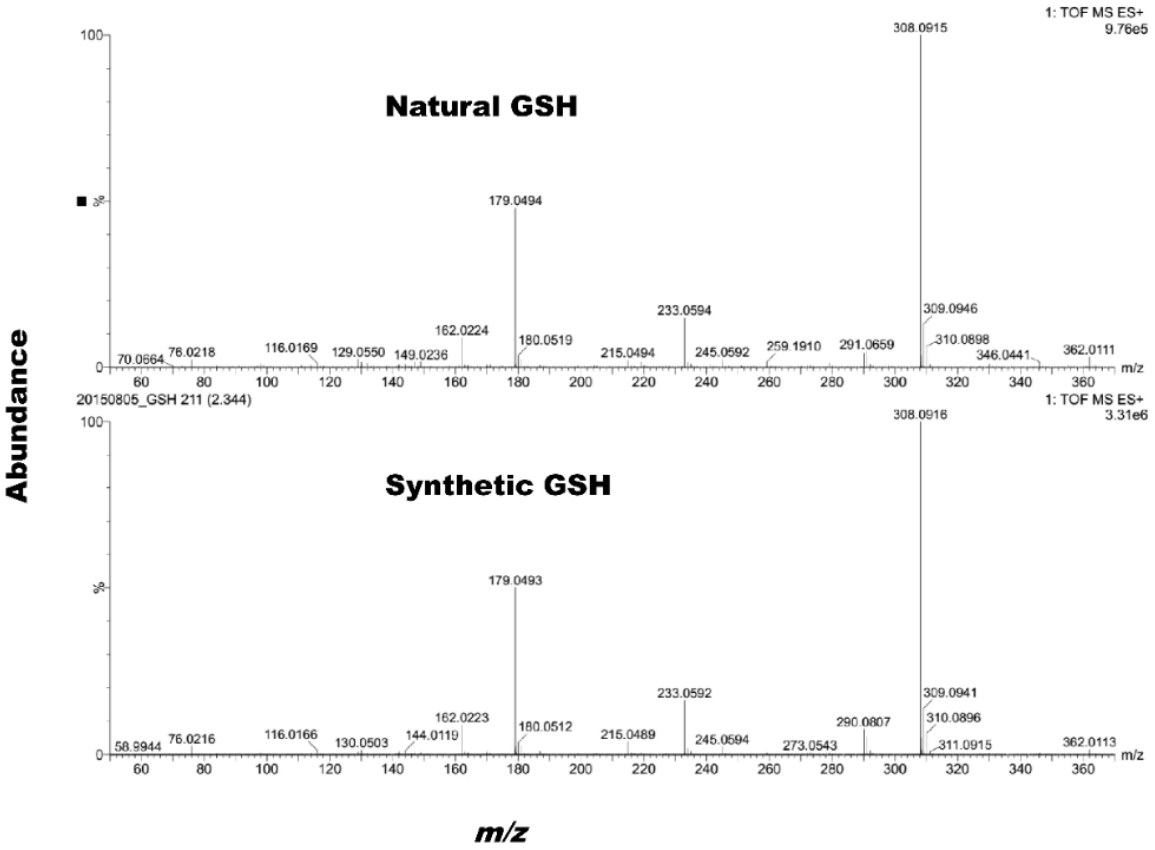


Figure 4.5: Representative mass spectrum showing natural and synthetic GSH

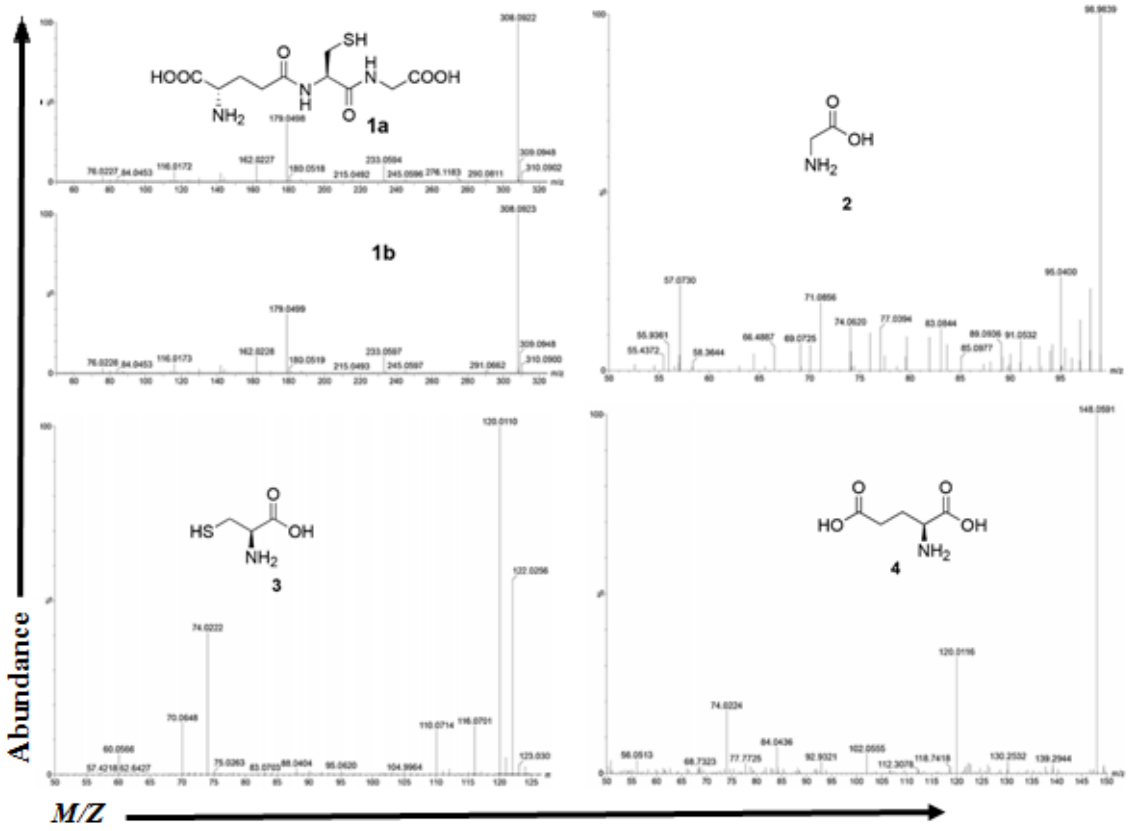


Figure 4.6: Representative mass spectrum showing (1a) natural GSH, (1b) synthetic GSH, (2) glycine, (3) cysteine and (4) glutamic acid

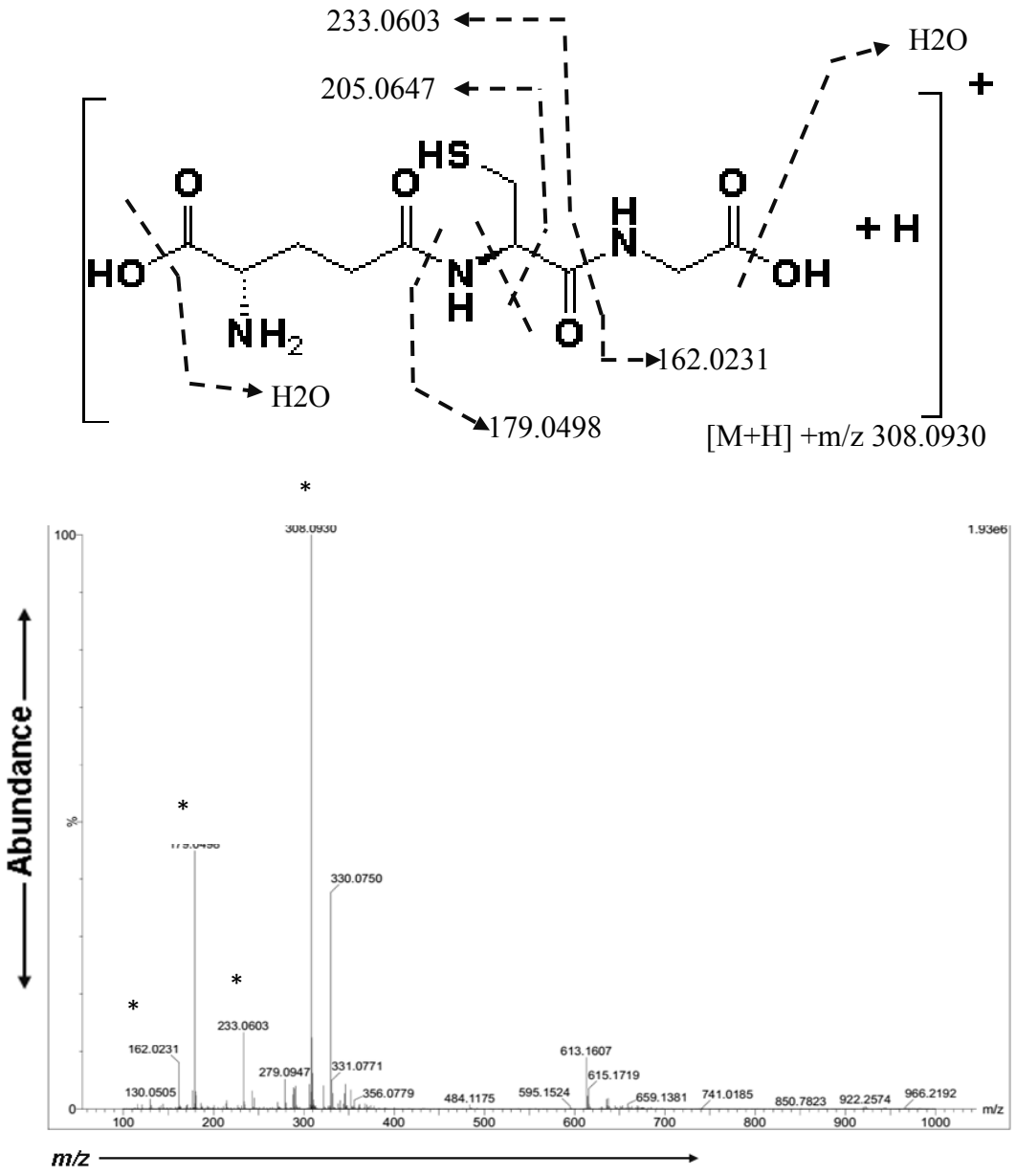


Figure 4.7a: QToF-MS fragmentation of $[M+H]^+$ for identification of GSH

Fragment Ion Calculator Results

Sequence: **ECG**, pI: **3.99976**

Fragment Ion Table, monoisotopic masses

Seq	#	A	B	C	X	Y	Z	# (+1)
E	1	102.05555	130.05046	147.07701	-	308.09168	291.06513	3
C	2	205.06473	233.05965	250.08620	205.02835	179.04908	162.02253	2
G	3	262.08620	290.08111	-	102.01916	76.03990	59.01335	1

Mass/Charge Table

	Mass	
	Mono	Avg
(M)	307.08385	307.32153
$(M+H)^{+}$	308.09168	308.32947
$(M+2H)^{2+}$	154.54977	154.66872
$(M+3H)^{3+}$	103.36914	103.44848
$(M+4H)^{4+}$	77.77882	77.83836

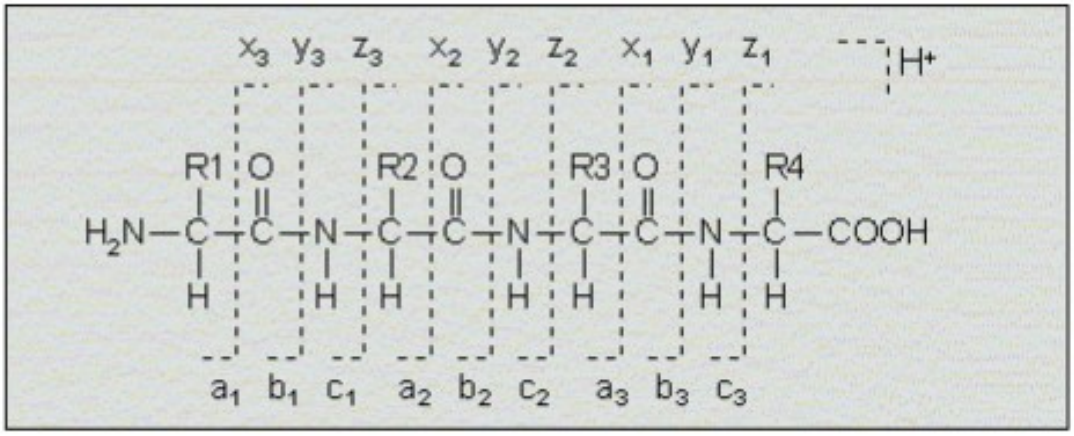


Figure 4.7b: Fragmentation pattern of peptides showing major monoisotopic masses for z_2, y_2 and b_2 in GSH

4.4.4 Bioactivity of *C. cosyra* HMP glutathione.

In bioassays, glutathione reduced oviposition responses of the five fruit fly species *C. cosyra, C. fasciventris, C. rosa, C. capitata* and *Z. cucurbitae* in a dose-response manner (Figures 4.8A-C). The oviposition reducing response was both concentration and species-dependent, especially at the highest concentration of 10 mg/mL; with *C.*

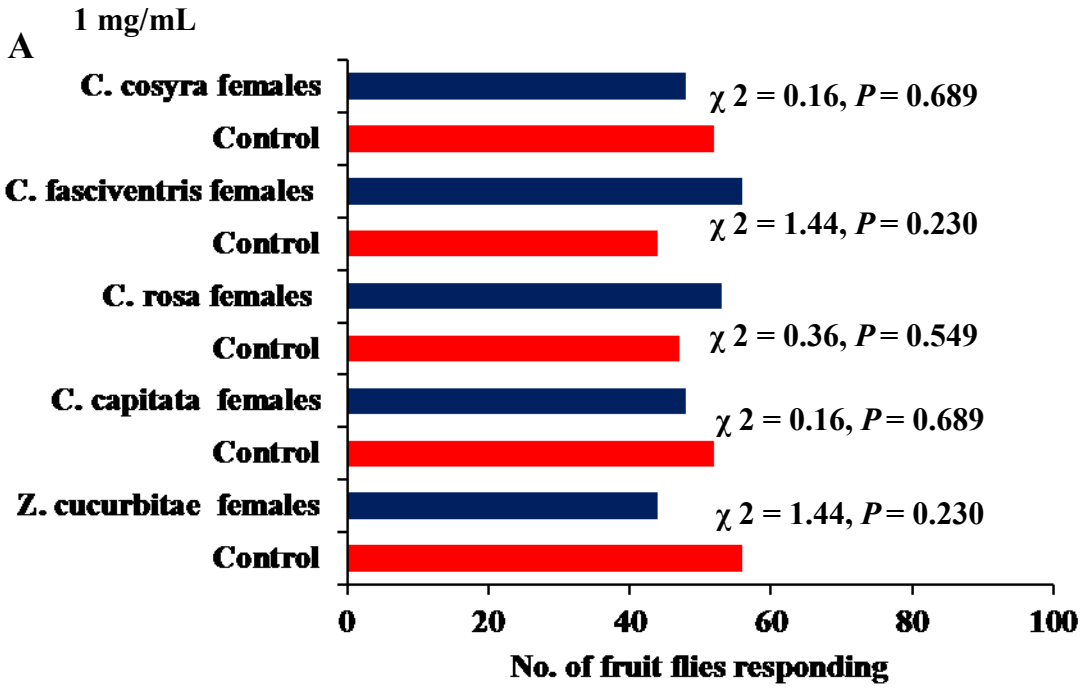
cosyra (χ^2 = 54.76; df = 1, $P < 0.001$), *C. fasciventris* (χ^2 = 64.00; df = 1, $P < 0.001$), and *C. rosa* (χ^2 = 60.84; df = 1; $P < 0.001$), being more responsive to the HMP than *C. capitata* (χ^2 = 10.24; df = 1; $P = 0.001$) and *Z. cucurbitae (*χ^2 = 31.36; df = 1; $P <$ 0.001) (Figure 4.8C). Consistent with our previous results on oviposition responses of *C. cosyra*, the ovipositing heterospecific fruit flies also required on the average an additional 1 to 3 min longer to assess the suitability of the oviposition substrate treated with increasing dose of GSH (Table 4.2). The GSH concentration of 10 mg/mL elicited the longest assessment time from ovipositing females compared to the control.

Table 4.2: Oviposition time of *C. cosyra*, *C. fasciventris C. rosa*, *C. capitata* and *Z. cucurbitae* on oviposition substrates treated with (a) 1 mg/mL, (b) 5 mg/mL (c)10 mg/mL aqueous solution of GSH and a control (n=100 females/ treatment)

Concentration GSH (mg/mL)	responding fruit fly	treatment median (range) (minutes)	control Median (range) (minutes)	*P* value
A	*C. cosyra*	14.0 (4.0-29.3)	10.3 (3.0-29.5)	0.5852
A	*C. fasciventris*	12.3 (5.1-29.3)	10.5 (2.5-29.2)	0.4587
A	*C. rosa*	12.4 (3.4-29.4)	10.5 (1.6-29.6)	0.4123
A	*C. capitata*	10.4 (1.3-20.0)	9.4 (3.9-25.6)	0.287
A	*Z. cucurbitae*	11.3 (2.2-23.5)	6.8 (3.7-24.9)	0.3526
B	*C. cosyra*	13.6 (6.0-29.2)	17.1 (2.0-29.3)	0.2589
B	*C. fasciventris*	13.5 (4.0-29.1)	14.1 (2.4-29.5)	0.1587
B	*C. rosa*	13.8 (4.0-24.0)	13.9 (2.4-29.5)	0.2452
B	*C. capitata*	14.7 (5.2-25.6)	12.5 (2.6-29.6)	0.3214
B	*Z. cucurbitae*	9.3 (4.3-27.8)	10.3 (4.7-23.6)	0.2854
C	*C. cosyra*	16.0 (3.0-28.8)	7.0 (2.0-16.6)	< 0.001
C	*C. fasciventris*	19.4 (4.2-29.1)	9.9 (3.0-19.2)	< 0.001
C	*C. rosa*	16.1 (3.1-28.6)	10.3 (3.0-18.5)	< 0.001
C	*C. capitata*	17.4 (3.3-29.4)	10.5 (1.6-17.2)	< 0.001
C	*Z. cucurbitae*	15.3 (4.5-23.9)	12.7 (4.7-15.1)	< 0.001

Our results showed that the five fruit fly species *C. cosyra*, *C. fasciventris, C. rosa, C. capitata* and *Z. cucurbitae* responses to GSH treated mango slices was both

concentration- and species-dependent. *C. cosyra*, *C. fasciventris, C. rosa* were more sensitive to GSH at the most effective concentration of 10 mg/mL than *C. capitata* and *Z. cucurbitae*. It has been reported that the host range of *C. cosyra* is narrow, mainly infesting mango and marula, (Steck, 2012) whereas the relationship between other fruit fly species and host fruits may be less specific. Thus, intra- and interspecific recognition and sensitivity to GSH by these fruit fly species may be influenced also by host factors including semiochemicals. Future studies on the role of host semiochemicals on fruit fly discrimination of pheromone-marked hosts for oviposition are warranted. Furthermore, since chemoreceptors have been shown to play a role in the detection of semiochemicals (Depetris-Chauvin *et al*., 2015), elucidating the detection mechanism of *C. cosyra* HMP by conspecifics and heterospecifics is recommended.



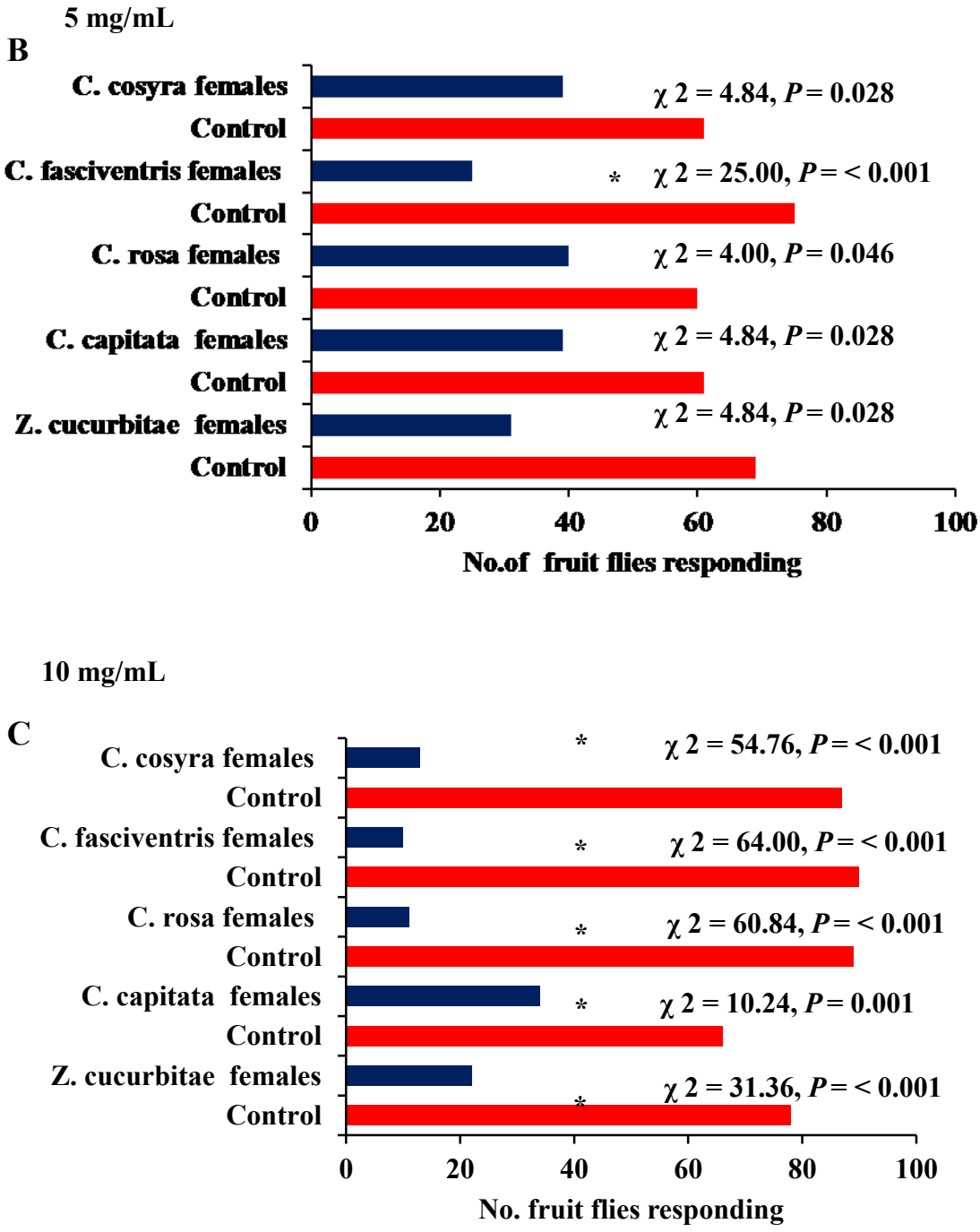


Figure 4.8: Discrimination of oviposition substrates treated with (A) 1 mg/mL (GSH) by *C. cosyra*, *C. fasciventris*, *C. rosa*, *C. capitata* and *Z. cucurbitae* (B) 5 mg/mL, and (C) 10 mg/mL of aqueous solutions of synthetic glutathione (GSH). *Denotes significantly different at 0.05

4.4.5 Relationship between glutathione amount in fecal matter and *C. cosyra* age.

To determine the relationship between amount of GSH in fecal matter and age of *C. cosyra*, fecal matter was collected and analyzed from 1, 3, 6, 9, 12, 15, 18, 21, 24, 27- and 30 days old adult females. GSH amount increased from 93.4 ± 0.36 µg/mg, detected in the fecal matter of 6 d old gravid females to an optimal amount of 104.1 ± 0.4 µg/mg and 106.0 ± 1.18 realized in 12 d and 15 d old females respectively before dropping. Overall, the amount of GSH in fecal matter correlated positively (R^2= 0.8817) with the age of gravid females (Figure 4.9).

This implies that age is an important factor for the successful release of enzymes that catalyze the biosynthesis of the HMP. Thus, HMP is a critical factor in the reproductive biology of sexually mature females of this fruit fly species, confirming previous findings by other researchers (Diamantidis *et al*., 2008; Edmunds *et al*., 2010; Noctor *et al*., 2011). The findings of this also suggest the potential wider applicability of the host marking pheromone of *C. cosyra* in fruit fly management in important agricultural commodities. Furthermore, detection of the GSH in the ovipositor and hemolymph of 10 d old females suggests that this pheromone is synthesized in the gut of *C. cosyra* and thereafter transported into the hemolymph and into the ovipositor. The fact that GSH level was 5-10 times higher in the aqueous extract of the fecal matter than in similar extracts of the ovipositor and hemolymph of females, suggests that apart from the amounts of the pheromone deposited via the ovipositor after egg laying, females can also protect their progeny from conspecifics and heterospecifics using their fecal matter directly or indirectly on or near the host.

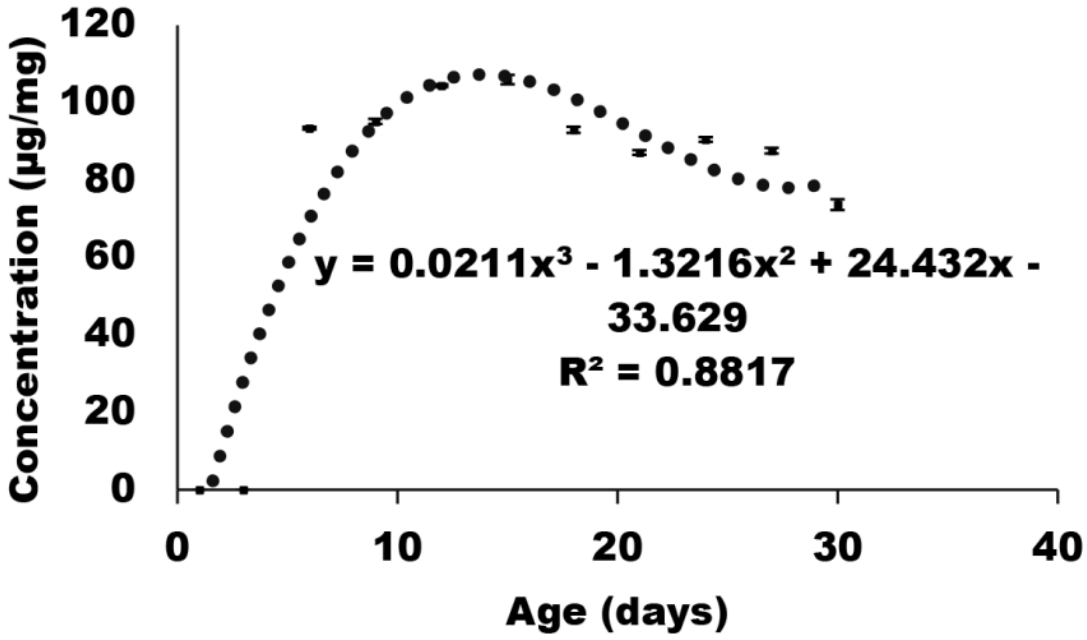


Figure 4.9: Relationship between female age (days) and the amount of glutathione detected in fecal matter of *C. cosyra*. Concentrations (µg/mg) of FSC for the various insect age bearing the same letter are not significantly different (P = 0.05, Tukey's, HSD test)

Several studies have identified GSH as a generalist antioxidant compound in many organisms, including plants, animals, fungi, some bacteria and archaea (Meister *et al*., 1983; Noctor *et al*., 2011). In addition to the role it plays in the synthesis of proteins and DNA, transport, enzyme activity, metabolism, and protection of cells, it has also been shown to play a major role in the excretion of xenobiotics and toxic metals in many organisms (Noctor *et al*., 2011).

Cysteine-glutathione disulfide, a GSH derivative has also been reported as a sex pheromone in the marine polychaete *Nereis succinea* (Zeeck, Müller, *et al*., 1998). In insects GSH has been found to play a role in insecticide resistance through its conjugation with the insecticide to produce water-soluble metabolites that are readily excreted (Ranson *et al*., 2005), and in the detoxification of toxic plant metabolites and allelochemicals including glucosinolates, hydroxamic acids, and furocoumarins ingested by herbivorous insects (Ranson *et al*., 2005; Simon, 1996). In addition to

these roles, the present study has found GSH as a HMP released by ovipositing females of *C. cosyra* to reduce the over-exploitation of the same host for egg laying by conspecifics and heterospecifics.

4.4.6 Effect of Glutathione on the egg parasitoid *Forpius arisanus*

Fopius arisanus (Sonan) is an important parasitoid of Tephritid fruit flies for at least two reasons. First, it is the one of only three *opiine* parasitoids known to infect the host during the egg stage. Second, it has a wide range of potential fruit fly hosts. Perhaps due to its life history, *F. arisanus* has been a successfully used for biological control of fruit flies in multiple tropical regions (Quilici *et al*., 2013).

Behavioral assays with females of *F. arisanus* (Figure 4.10) and GSH were conducted in a glass petri dish with the base dish measuring 14 cm diameter × 1.5 cm-high and the lid having a 0.5 cm diameter hole for ventilation and insect introduction point (Egonyu *et al*., 2013).

Figure 4.10: Fruit fly egg parasitoid *F. arisanus*

Two pieces of filter paper (Whatman No. 1, 1001-090, 1.5 cm diameter), one loaded with either 1, 5 or 10 mg/mL glutathione and the other loaded with distilled water as a control, (vol = 500 µl), were placed at opposite sides of the petri dish arena. Before assays, the solvent was allowed to evaporate for 10 min. For this experiment, the tests were replicated 25 times for each of the different GSH concentration (1, 5 or 10 mg/mL) using fresh samples in a clean petri dish and each insect used only once. To

minimize possible positional bias, locations of test and control filter papers were switched after five replicates.

Time spent by the parasitoid moving inside the Petri dish relative to the 2-filter papers was recorded as a) source approach; this is when the insect attached on the roof of the Petri dish lid and spent more time on test side, b) source contact: this is when the insects move on the base of the arena including the surface of the test filter papers, c) non-respondent: this is when the insects flew and randomly moved around the arena for the entire recording time. Data recording from items a and b only were considered positive response and were used in the calculation.

Behavioral responses of individual 10-d old *F. arisanus* female were tracked by video recording using EthoVision XT version 8.0 video-tracking system (Plate 4.1) (Grieco *et al*., 2010). The movements of individual insects released at the center of the petri dish arena were video-recorded for 10 min. Positions of each test insect were monitored every 0.5 s (Plate. 4.2), and the mean distances of the insect to either odor sources were generated by using the EthoVision computer software.

Plate 4.1: EthoVision video recording system

In dose-response tests at concentrations of 1, 5 and 10 mg/mL, the arresting behavior increased with increasing dose with 10 mg/mL GSH eliciting the highest arresting and searching behavior in *F. arisanus* females (Table 4.3 – 4.5)

For GSH 1 mg/mL dose (Table 4.3A), 25 trials were made with *F. ari*sanus allowed to survey the arena for an average of 10 min (600 s). The insect covered an average distance of 113.3 cm with an average time of 144.1 s being mobile and 454.1 s being immobile in the arena. Out of the 25 trials conducted, the insect did not make a choice between the test and the control in 9 trials. In the remaining 16 trials the insects spent an average of 8.7 s on the test filter paper disc and an average of 51.4 s on the control disc (Table 4.3A).

For GSH 5 mg/mL dosage (Table 4.3B), 25 trials were made with the *F. ari*sanus allowed to survey the arena for an average of 10 min (600 s). The insect covered an average distance of 109.5 cm with an average time of 82.3 s being mobile and 517.7 s being immobile in the arena. Out of the 25 runs performed, the insect did not make a choice between the test and the control in 10 trials. In the remaining 15 trials the insects spent an average of 42.8 s on the test filter paper disc and an average of 13.4 s on the control disc (Table 4.3B).

For GSH 10 mg/mL dosage (Table 4.3C), 25 trials were made with the *F. ari*sanus allowed to survey the arena for an average of 10 min (600 s). From the 25 replicates/ trials conducted for this dosage, 8 displayed *F. arisanus* moving on the base of the arena including the surface of the two filter papers (source contact), 7 showed the insect attaching itself on the roof of the lid and spend more time on test side (source approach) (this two were considered positive response and their values used in the calculation). The remaining 10 exhibited no distinct response as the insects flew and randomly moved around the arena for the entire recording time hence categorized as non-respondent. The insect covered an average distance of 115.0 cm with an average time of 418.0 s being mobile and 182.2 s being immobile in the arena. In the responding trials the insects spent an average of 356.4 s on the test disc (filter paper B, Plate 4.2) and an average of 61.5 s on the control disc (filter paper A, Plate 4.3C).

Table 4.3: Arresting and searching behavior recordings for *F. arisanus* females

GSH Conc (mg/mL)	Trial	Distance covered (cm)	Duration(s)			Mobility(s)	
A			**Arena**	**Test**	**Control**	**Mobile**	**Immobile**
1	1	2.3	600.2	0.0	0.0	0.8	599.2
	2	47.8	600.2	0.0	29.0	98.2	501.8
	3	6.4	600.2	0.0	0.0	13.0	587.0
	4	9.9	600.2	0.0	0.0	26.6	573.4
	5	18.1	600.2	0.0	600.2	22.4	577.6
	6	3.5	600.2	0.0	0.0	4.6	595.4
	7	2.9	600.2	0.0	0.0	11.2	588.8
	8	49.8	600.2	19.0	0.0	140.0	460.0
	9	162.7	600.2	8.0	264.6	209.2	390.8
	10	219.0	600.2	1.6	94.4	165.8	432.2
	11	360.6	600.2	71.2	26.0	341.0	259.0
	12	179.7	600.2	1.0	30.4	230.2	369.8
	13	50.0	600.2	7.6	0.0	50.6	539.4
	14	34.0	600.2	1.8	1.6	37.8	562.2
	15	608.4	600.2	0.0	0.0	126.4	473.6
	16	5.0	600.2	0.0	0.0	14.0	555.6
	17	406.8	600.2	50.6	25.2	384.4	214.0
	18	2.3	600.2	0.0	0.0	1.6	598.4
	19	31.2	600.2	0.0	7.4	39.6	560.4
	20	240.8	600.2	3.2	192.2	430.6	169.4
	21	42.6	600.2	0.0	7.0	57.2	542.8
	22	137.5	600.2	2.0	4.8	498.2	101.8
	23	31.3	600.2	33.0	0.0	345.8	254.2
	24	148.6	600.2	18.0	1.6	151.4	448.6
	25	31.3	600.2	0.0	0.0	203.1	397.1
	Total	**2832.3**	**15005**	**217.0**	**1284.4**	**3603.7**	**11352.5**
	Mean	**113.3**	**600.2**	**8.7**	**51.4**	**144.1**	**454.1**
B							
5	1	4.9	600.2	0.0	0.0	7.8	592.2
	2	289.5	600.2	0.0	0.0	145.0	455.0
	3	943.8	600.2	513.8	0.0	80.8	519.2
	4	712.6	600.2	0.0	0.0	111.8	488.2
	5	15.8	600.2	0.0	7.4	21.0	579.0
	6	16.5	600.2	409.6	0.0	24.8	575.2
	7	10.1	600.2	4.2	0.0	15.8	584.2
	8	101.4	600.2	6.4	50.4	100.2	499.8

	9	73.7	600.2	2.4	16.4	88.0	512.0
	10	16.4	600.2	32.2	0.0	18.6	581.4
	11	30.4	600.2	0.0	0.0	39.8	560.2
	12	133.3	600.2	40.2	124.6	187.2	412.8
	13	20.9	600.2	0.0	14.4	30.0	570.0
	14	2.1	600.2	0.0	0.0	3.4	596.6
	15	140.7	600.2	1.2	7.8	88.6	511.4
	16	36.1	600.2	47.6	0.6	127.6	472.4
	17	8.4	600.2	0.0	0.0	12.6	587.4
	18	13.7	600.2	0.0	0.0	384.0	216.0
	19	22.9	600.2	0.0	0.0	104.4	495.6
	20	14.2	600.2	0.0	0.0	257.8	342.2
	21	5.8	600.2	0.0	0.0	7.8	592.2
	22	2.1	600.2	0.0	0.0	6.0	594.0
	23	12.4	600.2	0.0	72.0	15.8	584.2
	24	39.7	600.2	0.0	35.0	81.6	518.4
	25	69.2	600.2	11.6	6.6	97.6	502.4
	Total	**2736.6**	**15005.0**	**1069.2**	**335.2**	**2058.0**	**12942.0**
	Mean	**109.5**	**600.2**	**42.8**	**13.4**	**82.3**	**517.7**
C	1	4.9	600.2	502.0	12.3	514.3	85.9
10	2	289.5	600.2	220.6	320.3	540.9	59.3
	3	943.8	600.2	513.8	27.5	541.3	58.9
	4	712.6	600.2	120.5	136.5	257.0	343.2
	5	15.8	600.2	369.2	7.4	376.6	223.6
	6	16.5	600.2	409.6	12.3	421.9	178.3
	7	10.1	600.2	125.3	100.2	225.5	374.7
	8	101.4	600.2	307.5	50.4	357.9	242.3
	9	73.7	600.2	487.2	16.4	503.6	96.6
	10	16.4	600.2	132.2	3.2	135.4	464.8
	11	30.4	600.2	560.3	5.6	565.9	34.3
	12	133.3	600.2	486.2	2.3	488.5	111.7
	13	20.9	600.2	63.3	14.4	77.7	522.6
	14	2.1	600.2	258.7	230.5	489.2	111.0
	15	140.7	600.2	1.2	7.8	9.0	591.2
	16	36.1	600.2	47.6	0.6	48.2	552.0
	17	8.4	600.2	507.3	29.3	536.6	63.6
	18	13.7	600.2	587.2	10.8	598.0	2.2
	19	22.9	600.2	298.3	254.2	552.5	47.7
	20	14.2	600.2	493.6	12.2	505.8	94.4
	21	59.0	600.2	498.3	20.1	518.4	81.8
	22	87.0	600.2	250.0	220.3	470.3	129.9
	23	12.4	600.2	550.6	2.3	552.9	47.3

	24	39.7	600.2	562.9	35.0	597.9	2.3
	25	69.2	600.2	557.1	6.6	563.7	36.5
	Total	**2874.8**	**15005.0**	**8910.5**	**1538.5**	**10449.0**	**4556.1**
	Mean	**115.0**	**600.2**	**356.4**	**61.5**	**418.0**	**182.2**

A, 1 mg/mL; B, 5 mg/mL, C, 10 mg/Ml

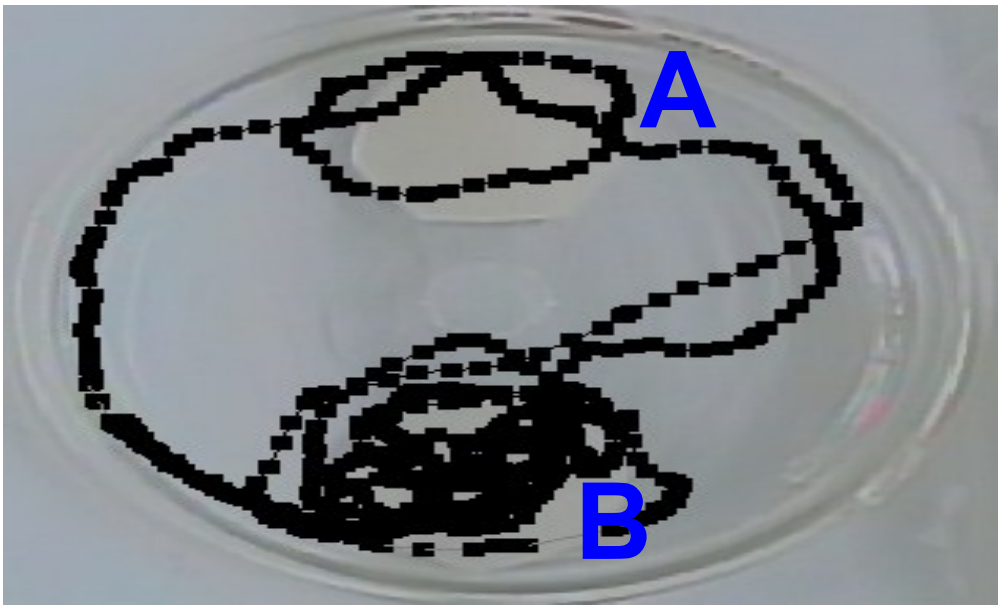


Plate 4.2: Representative track movement of F. arisanus in a petri dish arena with filter papers A, containing blank and B containing glutathione; black squares represent positions of the insect during the trial

The HMP is known to provide ovipositing females with the means of identifying hosts that have already been attacked and warning conspecific and/ heterospecifics that a further developed and therefore competitively-superior egg is already present in the host. In carrying out this study, we hypothesized that *C. cosyra* HMP is not cryptic to the marker. This hypothesis was proven correct not only by it being detected by five fruit fly species *C. cosyra*, *C. fasciventris, C. rosa, C. capitata* and *Z. cucurbitae* but also by the egg parasitoid *F. arisanus*.

These results indicated that *F. arisanus* has the ability to distinguish the odors (i.e., the HMPs) from the control suggesting the occurrence of an associated learning process a

key and useful trait in parasitism efficiency. This knowledge is useful in reducing the loss of the parasitoids in searching for the hosts and in encouraging the localization of the parasitoids in the fruits which could increase the efficiency of this biological control strategy of the pest (Silva *et al*., 2012).

Therefore, the identification of GSH as an oviposition reducing semiochemical in five fruit fly species and its effect on egg parasitoid, suggests that it can be deployed as a component for the integrated management of some species of fruit flies. Further development and optimization of *C. cosyra* HMP for use in fruit fly IPM programs in Africa is warranted, as well as investigation of the applicability of this HMP for other fruit fly genera.

CHAPTER FIVE

HOST MARKING PHEROMONE IN C. ROSA AND C. FASCIVENTRIS

5.1 Introduction

African fruit fly species such as *C. cosyra C. capitata*, *C. rosa*, *C. fasciventris* deposit HMP on the surface of fruit immediately after oviposition to suppress oviposition in conspecifics (Kachigamba *et al*., 2012). In chapter four, the isolation and characterization of *C. cosyra* HMP was reported in the females of *C. cosyra* as glutathione (Cheseto *et al*., 2017). However, exhibition of this behavior varies in different species including the compounds involved. In this chapter, the same experimental design as those for *C. cosyra*, was used to determine if other *Ceratitis* species which exhibit host marking behavior share the same HMP to *C. cosyra*.

This hypothesis was tested by determining the HMP of two African cryptic species complex *C. rosa* and *C. fasciventris*. The specific objectives were to: (i) confirm the bioactivity of aqueous extracts of the fecal matter of *C. rosa* and *C. fasciventris* against conspecifics and heterospecifics (ii) identify the female specific compound (FSC) and test its synthetic equivalent for bioactivity against conspecifics and the heterospecific (iii) assess the distribution of the HMP in *C. rosa* and *C. fasciventris* specific body tissue (ovipositor and hemolymph) and determine the relationship, if any, between HMP concentration and the fruit fly age.

5.2 Materials and methods

5.2.1 Rearing of insects and collection of fecal matter

The stock colonies of *C. rosa* and *C. fasciventris* previously identified (Marc *et al*., 2002) were obtained and raised using the methods described in detail in section 3.5

5.2.2 Bioassays.

For all the bioassays, sexually mature (10-21day old) female fruit flies were used (Kachigamba *et al*., 2012) and a ripe mango of apple variety selected and prepared as an oviposition substrate as explained in chapter four (Griesbach, 2003). Oviposition

reduction response in the fruit flies was studied in dual-choice tests as described (Kachigamba *et al*., 2012). Female fruit flies (100) of each species were observed to choose between ovipositing on:

a) a marked mango slice treated with 1 mL of aqueous fecal matter solution (10 mg/mL; 100 insect's equivalent) or a control (treated with 1 mL distilled water). The tests were conducted for; i) conspecifics, *C. rosa* female aqueous fecal matter extract using *C. rosa* females and *C. fasciventris* aqueous fecal matter extract using *C. fasciventris* females and ii) heterospecifics, *C. rosa* female aqueous fecal matter extract using *C. fasciventris* females and *C. fasciventris* aqueous fecal matter extract using *C. rosa* females

b) a marked mango slice treated with 1 mL known concentration of glutamic acid (GA) (1 mg/mL, 5 mg/mL or 10 mg/mL and a control (treated with 1 mL distilled water) using *C. rosa*, and *C. fasciventris* females.

Test samples and controls soaked in cotton swabs were applied to the mango substrate and the fruit flies grouped and treated as previously reported in chapter four. In all experiments involving oviposition, total oviposition time was measured beginning 30 s after fruit fly introduction into the observation cage and the session ended only if the female lays the egg and displays and deposit HMP. After an observation session was terminated for a female, she was returned to the rearing cage. The maximum total oviposition time allowed per observation was 30 min. The fruit flies which failed to make a choice between the treated substrate and control after 30 min were deemed to be non-responsive and were replaced by fresh fruit flies.

It was observed that immediately following oviposition *Ceratitis* female fruit flies marked a surface area of ca. 2 cm^2. Therefore, we sampled an equivalent surface area in treated and control mango slices and analyzed for glutamic acid (GA) to determine the concentration of the compound that female fruit flies encounter during oviposition. The mango slices were prepared as described for the dual choice oviposition assay and 10 mg/mL of glutamic acid in (1% formic acid/dd H_2O), 1 mL) evenly applied to the entire outer surface using cotton swabs and allowed to dry. Each mango slice was further cut into small pieces each measuring 2 cm^2 (one half of the mango slice produced 18-22 2 cm^2 pieces). The 2 cm^2 pieces of mango were each held with a pair

of forceps and thoroughly rinsed 30 times on the outer surface with 1 mL (1% formic acid/dd H_2O) into a clean 50 mL beaker using 1 mL 200-1000 µL Eppendorf pipette. This was repeated 10 times using different 2 cm^2 pieces randomly selected giving rise to 10 samples. The samples were transferred into a 1.5 mL Eppendorf tubes, vortexed for 30 s, sonicated for 10 min and centrifuged at 14,000 rpm for 5 min to remove any insoluble material after which 1 µL of the supernatant was analyzed by LC-MS.

5.2.3 Chemical analyses

5.2.3.1 Fecal matter, Ovipositor and Hemolymph

C. rosa females or *C. fasciventris* (1 mg) fecal matter previously collected from both males and female of specific age was separately dissolved in 1 mL 0.01% formic acid/acetonitrile (95: 5, LC-MS grade Chromasolv, Sigma-Aldrich, St. Luis, MO), vortexed for 30 s, sonicated for 30 min and centrifuged at 14,000 rpm for 5 min to remove any insoluble material after which 0.2 µL of the supernatant was analyzed using LC-QTOF-MS. Ovipositor and hemolymph were also collected, prepared and analyzed using the procedure described in Chapter four (section 4.2.3). The empirical formula generated was used to predict structures that were proposed based on the online database (METLIN, ChemSpider and ChemCalc, fragment ion calculator), fragmentation pattern, literature and authentic standard (L-glutamic acid, >99 % purity), (Sigma-Aldrich, St. Louis, MO). The application manager ChromaLynx, also a module of MassLynx software, was used to investigate the presence of non-target compounds in samples. Library searching was performed using the commercial NIST -MS/MS library

5.2.3.2 Relationship between glutamic acid amount in fecal matter and age of *C. rosa* & *C. fasciventris* females

The fecal matter (10 mg) of either *C. rosa* or *C. fasciventris* females was obtained as earlier described in chapter three starting from day one after eclosion and stopped at day 30 when majority of the flies were dead. On a daily basis, the fecal matter for both males and females were separately washed off the jar with 5 mL distilled water, freeze dried, re-dissolved in 1 mL 0.01% formic acid/acetonitrile (95: 5), vortexed for 30 s,

sonicated for 30 min and centrifuged at 14,000 rpm for 5 min after which 10 µL of the supernatant were analyzed by LC-MS. The same procedure was used to analyze the extracting solvents and three different samples (10 mg each) obtained from the rearing diet. The rearing diet was analyzed in using two procedure a) dissolved and prepared in the same solvent as the one used in fecal matter extraction and analyzed by LC-QTOF-MS to establish any free amino acids present b) acid hydrolysis before analysis. 10 mg of the sample was transferred into a 5 mL micro-reaction vial into which 2 mL of 6N HCl was added and closed after careful introduction of nitrogen gas. The sample was hydrolyzed for 24 h at 110 °C. After the hydrolysis, the mixtures were evaporated to dryness under vacuum. The hydrolysates were reconstituted in 1 mL 0.01% formic acid/acetonitrile (95: 5), vortexed for 30 s, sonicated for 30 min, and then centrifuged at 14,000 rpm and the supernatant analyzed by LC-QTOF-MS. This was repeated three times using different samples.

The LC-MS operating conditions have been described in Chapter four. Serial dilutions of glutamic acid standard (1-100 ng/µL) were analyzed by LC-MS in full scan mode to generate linear calibration curve (peak area vs. concentration) with the following equation; [$y = 3137x - 1353.1$ ($R^2 = 0.9993$)] which served as a basis for external quantification.

5.3 Statistical Analyses

The number of insects responding to the treatments and controls in the dual choice assays was analyzed by Chi-square goodness of fit to assess (a) *C. rosa* females discrimination to aqueous females fecal matter of conspecifics and heterospecifics compared to control (b) *C. fasciventris* females discrimination to aqueous females fecal matter of conspecifics and heterospecifics compared to control (c) *C. rosa* and *C. fasciventris* females discrimination to different doses of GA against control. Non-respondents were not included in the analysis. Analysis of LC-QTOF-MS profiles involved comparison of retention times of peaks for individual chemical components and mass spectra for both the males and females extract. The peak area from the total ion chromatogram corresponding to GA obtained from LC-MS analysis was used to compute concentrations from the calibration curve. Concentration of GA determined

at different insect ages was expressed as mean ± standard error. Analysis of variance was carried for all the concentrations for the various ages and means were separated using Tukey's studentized HSD. R-statistical program version 2.11.0 software (R Development Core Team, 2012) was used to perform the statistical analyses and all tests were performed at 5% significance level.

5.4 Results and Discussion

5.4.1 Bioactivity of *C. fasciventris* and *C. rosa* fecal matter extract confirmed

Females of *C. rosa* and *C. fasciventris* significantly preferred to oviposit into mango slices treated with water than into mango slices treated with 10 mg/mL fecal matter extract of conspecifics (χ^2 = 17.64 , *df* = 1, *P* < 0.001) and (χ^2 = 64.00 , df = 1, *p* < 0.001) for *C. rosa* and *C. fasciventris* females respectively (Figure 5A) in agreement with previous results (Arredondo & Díaz-Fleischer, 2006; Kachigamba *et al*., 2012). For heterospecifics assay, (example, *C. rosa* female's response to oviposition substrate treated with *C. fasciventris* female fecal matter extract) the flies still preferred the water treated dome but the oviposition deterrent effect reduced by half compared to conspecific assay (Figure 5.1 B). This suggests that the ovipositing flies recognized the compounds present in its own feces and to a lesser extent that of the sister fly which provoked the oviposition deterrence behavior aimed at ensuring survival of subsequent generation (Papaj *et al*., 1992). Also, GA was the major female unique compound identified in the fecal matter extract of *C. rosa* and *C. fasciventris*, other the minor components which were species specific were not identified and this might explain why the non-discrimination in oviposition behavior dropped by half for *C. fasciventris, and C. rosa* against hosts marked by heterospecifics fecal matter.

As expected, ovipositing females required twice as long to assess the suitability of the treated oviposition substrate than controls (two sample Wilcoxon test, *W*= 259, *P* < 0.001 and *W*= 702, *P* < 0.001), for *C. rosa* and *C. fasciventris* females respectively (Table 5.1).

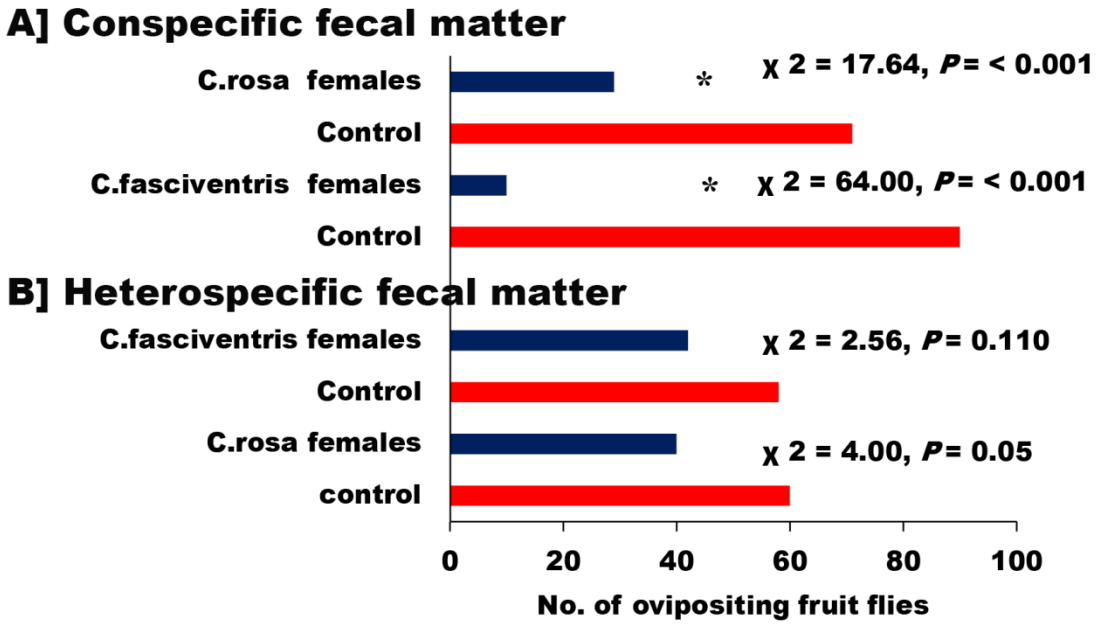


Figure 5.1: Discrimination of oviposition substrates treated with *C. rosa* or *C. fasciventris* fecal matter extract**,** A, conspecific fecal matter by conspecific females B, heterospecific fecal matter by heterospecific females. *Denotes significantly different at 0.05

Thirty eight percent of *C. rosa* and 23% of *C. fasciventris* ovipositing fruit flies chose to lay eggs in mango slices treated with fecal matter extract, but they took relatively double the time to assess the oviposition substrate compared to controls. This indicates that females recognized the treated hosts but factors such as innate sensitivity to HMP (Addesso *et al*., 2007), and physiological state of the fly could have contributed to the observed oviposition pattern. Generally, the more mature eggs carried by the females or the longer the time elapsed since the last oviposition, the more females become defiant to the HMP. It is also known that the nature of the fly, for instance, laboratory reared flies of *C. capitata* females kept for over 200 generations were found to be 3-fold less sensitive to the same concentration of HMP compared to the wild-caught fruit flies. Additionally, the type of host substrate used could play a role in oviposition site selection (Dimbi *et al*., 2009). The preferred host for *C. rosa* is *Lettowianthus stellatus* (Dimbi *et al*., 2009). Future studies should investigate the influence of the preferred host on HMP responses in both wild- and laboratory-reared fruit flies.

Table 5.1: Oviposition time of *C. fasciventris* and *C. rosa* females on mango slices treated with aqueous solution of crude fecal matter extract (n = 100 females/ treatment)

Test Fecal matter From	Responding Fruit fly	Treatment Median (range) (minutes)	Control Median (range) (minutes)	*p*-value
C. fasciventris	*C. fasciventris*	8.1 (10.5-29.7)	5.4 (4.2-15.3)	< 0.001
	C. rosa	11.9 (9.8-26.6)	6.6 (5.1-18.7)	0.033
C. rosa	*C. fasciventris*	10.9 (9.5-28.2)	5.7 (2.8-16.2)	0.012
	C. rosa	8.4 (7.6-27.0)	4.4 (3.3-21.4)	< 0.001

5.4.2 LC-QTOF-MS identification of *C. fasciventris* and *C. rosa* HMP

Chemical analysis of the aqueous fecal matter extracts of both females and males, by LC-QTOF-MS, identified glutamic acid, as specific to the fecal matter extract of females. GA eluted from the column at 1.7 min (Figure 5.2) with a molecular ion peak $[M+H]^+$ at *m/z* 148.0607, corresponding to a molecular formula of $C_5H_{10}NO_4$. The amino acid identity was supported by the presence of the expected fragments with monoisotopic mass ions at *m/z* 102.0549 $[M+H-HCOOH]^+$ and 130.0503 $[M+H-H_2O]^+$ (Qu *et al*., 2002), (Figure 5.4). The identity of GA was confirmed by comparison of mass spectrometric data, retention time and co-injection of the natural extract with an authentic standard (Figure 5.4). Additionally, eleven other compounds common to fecal matter extracts of males, females and the undigested rearing diet were identified (Figure 5.3, Table 5.3), (shown only for *C. rosa* as an example).

Comparison of the LC-QTOF-MS amino acid profiles of the non-hydrolyzed rearing diet and acid digested rearing diet, revealed the presence of several amino acids including glutamic acid, cysteine, glycine, alanine, arginine, lysine, histidine, proline, valine, methionine, tyrosine, isoleucine, leucine and phenylalanine in the acid digested diet (Figure 5.3, Table 5.2). These amino acids were not detected in the non-hydrolyzed rearing diet (Figure 5.2, 5.3, Table 5.3). This indicates that GA is likely a

product from digestion of a protein derived from the rearing diet, which may occur in the gut of females.

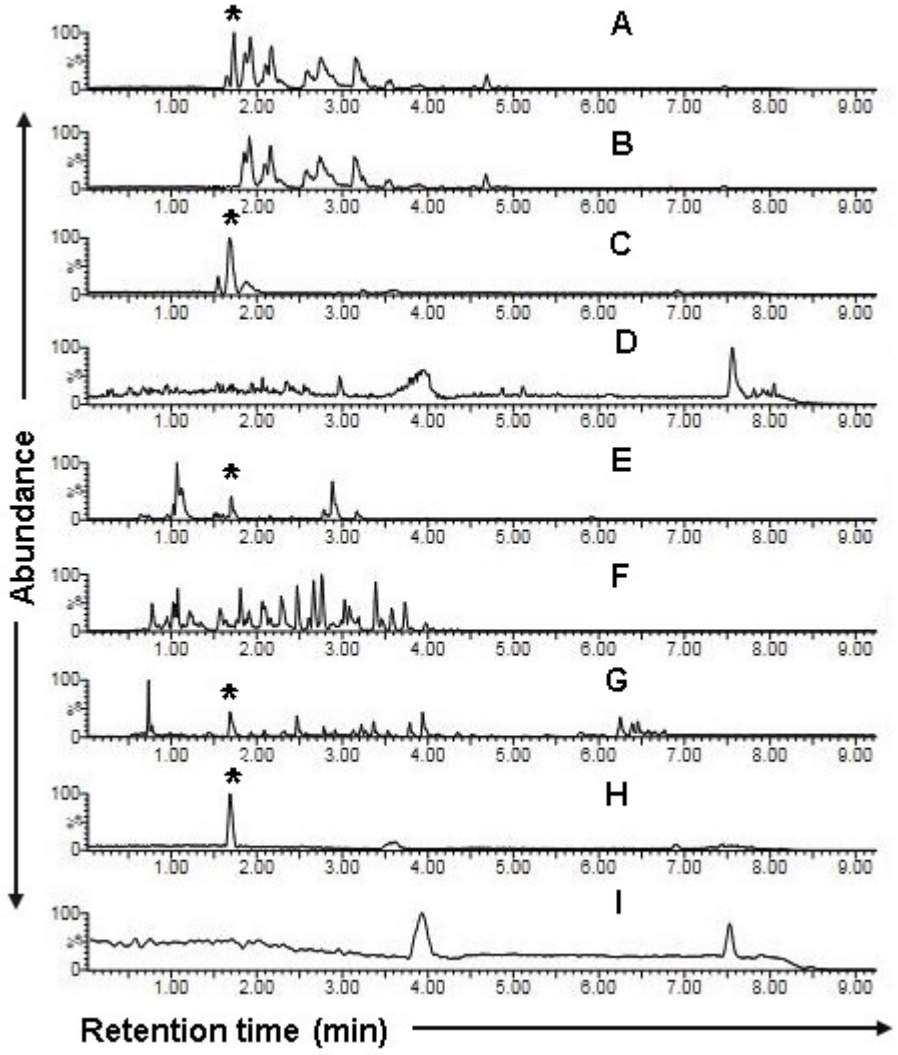


Figure 5.2: Representative overlaid total ion chromatogram showing secretions and tissues extracts of males and females of *C. rosa* (A). female fecal extract, (B). male fecal extract, (C). ovipositor extract, (D). male hemolymph extract, (E). female hemolymph extract, (F). undigested rearing diet, (G). digested rearing diet, (H). glutamic acid standard, and (I). extracting solvent. * glutamic acid peak.

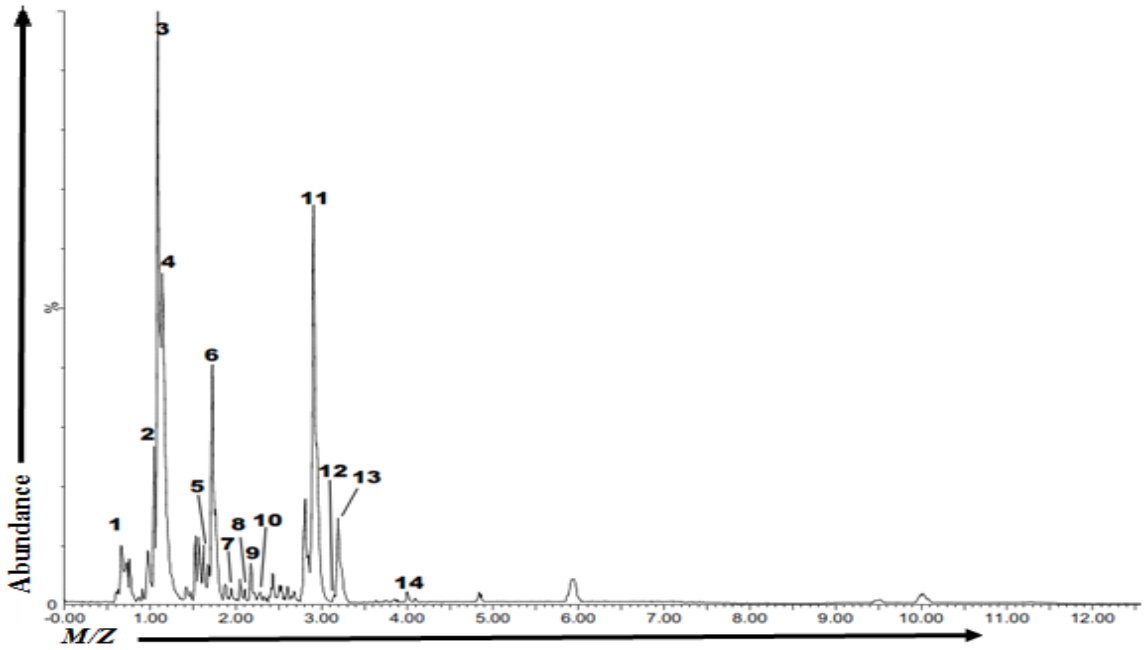


Figure 5.3: Representative total ion chromatogram showing amino acids detected in acid digested rearing diet

Table 5.2: Amino acids identified in acid digested rearing diet

peak no.	amino acid	$[M+H]^+$
1	glycine	76.0388
2	alanine	90.0940
3	arginine	175.1195
4	lysine	147.1132
5	histidine	156.1570
6	glutamic acid	148.0607
7	proline	116.0711
8	valine	118.0862
9	cysteine	122.0938
10	methionine	150.0580
11	tyrosine	182.0815
12	isoleucine	132.1022
13	leucine	132.1024
14	phenylalanine	166.0870

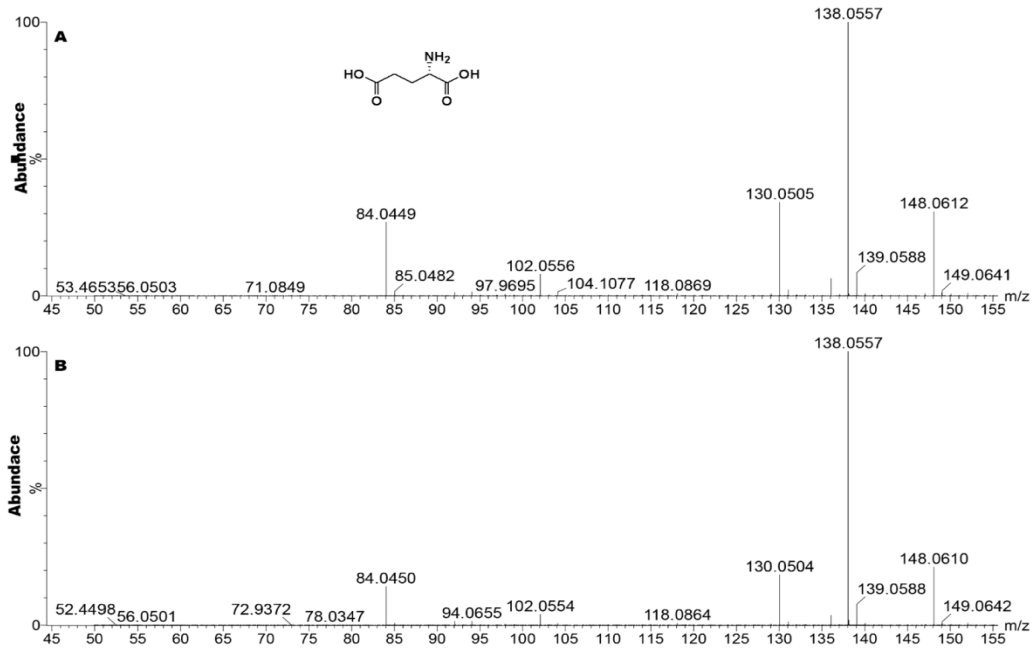


Figure 5.4: Representative mass spectrum of (A) natural glutamic acid from fecal matter and (B) synthetic glutamic acid

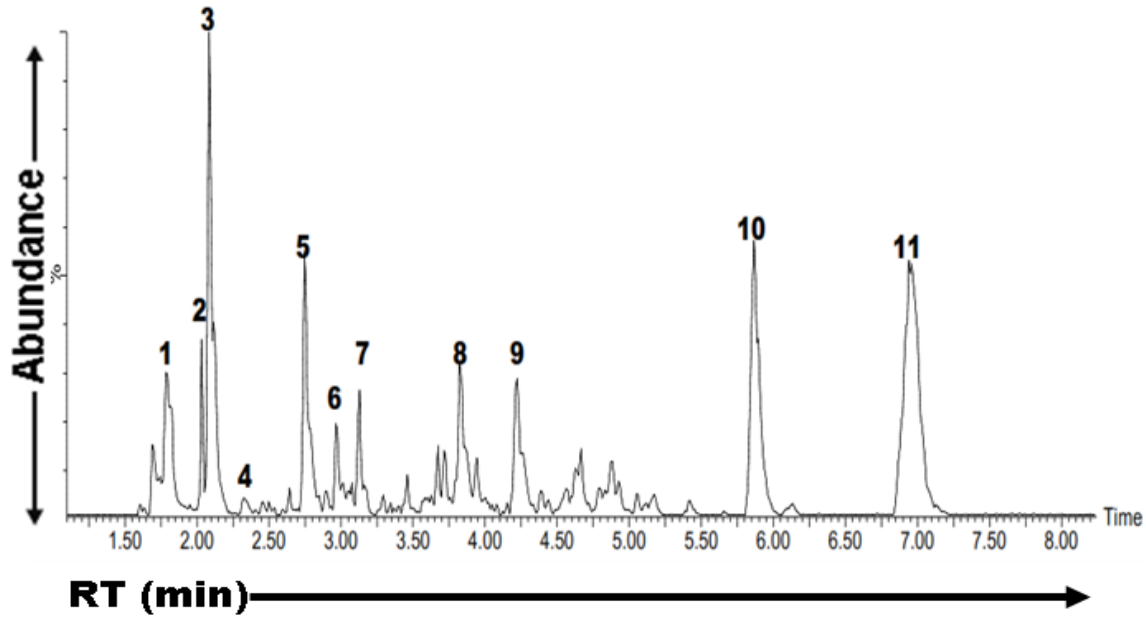


Figure 5.5: Representative total ion chromatogram showing compounds detected in undigested rearing diet

Table 5.3: Compounds identified in fecal matter of females, males and undigested rearing diet

Peak No.	t_R (min)	Compound	Molecular Formula	$[M+H]^+$	Key fragment ions	F	M	U
1	1.68	Glycylglycylglycyl-*N*-ethylglycinamide	$C_{10}H_{19}N_5O_4$	274.1516	84.9628,110.0131, 182.9704, 184.969, 198.9487, 214.9256, 238.1289	+	+	+
2	1.70	Glutamic acid	$C_5H_9NO_4$	148.0607	84.0449, 102.0549, 130.0503, 102.0550	+	-	-
3	1.76	bis-Demethoxycurcumin	$C_{19}H_{17}O_4$	309.1790	175.1265, 180.1091, 225.133, 273.1562,291.1675,295.1631, 310.1827,319.1748	+	+	+
4	1.88	Ser-Ala-Ala	$C_9H_{17}N_3O_5$	248.1239	105.0659, 161.1191, 202.0797, 220.0909, 230.112, 249.1265, 266.135, 267.1383, 231.0986	+	+	+
5	2.12	Asp-Lys	$C_{10}H_{19}N_3O_5$	262.1398	116.0342, 130.0917, 133.0608, 244.1292, 245.132	+	+	+
6	2.19	Uridine	$C_9H_{13}N_2O_6$	245.1713	137.0512, 179.0566, 229.1638, 235.1288	+	+	+
7	2.61	Saccharopine	$C_{11}H_{21}N_2O_6$	277.1458	132.1011, 185.0909, 230.1373, 294.1532	+	+	+
8	3.17	Cinnamoyl glucose	$C_{15}H_{19}O_7$	311.1301	166.0852, 201.1699, 264.121	+	+	+
9	3.88	Unknown		555.2967	144.0799, 197.1266, 217.0965, 415.2507, 437.2318, 441.2218	+	+	+
10	4.71	Unknown		416.1715	251.1991, 326.2315, 344.2414, 384.2336	+	+	+
11	7.49	1,4-Diaminonaphthalene	$C_{10}H_{11}N_2$	159.1575	102.0910, 136.0198, 158.1526	+	+	+

F = females, M = males, U = undigested rearing diet, + = present and - = not detected

5.4.3 Bioactivity of glutamic acid

Glutamic acid (GA) reduced oviposition responses of females of *C. rosa* and *C. fasciventris* following a concentration-dependent response (Figure 5.7). There was a significant preference for the mango slices treated with water compared to the mango slices treated with glutamic acid, especially at the highest concentration of 10 mg/mL (χ^2 = 40.96, *df* = 1, *P* < 0.001) and (χ^2 = 29.2 , df = 1, *p*= < 0.001) for *C. rosa* and *C. fasciventris* respectively (Figure 5.3). Ovipositing females on average required five times longer to assess the suitability of the treated mango slices than controls (two sample Wilcoxon test, *W* = 369, *P* <0.001 and *W*= 797, *P* < 0.001), for *C. rosa* and *C. fasciventris* females respectively), (Table 5.4). These results confirmed the bioactivity previously observed for the fecal matter extract and presence of a HMP in the extract.

In recovery studies, glutamic acid concentrations recovered ranged 3.5 – 4.4 µg/2 cm^2 of mango. This amount of GA is 1.3 and 4.2 times less than the amount found in 1 mg of ovipositor and fecal matter extract respectively. Although, the observed effective oviposition deterrence concentration of GA against *C. rosa* and *C. fasciventris* is 10 mg/mL for a mango slice, the actual concentration encountered by a fruit fly during oviposition is 4.20 ± 0.38 µg/2 cm^2. This result corroborates previous studies (Kachigamba *et al*., 2012), which reported that up to 90 fruit flies make oviposition attempts in a mango fruit when deprived of an oviposition substrate for 24 h. Additionally, other research has reported effective HMP concentrations of 1-100 mg/mL (Aluja & Díaz-Fleischer, 2006; Arredondo & Díaz-Fleischer, 2006), and that more than 75 ovipositor dragging circles are made by laboratory reared *Ceratitis* female fruit flies before detection of the presence of HMPs. This insensitivity of laboratory-reared fruit flies to HMP has been linked to population selection which favors high fecundity at the expense of HMP detection. These findings suggest that wild caught fruit flies may be more sensitive to HMPs (Prokopy *et al*., 1989). It would be interesting to compare responses of wild-caught and laboratory-reared fruit flies to field-realistic doses of HMPs, such as GA, to confirm previous work (Prokopy *et al*., 1989), and to also determine if

contextual host plant volatiles are needed as background odors or for possible enhancement of the HMPs.

The fact that non-responders to fecal matter extract and GA ranged from 20-30% is consistent with most laboratory assays involving semiochemicals using insects; 50% non-responders in *Rhagoletis mendax* in oviposition assays (Faraone *et al*., 2016), and 5-40 % in the bark beetle parasitoid *Roptrocerus xylophagorum* and sand fly *Lutzomyia longipalpis* (Bray *et al*., 2007; Sullivan *et al*., 2000).

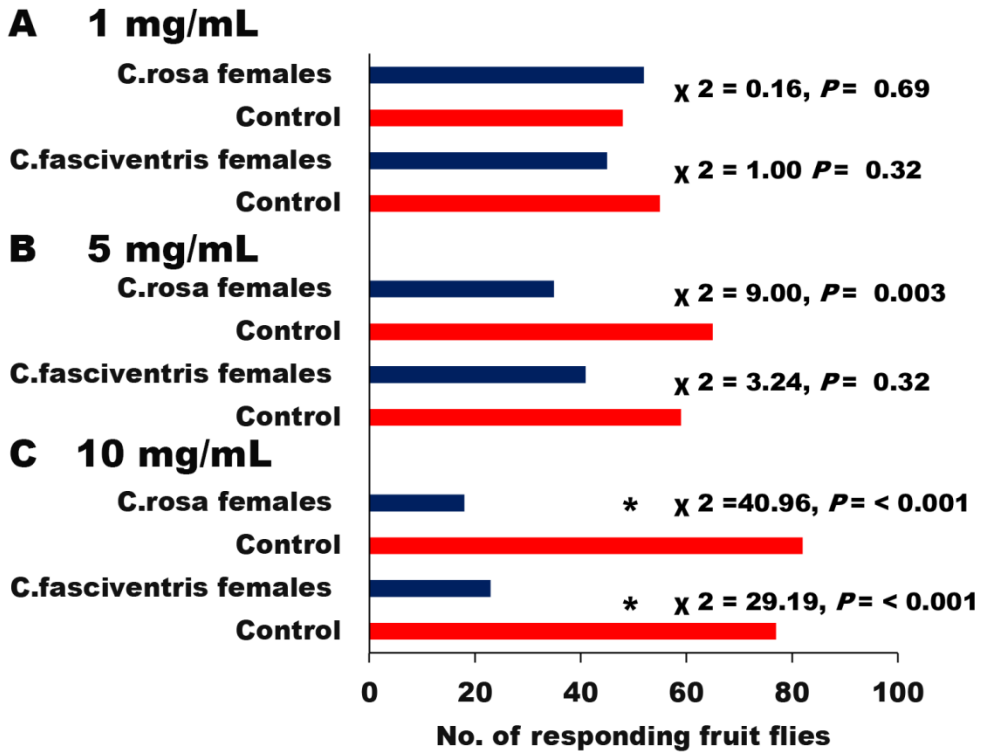


Figure 5.6: Discrimination of oviposition substrates treated with varying concentration solution of synthetic GA by *C. fasciventris* and *C. rosa* females (A) 1 mg/mL, (B) 5 mg/mL, and (C) 10 mg/mL. *Denotes significantly different at 0.05

Table 5.4: Oviposition time of *C. fasciventris* and *C. rosa* females on mango slices treated with solution of glutamic acid (n = 100 females/ treatment)

Test Glutamic acid concentration (mg/mL)	Responding Fruit fly	Treatment Median (range) (minutes)	Control Median (range) (minutes)	*p*-value
1	*C. rosa*	8.0 (3.6-20.3)	6.2 (3.7-18.0)	0.312
1	*C. fasciventris*	6.1 (3.8-19.1)	8.0 (3.8-19.0)	0.432
5	*C. rosa*	7.1 (4.2-23.2)	6.9 (2.8-17.5)	0.294
5	*C. fasciventris*	10.0 (4.0-18.9)	6.4 (2.5-20.6)	0.245
10	*C. rosa*	9.5 (7.3-28.6)	3.3 (2.2-15.7)	< 0.001
10	*C. fasciventris*	9.0 (8.0-29.2)	4.7 (4.0-16.5)	< 0.001

5.4.4 Relationship between glutamic acid amount in fecal matter and age of *C. rosa* and *C. fasciventris*

Glutamic acid was detected in the fecal matter extracts of 6 to 30 d old females of *C. rosa* and *C. fasciventris*. The concentrations of GA correlated positively with the age of females [(R^2 = 0.8799); 25.93 ± 1.24 µg/mg and 32.62 ± 0.40 µg/mg] and [(R^2 = 0.8821); 14.31 ± 0.79 µg/mg and 17.78 ± 1.18 µg/mg] (optimal amount), detected in fecal matter extracts of 6 d old and 12 d old *C. rosa* and *C. fasciventris* females respectively before slightly dropping by a factor of between 1.5-2.5 (Figure 5.7).

These results indicate that ovipositing females exploit GA as a HMP to coincide with sexual maturity, when female sensilla may also exhibit the highest sensitivity to detect the pheromone. More experiments are needed, including elucidating how females detect HMPs to establish the relationship, if any, between age of female and detection of HMP. Since the average lifespan of a *Ceratitis* male or female is about 30 d, it is likely that the highest consumption of food and utilization of nutrients may occur at 12-20 d, when both sexes exhibit a strong inclination to mate. The fruit flies of age 14-20 d old have been shown to produce pheromones because they are sexually mature compared to older flies

(more than 28 day (d) old) or younger flies (less than 6 d old) (Aluja & Boller, 1992; Diamantidis *et al*., 2008; Edmunds *et al*., 2010).

As such, if GA is a byproduct of the metabolism of food ingested by ovipositing females at this age, then its concentration would be expected to be high in both the ovipositor and fecal matter excreted by these females. If so, this may be advantageous to females since it would provide them with the opportunity to deposit the most potent levels of the HMP present in both the ovipositor and fecal matter on the oviposition substrate. Further research is needed to determine the possible physiological and/or behavioral mechanisms involved. As previously found for *C. cosyra* (Cheseto *et al*., 2017), GA level was 10-20 times higher in fecal matter extract than in similar extracts of the ovipositor and hemolymph of females (Figure 5.2). These results suggest that GA is transferred from the gut into the ovipositor via the hemolymph, with the excess amount excreted with the fecal matter. Experiments elucidating the transportation, stereochemistry and excretion of GA in *C. rosa* are highly recommended.

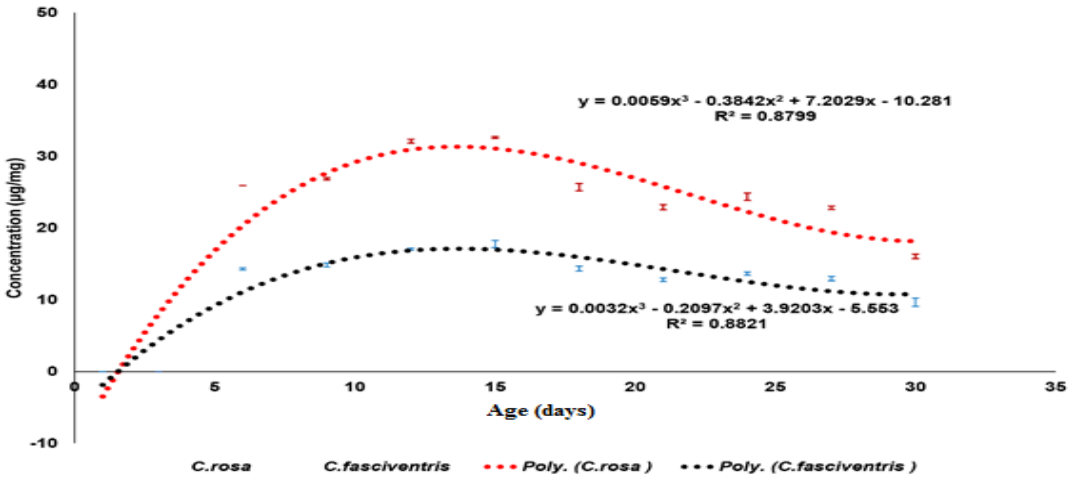


Figure 5.7: Relationship between female age (days) and the amount of glutamic acid detected in fecal matter of *C. rosa* and *C. fasciventris***.** Concentrations (µg/mg) of GA for the various insect age bearing the same letter are not significantly different (P = 0.05, Tukey's, HSD test)

The present study identified glutamic acid as the HMP from the fecal matter extract of females of *C. rosa* & *C. fasciventris* and that GA concentration influenced female oviposition response. This study also showed that GA is present in the hemolymph and ovipositor of females and that age significantly affected female HMP production.

The presence of GA in organisms has been of interest to researchers in the past. For example, GA together with inosine and glutamine have been reported as components of the sex pheromone of the marine polychaete *Nereis succinea* (Zeeck, *et al*., 1998), GA is a constituent of volicitin, *N*-(17-hydroxylinolenoyl)-L-glutamine and other fatty acid amino acid conjugates found in the gut of *Manduca sexta,* and it is known to trigger elicitor activity in maize (Yoshinaga *et al*., 2014). In chemical synthesis, GA has been employed as a useful chiral synthon in the synthesis of many pheromones (Smith *et al*., 1979). For example, synthesis of γ-caprolactone, a sex pheromone component of the dermestid beetle (Cross *et al*., 1977), 2-ethyl-1,6-dioxaspiro[4,4] nonane (chalcogran), an aggregation pheromone of the six-toothed spruce bark beetle (Francke *et al*., 1977), and (*Z*)-5-tetradecen-4-olide, a sex pheromone of the Japanese beetle (Midland & Nhan, 1981). Additionally, in the silkworm and locust, GA functions either as an agonist for muscle contraction, or as an inhibitor, depending on the isomeric form (Kandel *et al*., 2000; Sekimizu *et al*., 2005).

Chapter four and Five provide strong evidence of the utilization of amino acids and their derivatives as HMPs in *Ceratitis* species and other fruit flies (US 6,555,120 B1, 2003; Cheseto *et al*., 2017; Edmunds *et al*., 2010). Interestingly, in *C. cosyra*, we identified the tripeptide glutathione as the HMP of ovipositing females, yet no glutamic acid was detected in the fecal matter. Glutathione is composed of glutamic acid, cysteine and glycine, whereas in *C. rosa* and *C. fasciventris*, the HMP is glutamic acid. Both HMPs are thought to be produced from digestion of the rearing diet in the gut of both insects. Indeed, acid hydrolysis of the rearing diet identified glutamic acid, cysteine, glycine, and eleven other amino acids (alanine, arginine, lysine, histidine, proline, valine, methionine, tyrosine, isoleucine, leucine and phenylalanine), which would possibly explain the exploitation of amino acids and their derivatives as HMPs by this group of fruit flies.

These findings suggest that more experiments are needed, especially on other *Ceratitis* species to confirm their use of amino acids for biosynthesis of HMPs.

Furthermore, the Mexican fruit fly *Anastrepha ludens* utilizes *N*-[2,14-dimethyl-1-oxopentadecyl)-glutamic acid,(US 6,555,120 B1, 2003; Edmunds *et al*., 2010) as its HMP, whereas the HMP of the cherry fruit fly *Rhagoletis cerasi* is *N*-[15(β-glucopyranosyl)oxy-8-hydroxypalmitol]-taurine(Aluja & Boller, 1992). Collectively, these results suggest that sibling species such as found in *Ceratitis* species may utilize the same pathway to make their HMPs, whereas non-related species may utilize different pathways, which may be associated with the evolution of fruit flies. Additional research would be needed to confirm this scenario.

Our studies on HMPs of *C. cosyra*, *C. rosa* and *C. fasciventris* (Cheseto *et al*., 2017), revealed that the HMP of *C. cosyra* reduced oviposition responses in conspecifics and the heterospecific species *C. rosa*, *C. fasciventris*, *C. capitata* and *Zeugodacus cucurbitae*, while the HMP of *C. rosa* and *C. fasciventris* reduced oviposition responses in this two species only in agreement with previous findings using fecal matter (Kachigamba *et al*., 2012). These findings suggest that over time *C. cosyra* females have evolved to produce the enzymes required for the biosynthesis of amino acids into the tripeptide glutathione, which has the advantage to reduce oviposition responses, not only in conspecifics but also heterospecifics. Evolutionary studies may provide an explanation for the utilization of similar molecular subunits in HMP production in sibling species. For instance, phylogenetic studies of the genus *Ceratitis* based on mitochondrial and nuclear gene found a more recent divergence of *C. rosa* and *C. fasciventris* compared to *C. cosyra* (Barr *et al*., 2006). Therefore, it appears that *C. cosyra* is at a more advanced stage than *C. rosa* in the evolutionary development of *Ceratitis* species. As such, it is not surprising that the advanced and complex evolution and pre-adaptive nature of *C. cosyra* compared to *C. rosa* and *C. fasciventris* (Barr *et al*., 2006; Mwatawala *et al*., 2015) could have contributed to the rapid spread of *C. cosyra* across Africa, attacking a wide range of fruits, which is second to the invasive fruit fly species *B. dorsalis*.(Mwatawala *et al*.,

2015) Future research on competitive displacement and population genetics of other *Ceratitis* species is needed to validate this.

The identification of glutamic acid as a host marking pheromone in *C. rosa* and *C. fasciventris* and the previous identification of GSH as a HMP of *C. cosyra* (Cheseto *et al*., 2017) improves our understanding of the chemical ecology of fruit flies, particularly in *Ceratitis* species. It remains to be established whether this pattern occurs in other *Ceratitis* species, and to assess their usefulness in the integrated management in fruit flies.

CHAPTER SIX

EFFECT OF GLUTATHIONE AND SELECTED FRUIT FLY LARVAE ON VOLATILE PROFILE OF RIPENING MANGO FRUIT

6.1 Introduction

Mangoes (*Mangifera indica* Linn.), popularly known as "The King of Fruits" is a dicotyledonous fruit of the family Anacardiaceae. Mango is grown in tropical and subtropical regions around the world (Ploetz, 1994), and is one of the most important fruits in the world market (Yashoda, 2003), with a total production estimated at 40 million tons in 2016 (Mitra, 2016).

In Kenya, mango is an important fruit that provides both income and nutritional security for many people (*icipe*, 2007). Despite it being a key fruit, its production and utilization faces a number of challenges, one of them being infestation by Tephritid fruit flies (*icipe*, 2007). It is estimated that Kenya produces 183,486 tons of mango annually, with more than 50 % of this being lost to fruit flies which use it as a breeding ground (Griesbach, 2003).

Finding a suitable oviposition site is crucial not only for fruit flies but to the general phytophagous insects (Thompson, 1999). Olfaction plays an important role in enabling the insect to recognize host plants at a distance (Picker *et al*., 2002; Pickett and Cadenasso, 2002) and it determines the probability of alighting on a given host. After landing, a combination of contact chemoreception, visual and physical cues provide further sensory input leading to either acceptance or rejection of the oviposition site (Bruce *et al*., 2011).

Since herbivory often induces a variety of chemical changes in plants, it is reasonable to assume that the same changes will also take place when the same insects oviposit and their off springs develop inside a fruit (Bruce *et al*., 2011). In this context, volatile chemicals emitted by plants and fruit may act as cues to both herbivores and the natural

enemies of herbivores. The plant's role in this interaction is now considered to be active, with the plant 'signalling' that it has been attacked by herbivores through changes in the emission of volatiles (Pare *et al*., 1999).

To date, enormous amount of research has been geared towards identifying kairomones from mango fruit for managing fruit flies (Lalel *et al*., 2003). More than 270 volatile compounds have been identified from mango in the last century with monoterpene and sesquiterpene hydrocarbons being the most abundant. Esters and lactones have also been found to play a part in the unique volatile chemistry of certain cultivars (Lalel *et al*., 2003; Moalemiyan *et al*., 2006; Rodrıguez *et al*., 2013).

Since chemical cues are generally considered to play a central role in the location, evaluation and utilization of hosts by frugivorous insects, the current Chapter sort to evaluate the exogenous effect of glutathione, a HMP of *C. cosyra,* and endogenous effect of selected fruit fly larvae on volatile profile from ripening mango fruit in order to have an in-depth understanding of fruit fly-mango chemical ecology.

6.2 Materials and methods

6.2.1 Plant material

In all experiments, unblemished, ripe apple mango (*M. indica L.*) varieties of ≈ 25 cm^2 (surface area) were purchased from local markets in Nairobi. In order to exclude the effect of fruit maturation, all volatile collections were made using a single batch of fruit purchased the same day (Hern *et al*., 2002).

The mangoes were first washed with distilled water to remove debris, and dried on paper towel. Headspace volatiles were collected from two categories of mango based on the target output:

Category 1; the target output was to analyze the effect of topical application of glutathione, a *C. cosyra* HMP, on mango fruit volatiles. To achieve this, different mangoes were treated as follows a) intact mango b) intact mango evenly spread with 10

mg/ml glutathione (volume 1 mL) c) spiked mango (1 mm holes, 1 cm distant apart ≈ 50 holes/fruit) evenly spread with 10 mg/ml glutathione (volume 1ml) d) spiked mango (1mm holes, 1cm distant apart ≈ 50 holes/fruit) evenly spread with distilled water (volume 1ml) and e) a blank (empty oven bag). The trapping was done for a maximum period of 12 h for the various treatment listed a-e at 0 h, 12 h, 24 h and 48 h. After each treatment the adsorbent was removed, eluted and analyzed immediately to avoid loss of volatiles.

Category 2; the target output was to analyze the effect of developing fruit fly larvae on mango volatiles. A single fruit from the same batch was exposed to the cages containing ca. 500 fruit flies (200 males and 300 females) for a period of 24 h before commencement of trapping as follows 1. *B. dorsalis*, 2. *C. cosyra* 3. *C. rosa* 4. *C. fasciventris* and 5. mango placed in an empty cage as control. After 24 h, the mangoes were removed from the respective cages, wiped with a different piece of paper towel to remove surface eggs and dirt. The headspace volatiles were trapped on to a cleaned adsorbent for a maximum period of 24 h after which the loaded adsorbent was removed and replaced with fresh one. The removed adsorbent was eluted and analyzed immediately to avoid loss of volatile. This cycle continued for a total period of 10 days (duration taken by this fruit fly species to reach 4^{th} instar).

Headspace environment for the two categories of mangoes was created by enclosing each treated sample in a Reynolds® oven bag (Richmond, VA, USA) (482×596mm) conditioned by heating in an oven at 110°C for 24 h; supplied with charcoal filtered and humidified environmental air (flow rate 260 ml/min) using a push-pull Gast pump (model DAA-V174-EB; Gast Manufacturing, Inc., Benton Harbor, MI, USA). The volatiles were trapped on to pre-cleaned with dichloromethane (DCM) adsorbent Super-Q traps (30 mg, Analytical Research System, Gainesville, Florida, USA) at a flow rate of 170 mL/min using a Vacuubrand CVC2 vacuum pump (Vacuubrand, Wertheim, Germany).

The Super-Q traps were eluted with DCM 200 µL GC-grade (Sigma Aldrich, Gillingham, UK) in to micro-injection insert vials (Agilent Technologies, Palo Alto, CA) and immediately analyzed using GC-MS.

6.2.2 Analysis of volatiles

GC-MS in full scan mode was used to detect and profile all the compounds present in the volatiles. A 5-point serial dilutions of authentic standards of 1,8-cineole and β-caryophyllene (1-280 ng/µL) were also analyzed by GC-MS in full scan mode to generate linear calibration curves (peak area vs. concentration) with the following equations; [y = 203482x - 451578 (R^2 = 0.9997) and y = 199907x - 674423 (R^2 = 0.9990) for 1,8-cineole and β-caryophyllene respectively] which served as the basis for the external quantification.

6.2.3 GC-MS conditions

Volatiles (1.0 µL) were analyzed by GC-MS on a 7890A gas chromatograph linked to a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC was fitted with a HP5 MS low bleed capillary column (30 m × 0.25 mm i.d., 0.25 µm) (J&W, Folsom, CA, USA). Helium at a flow rate of 1.25 ml min^{-1} served as the carrier gas. The oven temperature was programmed from 35 to 285 °C with the initial temperature maintained for 5 min then 10 °C min^{-1} to 280 °C, held at this temperature for 20.4 min. The mass selective detector was maintained at ion source temperature of 230 °C and a quadrupole temperature of 180 °C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. Fragment ions were analyzed over 40–550 *m/z* mass range in the full scan mode. The filament delay time was set at 3.3 min.

A HP Z220 SFF intel Xeon workstation equipped with ChemStation B.02.02. acquisition software was used. The mass spectrum was generated for each peak using Chemstation integrator set as follows: initial threshold = 5, initial peak width = 0.1, initial area reject = 1 and shoulder detection = on. The compounds were identified by comparison of mass spectrometric data and retention times with those of authentic standards and reference

spectra published by library–MS databases: National Institute of Standards and Technology (NIST) 05, 08, and 11

Identification of the VOCs was achieved on the basis of their retention indices (RI) (determined with reference to a homologous series of normal alkanes C_5-C_{31}) and calculated based on the equation of Van den Dool and Kratz and comparison with what is documented in literature (Adams, 2007; van Den Dool *et al*., 1963), as shown below:

$$\mathbf{RIx = 100\ n_0 + 100\ (R_Tx - R_Tn_0) / (R_Tn_1 - R_Tn_0)}$$

With:

x = the name of the target compound

n_0 = n-alkane $C_{n0}H_{2n0+2}$ directly eluting before x

n_1 = n-alkane $C_{n1}H2_{n1+2}$ directly eluting after x

R_T = retention time

RI = retention index

6.2.4 Data collection

Data collected were total ion chromatogram and mass spectral data of the various volatile organic compounds (VOCs) and their concentrations present in various sample treatment. The peak areas of total ion chromatogram corresponding to VOCs obtained by GC-MS analysis were used to compute concentrations from the calibration curve.

6.3 Results

6.3.1 Category 1: Volatile organic compounds (VOCs) of apple mango treated with glutathione

Head space analysis of apple mango fruit VOCs after subjection to various treatment a-d yielded a total of 82 compounds where a) VOCs trapped from intact mango b) VOCs trapped from intact mango evenly spread with 10 mg/ml glutathione (volume 1ml) c)

VOCs trapped from spiked mango (1mm holes, 1cm distant apart ≈ 50 holes/fruit) evenly spread with 10 mg/ml glutathione (volume 1ml) d) VOCs trapped from spiked mango (1mm holes, 1cm distant apart ≈ 50 holes/fruit) evenly spread with distilled water (volume 1ml) and e) a blank (empty oven bag) which served as a control whose results are not reported here. Table 6.1 shows (RT) the retention time (min), chemical identity and concentration (ng/h) of the VOCs identified for category one while Table 6.7 shows the structures and major fragments for the VOCs observed. Apart from detection of acetoin in spiked mango and spiked mango + HMP there was no qualitative difference in VOCs produced across the four treatments. Of the total 82 VOCs identified, 5.8, 37.8, 23.2, 13.4 and 9.8 % represented esters, hydrocarbons (straight chains, branched and cyclic), monoterpenes, sesquiterpenes and others (aldehydes, ketones and alcohols) respectively. The monoterpenes α-pinene and myrcene were the most abundant compounds in the four trapped VOCs across the four different times, whereas *(E)*-β-caryophyllene was the most abundant sesquiterpene. When the total VOCs for each treatment for 0, 12, 24 and 48 h was summed up c) intact mango + HMP tops the list (22668 ng/h) followed by b) spiked mango + HMP (9697 ng/h), a) spiked mango (8444 ng/h) and lastly d) intact mango (7356 ng/h).

Table 6.1: VOCs concentration (ng/h) identified in apple mango treated with glutathione

				Spiked mango (d)				Spiked mango + HMP (c)				Intact mango + HMP (b)				Intact mango (a)			
No	**RT**	**Compound Name**	**RI**	**0 h**	**12 h**	**24 h**	**48 h**	**0 h**	**12 h**	**24 h**	**48 h**	**0 h**	**12 h**	**24 h**	**48 h**	**0 h**	**12 h**	**24 h**	**48 h**
1*	3.91	Acetoin	653	9.5	10.3	11.1	12.0	4.8	5.1	5.4	5.7								
2*	4.00	Ethyl propanoate	687	3.0	3.0	3.1	3.2	10.3	11.1	12.0	13.0	11.4	11.3	6.8	10.4	6.4	3.1	2.4	2.7
3*	4.24	Methyl butanoate	707	7.6	8.2	8.7	9.4	7.5	8.0	8.6	9.3	11.6	11.6	6.9	10.7	6.5	3.1	2.4	2.7
4*	4.49	(*Z*)-Methyl-2-butenoate	717	5.0	5.2	5.5	5.9	23.7	25.9	28.2	30.8	9.0	8.9	5.6	8.3	5.3	2.8	2.3	2.6
5*	5.35	Toluene	748	4.6	4.8	5.1	5.4	5.5	5.9	6.2	6.6	5.6	5.6	3.3	5.3	3.2	2.4	2.3	2.3
6*	6.13	(*E,E*)-1,3,5-heptatriene	776	4.6	4.8	5.1	5.4	2.8	2.9	3.0	3.0	7.9	7.8	5.0	7.3	4.8	5.3	5.9	6.7
7*	6.56	Ethyl butanoate	792	38.9	42.6	46.6	51.0	6.5	6.9	7.4	7.9	3.7	3.7	3.7	3.6	3.6	4.0	2.8	3.4
8*	7.90	Ethyl-2-methylbutanoate	844	3.0	3.1	3.1	3.2	3.2	3.3	3.4	3.5	8.3	8.2	5.2	7.7	5.0	4.7	4.4	4.2
9*	7.99	Ethyl-3-methylbutanoate	847	2.8	2.9	3.0	3.0	3.1	3.2	3.3	3.4	11.7	11.7	7.0	10.8	6.5	6.1	5.7	5.4
10*	8.19	4-Methyl-2-hexanol	855	25.1	27.4	29.9	32.6	2.9	2.9	3.0	3.1	3.0	3.0	3.0	2.9	2.9	28.7	10.2	19.5
11θ	9.35	n-nonane	900	5.6	5.9	6.3	6.7	3.8	4.0	4.2	4.3	2.5	2.5	3.6	2.5	3.5	3.8	4.3	4.9
12θ	9.77	α-Pinene	920	1049.9	1154.7	1269.9	1396.7	526.2	578.6	636.2	699.6	1311.8	1298.7	232.1	1180.9	211.2	443.6	134.6	289.1
13*	9.98	Ethyl tiglate	928	3.5	3.6	3.8	3.9	12.8	13.9	15.0	16.3	6.4	6.4	4.3	6.0	4.1	4.3	4.5	4.8
14θ	10.10	Camphene	933	4.2	4.4	4.6	4.8	55.4	60.8	66.6	73.1	50.1	49.6	26.2	45.3	24.0	21.8	19.8	18.1
15*	10.19	2,4,4-Trimethyl-1-hexene	938	8.7	9.4	10.1	10.9	684.2	752.4	827.4	909.9	4.6	4.6	8.6	4.4	8.0	8.8	4.2	6.5
16θ	10.38	Benzaldehyde	946	3.3	3.5	3.6	3.7	18.0	19.5	21.3	23.2	10.3	10.3	6.3	9.5	5.9	3.3	2.6	2.3
17θ	10.64	Sabinene	956	171.4	188.3	206.9	227.4	26.0	28.3	31.0	33.8	16.3	16.1	4.1	14.9	3.9	6.2	3.4	4.8
18	10.69	β- Pinene	961	3.1	3.2	3.3	3.4	9.8	10.5	11.4	12.3	213.7	211.	35.7	192.	32.7	69.4	22.4	45.9

θ													5		5				
19 *	10.83	α-Methyl styrene	966	2.3	2.3	2.3	2.4	4.9	5.1	5.4	5.8	13.4	13.3	7.8	12.3	7.3	8.3	9.6	11.0
20 θ	11.00	Myrcene	974	54.3	59.6	65.3	71.6	120.6	132.5	145.5	159.8	1239.8	1227.4	238.2	1116.0	216.7	575.6	174.2	374.9
21 *	11.14	3,5-Diphenyl-1-pentene	980	17.4	18.9	20.6	22.4	10.1	10.9	11.7	12.7	8.7	8.7	5.5	8.1	5.2	5.8	6.5	7.3
22 *	11.18	Ethyl hexanoate	982	38.9	42.6	46.6	51.1	10.1	10.9	11.8	12.7	191.6	189.7	96.9	172.7	88.3	105.5	126.2	151.0
23 θ	11.25	α-Phellandrene	983	4.9	5.1	5.4	5.7	4.8	5.0	5.3	5.6	48.1	47.6	7.7	43.5	7.2	15.7	6.3	11.0
24 θ	11.49	α-Terpinene	997	23.1	25.2	27.5	30.0	4.8	5.1	5.3	5.7	5.5	5.5	5.2	5.2	4.9	10.5	5.3	8.9
25 θ	11.57	decane	1000	3.5	3.6	3.7	3.9	3.6	3.7	3.9	4.0	2.3	2.3	2.4	2.3	2.4	2.4	2.4	2.4
26 θ	11.64	*o*-cymene	1004	2.5	2.5	2.5	2.6	46.9	51.4	56.3	61.7	18.0	17.8	4.3	16.4	4.1	7.7	3.7	5.4
27 θ	11.65	*p*-Cymene	1004	2.2	2.3	2.3	2.3	5.2	5.5	5.9	6.2	5.5	5.5	4.1	5.2	3.9	9.3	4.3	6.8
28 θ	11.72	β-Phellandrene	1009	19.7	21.5	23.4	25.5	24.6	26.8	29.3	32.0	40.6	40.2	31.3	36.7	28.7	86.2	28.0	58.2
29 θ	11.73	Sabinene	1009	7.7	8.2	8.8	9.5	5.5	5.8	6.1	6.5	221.3	219.1	4.3	199.4	4.1	7.7	27.4	56.8
30 θ	11.89	(*Z*)-β-ocimene	1019	4.5	4.7	5.0	5.3	7.9	8.5	9.1	9.8	9.1	9.0	5.6	8.4	5.3	5.7	6.1	6.5
31 *	12.01	Indene	1026	4.0	4.2	4.4	4.6	3.6	3.8	3.9	4.1	30.9	30.6	16.6	28.0	15.2	16.7	18.3	20.1
32 θ	12.07	(*E*)-β-ocimene	1030	4.8	5.0	5.3	5.6	9.2	9.9	10.7	11.5	24.9	24.7	7.2	22.7	6.8	28.0	10.0	19.0
33 θ	12.27	ɣ-terpinene	1042	6.4	6.9	7.3	7.8	5.2	5.5	5.8	6.2	34.1	33.8	4.9	30.9	4.6	10.6	4.7	7.7
34 θ	12.79	Terpinolene	1074	2.3	2.3	2.3	2.4	4.8	5.1	5.4	5.7	13.5	13.4	3.3	12.4	3.2	5.3	3.1	4.2
35 *	12.94	Ethyl heptanoate	1082	3.1	3.2	3.3	3.4	7.2	7.7	8.2	8.8	14.7	14.6	8.5	13.5	7.9	8.5	9.1	9.8
36 θ	13.04	n-nonanal	1089	2.2	2.2	2.2	2.2	6.9	7.4	7.9	8.5	13.8	13.7	8.0	12.6	7.5	8.0	8.6	9.2

37 θ	13.23	Undecane	1100	3.4	3.5	3.6	3.7	3.2	3.3	3.4	3.6	2.2	2.2	4.8	2.2	4.6	4.8	5.1	5.4
38 *	13.37	Methyl octanoate	1108	2.2	2.2	2.2	2.2	5.2	5.4	5.8	6.1	16.5	16.3	9.3	15.1	8.7	9.3	10.1	10.8
39 θ	13.46	allo-ocimene	1114	10.6	11.5	12.4	13.4	15.6	16.9	18.4	20.0	8.2	8.2	5.2	7.6	4.9	5.5	6.1	6.9
40 *	13.82	2-Methylindene	1135	2.6	2.7	2.7	2.8	29.9	32.6	35.7	39.0	12.8	12.6	7.5	11.7	7.0	8.0	9.1	10.5
41 *	13.92	1,4-Dihydronaphthalene	1140	3.8	4.0	4.2	4.4	22.4	24.4	26.7	29.1	7.5	7.5	4.9	7.0	4.6	5.1	5.7	6.4
42 *	14.21	1,3,5,8-Undecatetraene	1158	3.1	3.2	3.2	3.3	29.5	32.3	35.3	38.6	4.3	4.2	3.2	4.0	3.1	3.3	3.5	3.8
43 θ	14.27	4-Terpeneol	1161	4.6	4.8	5.1	5.4	46.6	51.0	55.9	61.3	3.3	3.3	3.3	3.2	3.2	4.8	3.0	3.9
44 *	14.51	Ethyl octanoate	1175	6.2	6.6	7.1	7.5	10.2	11.0	10.5	108.6	708.2	701.2	755.7	637.6	3.5	13.2	5.5	9.4
45 θ	14.65	Tridecanal	1183	2.5	2.5	2.5	2.5	2.9	3.0	3.0	3.1	52.2	51.7	27.2	47.2	24.9	27.2	29.7	32.4
46 θ	14.93	Dodecane	1200	3.9	4.0	4.2	4.4	2.7	2.8	2.8	2.9	5.3	5.3	5.3	5.0	5.0	5.3	5.6	6.0
47 *	15.01	Benzothiazole	1205	4.5	4.6	4.7	4.9	4.0	4.1	4.1	4.2	24.5	24.3	13.9	22.4	13.0	13.9	15.0	16.2
48 *	15.30	Caprolactam	1224	4.4	4.5	4.6	4.7	7.7	8.2	8.7	9.2	55.1	54.6	11.4	49.9	10.7	50.5	17.5	34.0
49 θ	16.00	Tridecane	1271	3.8	3.8	3.9	3.9	4.4	4.5	4.6	4.7	4.3	4.3	3.9	4.2	3.8	3.9	3.9	4.0
50 *	16.58	1-propynyl-benzene	1311	4.7	4.8	5.0	5.1	4.3	4.4	4.5	4.6	8.5	8.4	5.9	8.0	5.7	5.9	6.2	6.5
51 *	17.10	Ethyl-(4*E*)-decenoate	1348	4.0	4.1	4.1	4.2	3.9	4.0	4.0	4.1	16.0	15.9	9.7	14.8	9.1	9.7	10.3	11.0
52 θ	17.18	α-copaene	1354	3.5	3.5	3.6	3.6	3.9	4.0	4.0	4.1	11.1	11.0	4.5	10.3	4.4	5.4	4.0	4.7
53 *	17.71	2-epi-α-Funebrene	1392	3.4	3.4	3.4	3.4	5.0	5.2	5.3	5.5	5.0	5.0	5.1	4.9	4.9	5.1	3.9	4.5
54 θ	17.79	(*E*)-β-Caryophyllene	1398	3.5	3.5	3.5	3.6	3.7	3.7	3.7	3.8	10.3	10.2	4.9	9.6	4.7	6.9	4.4	5.7
55	17.82	Tetradecane	140	3.4	3.4	3.4	3.5	7.2	7.6	8.0	8.4	5.2	5.1	5.2	5.0	5.0	5.2	5.3	5.5

θ			0															
56 *	17.99	α-Guaiene	1413	5.2	5.4	5.6	5.8	5.0	5.2	5.4	5.6	4.1	4.1	4.1	4.1	4.1	6.0	4.2	5.1
57 θ	18.23	α-Humulene	1431	4.5	4.6	4.8	4.9	3.9	4.0	4.0	4.1	6.6	6.6	5.0	6.3	4.9	5.0	5.2	5.3
58 *	18.33	2,6-Di-tert-butylquinone	1439	6.8	7.1	7.5	7.9	3.6	3.6	3.6	3.6	17.8	17.7	10.6	16.4	9.9	10.6	11.3	12.1
59 θ	18.57	β-Cubebene	1458	4.2	4.3	4.3	4.4	8.2	8.7	9.2	9.8	7.6	7.6	5.5	7.2	5.3	5.5	5.7	5.9
60 θ	18.77	α-Farnesene	1473	9.9	10.5	11.2	12.0	3.9	3.9	4.0	4.1	13.4	13.3	8.4	12.4	7.9	8.4	8.9	9.4
61 *	18.85	δ-guaiene	1480	3.4	3.4	3.4	3.4	7.9	8.4	8.9	9.4	11.0	11.0	7.2	10.3	6.9	7.2	7.6	8.0
62 *	18.86	β-Selinene	1480	3.5	3.5	3.5	3.6	4.0	4.0	4.1	4.2	4.1	4.1	4.9	4.0	4.7	8.3	4.9	6.6
63 *	19.05	δ-Cadinene	1495	3.5	3.5	3.5	3.5	6.1	6.4	6.7	7.0	4.4	4.4	4.4	4.3	4.3	7.2	4.6	6.0
64 θ	19.12	Pentadecane	1500	5.3	5.5	5.7	5.9	7.5	7.9	8.3	8.8	4.6	4.6	4.6	4.5	4.5	4.4	4.3	4.2
65 *	19.77	Ethyl dodecanoate	1554	5.2	5.4	5.6	5.8	5.0	5.1	5.3	5.5	27.5	27.3	15.4	25.1	14.3	13.2	12.3	11.4
66 θ	19.82	Hexadecane	1558	3.8	3.9	3.9	4.0	9.0	9.6	10.2	10.9	26.6	26.3	15.0	24.2	13.9	12.9	11.9	11.1
67 *	20.91	1-Pentadecene	1650	5.5	5.8	6.0	6.3	8.9	9.5	10.1	10.8	8.9	8.8	6.1	8.3	5.9	5.6	5.4	5.2
68 θ	20.97	Heptadecane	1656	4.2	4.3	4.4	4.5	14.0	15.1	16.3	17.6	30.4	30.1	16.9	27.7	15.7	14.4	13.3	12.3
69 *	21.99	Ethyl tetradecanoate	1763	4.9	5.0	5.2	5.4	17.3	18.6	20.2	21.9	16.0	15.9	9.7	14.7	9.1	8.5	8.0	7.6
70 θ	22.30	Octadecane	1800	7.0	7.3	7.7	8.1	27.5	29.9	32.5	35.5	3.6	3.6	3.6	3.5	3.5	3.5	3.5	3.5
71 θ	23.08	Nonadecane	1873	4.4	4.5	4.6	4.8	5.0	5.1	5.3	5.5	7.9	7.8	5.6	7.4	5.4	5.2	5.0	4.9
72 *	24.01	1-Octadecene	1969	27.0	29.4	32.0	34.8	12.5	13.4	14.4	15.5	64.7	64.1	34.0	58.5	31.2	28.5	25.9	23.7
73 θ	24.87	Hexadecanol	2062	13.7	14.7	15.9	17.1	4.5	4.7	4.8	4.9	298.9	296.0	151.1	269.4	137.7	124.3	112.2	101.3

74*	25.23	Heneicosane	2100	4.0	4.0	4.1	4.2	4.0	4.0	4.1	4.2	3.6	3.6	4.5	3.5	4.4	4.3	4.2	4.1
75*	25.66	2-methyl-eicosane	2147	5.4	5.6	5.9	6.1	3.9	4.0	4.1	4.1	79.2	78.4	41.3	71.6	37.8	34.4	31.3	28.5
76*	25.86	1-Docosene	2168	4.3	4.4	4.5	4.6	12.4	13.3	14.3	15.4	260.3	257.8	4.2	234.6	4.1	4.1	10.3	4.1
77θ	25.91	Tetracosane	2174	16.8	18.2	19.6	21.3	3.4	3.4	3.5	3.5	120.0	118.8	3.7	108.3	3.7	3.4	6.0	3.4
78*	26.10	(*E*)-5-Eicosene	2194	3.5	3.6	3.6	3.6	3.4	3.4	3.4	3.4	171.4	169.7	3.4	154.6	3.4	3.4	3.5	3.5
79θ	26.15	Docosane	2200	3.7	3.7	3.8	3.8	3.4	3.4	3.4	3.5	3.6	3.6	3.6	3.6	3.6	3.5	3.5	3.5
80θ	27.00	Tricosane	2300	6.3	6.6	6.9	7.2	3.5	3.5	3.5	3.5	427.2	423.0	3.7	384.8	3.6	3.6	3.6	3.6
81*	27.57	1-Eicosene	2368	34.2	37.3	40.7	44.4	3.8	3.9	3.9	4.0	365.1	361.5	184.2	328.9	167.8	151.4	136.6	123.2
82θ	27.60	Tetracosane	2373	14.4	15.5	16.7	18.0	3.6	3.6	3.6	3.7	389.0	385.1	196.2	350.4	178.6	161.1	145.3	131.1

RT, Retention time (min), the reliability of identification proposal is indicated by the following: θ mass spectrum and Kovat index agreed with standards, * mass spectrum and Kovat index agreed with literature. RI retention index

6.3.2 Category 2: VOCs composition of fruit fly larvae infested apple mango

6.3.2.1 Chemical composition of non-infested apple mango (control)

The retention time (min), chemical identity and concentration (ng/h) of the VOCs headspace analysis of non-infested apple mango fruit are highlighted in Figure 6.1 and Table 6.2 while Table 6.7 shows the structures and major fragments for the VOCs observed. Overly a total of 58 VOCs was detected for the entire 10 days of trapping. The number detected was distributed as follows: 51 VOCs in day 1- 10 except for day 4 which recorded a total of 54 VOCs.

Cumulatively for the entire 10 days, α- pinene was the most abundant compound 13318.8 ng/h followed closely by myrcene and ethyl octanoate with 5433.8 and 3522.4 ng/h respectively while the least was α-methylstyrene, 22.1 ng/h. In terms of frequencies, of the total 63 VOCs identified, esters accounted for 33.3 % followed by monoterpene 25.4 %, sesquiterpenes 20.6 %, while the remaining 20.6 % comprised of aldehydes, ketones, non-terpenoid hydrocarbons and alcohols.

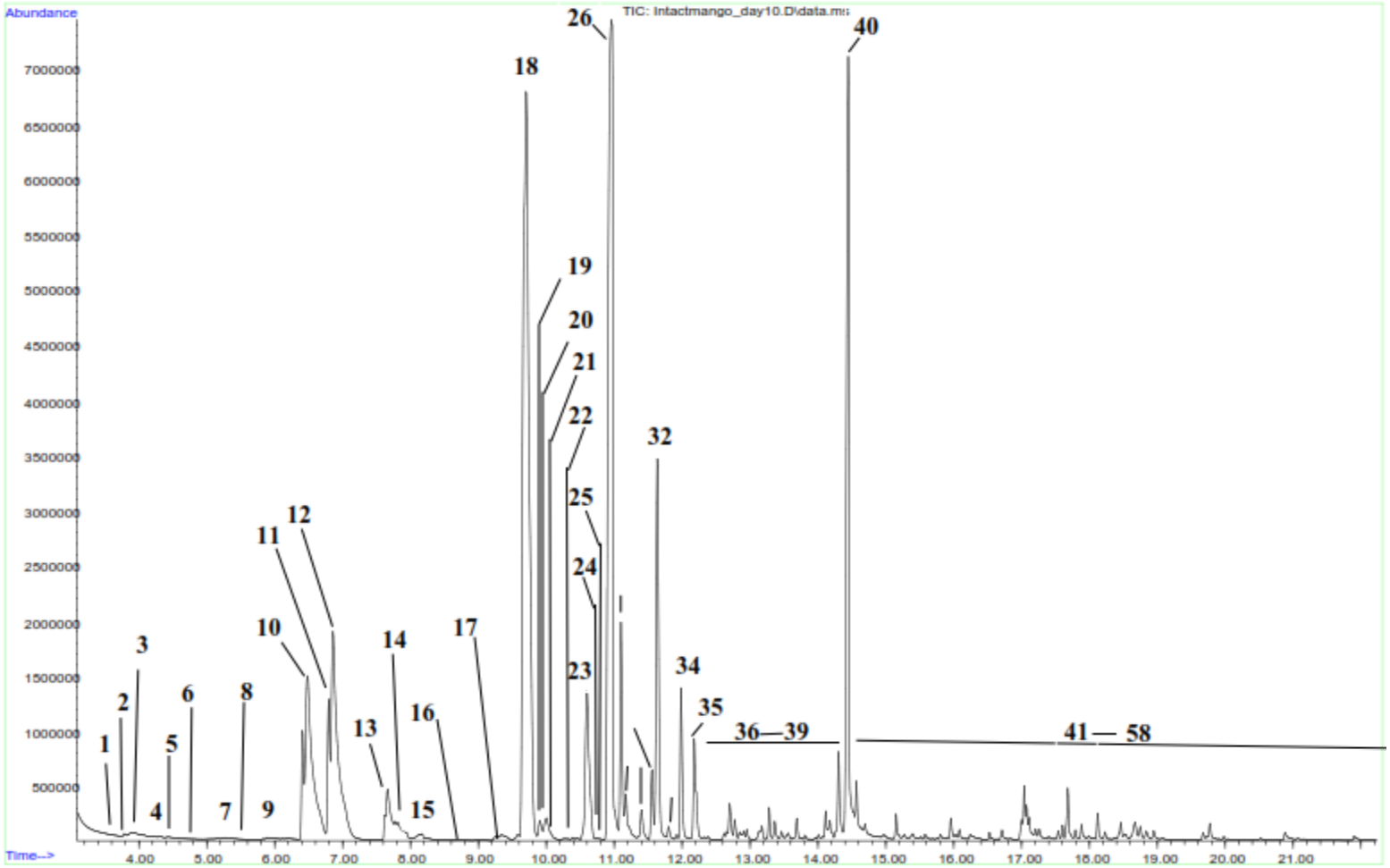


Figure 6.1: Representative total ion chromatogram showing VOCs detected in non-infested apple mango. Peaks 1-63 indicate the VOCs shown in Table 6.2

Table 6.2: Concentration of VOCs (ng/h) in non-infested apple mango

No	RT (min)	Compound name	RI	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1*	3.78	Acetoin	603					5.9	6.2	5.8	5.5	2.6	9.2
2*	4.00	Ethyl propanoate	687	3.7	3.4	3.2	4.4	7.4	7.9	7.3	6.8	2.7	12.1
3*	4.24	Methyl butanoate	708	3.5	3.3	3.0	3.6	4.3	4.2	4.0	3.8	2.9	5.6
4*	4.32	(*Z*)-methyl2-butenoate	710	3.9	3.5	3.3	4.5	6.7	3.5	3.3	3.2	9.0	4.4
5$^{\theta}$	5.35	Toluene	748	8.7	7.4	6.4	2.3	3.1	2.5	3.4	3.5	3.3	4.0
6*	5.89	2,3-Butanediol	768					4.3	3.8	3.6	3.5	3.6	4.9
7*	6.13	(*E,E*)-1,3,5-Heptatriene	776	5.7	5.0	4.5	6.1	8.5	3.7	3.4	3.2	4.4	7.4
8*	6.50	Ethyl butanoate	790	17.9	14.8	12.3	77.8	274.9	312.9	281.8	253.8	8.3	541.5
9*	7.90	Ethyl-2-methyl butanoate	844	17.4	14.4	12.0	6.1	10.6	10.8	9.9	9.1	4.2	17.1
10*	7.99	Ethyl-3-methyl butanoate	847	7.1	6.1	5.3	3.4	53.6	60.2	54.4	49.2	4.4	102.9
11*	8.15	5-methyl-2-hexanol	853	7.7	6.6	5.7	5.9						
12$^{\theta}$	8.18	Propanoic acid	854					14.0	14.1	12.9	11.9	5.1	22.9
13*	8.19	4-Methyl-2-hexanol	855	9.5	8.0	6.9							
14*	9.01	Propyl butanoate	887					2.8	2.9	2.9	2.8	14.4	3.5
15*	9.03	Malic Acid	887					441.1	14.1	12.9	11.8	859.4	22.8
16$^{\theta}$	9.77	α-Pinene	919	2145.2	1716.6	1373.8	1754.8	1034.4	1189.9	1071.1	964.2	4.6	2064.2
17*	9.98	Ethyl tiglate	928	8.1	6.9	6.0	18.6	32.3	19.2	17.5	16.0	32.9	31.7
18$^{\theta}$	10.10	Camphene	934	78.9	63.6	51.3	72.3	31.5	35.2	31.9	29.0	3.5	59.5
19*	10.38	Benzaldehyde	946	5.4	4.7	4.2	9.6						
20*	10.40	Ethyl 2,3-epoxybutyrate	947				5.1	73.2	4.2	4.0	3.8	140.7	5.7

21[θ]	10.60	β-Pinene	956	348.5	279.3	223.8	548.0	144.3	162.6	146.6	132.1	7.9	280.7
22*	10.83	α-Methylstyrene	967	7.1	6.2	5.4	3.4						
23[θ]	10.94	Myrcene	972	876.5	701.7	561.8	3279.2	14.7					
24*	11.11	Ethyl hexanoate	979	33.3	27.1	22.1	150.6	112.8	122.0	110.0	99.2	15.4	210.2
25[θ]	11.17	α-Phellandrene	982	35.1	28.5	23.2	152.7	10.7					
26[θ]	11.49	α-Terpinene	996	23.5	19.3	15.9	10.7	72.5	44.5	40.3	36.5	69.4	75.6
27[θ]	11.64	Sabinene	1004	170.1	136.5	109.7	14.7	227.9	259.0	233.3	210.2	7.8	448.0
28[θ]	11.65	*p*-Cymene	1005	16.2	13.4	11.2	134.5	38.0	43.2	39.1	35.4	2.8	73.3
29[θ]	11.72	β-Phellandrene	1009	3.7	3.4	3.1	827.4	2.5					
30[θ]	11.89	(*Z*)-β-ocimene	1019	17.5	14.5	12.0	189.2	14.3	12.8	11.7	10.8	8.1	20.6
31[θ]	12.07	(*E*)-β-ocimene	1030	16.9	14.0	11.6	34.6	77.0	83.9	75.7	68.4	10.1	144.0
32[θ]	12.27	ɣ-terpinene	1042	21.9	18.0	14.8	177.9	66.6	75.3	68.0	61.4	4.1	129.0
33[θ]	12.79	Terpinolene	1074	7.0	6.0	5.3	50.0	23.2	25.9	23.5	21.4	3.2	43.3
34*	12.94	Ethyl heptanoate	1083	4.6	4.1	3.7	6.3	6.2	6.8	6.3	5.9	2.5	10.1
35*	13.15	Phenylethyl alcohol	1095					7.9	8.6	8.0	7.4	2.6	13.3
36*	13.30	Methyl octanoate	1104	3.1	2.9	2.8	15.4	18.0	18.4	16.8	15.3	5.7	30.3
37[θ]	13.46	allo-ocimene	1114	11.0	9.3	7.8	26.3	13.0	14.1	12.9	11.9	3.0	22.9
38[θ]	14.27	4-Terpeneol	1161	4.2	3.8	3.5	31.1	17.7	18.6	17.0	15.5	4.6	30.7
39*	14.31	(*Z*)-ethyl 4-octenoate	1164	8.2	7.0	6.0	5.2	76.4	57.7	52.1	47.2	54.3	98.5
40*	14.51	Ethyl octanoate	1175	132.3	106.3	85.5	349.9	465.7	535.2	481.9	434.0	3.9	927.6
41*	15.17	Ethyl (*E*)-2-octenoate	1216	7.6	6.8	6.1	13.9	16.9	18.7	17.2	15.8	3.8	30.0
42*	17.02	Ethyl(*Z*)4-decenoate	1342	4.4	4.2	4.1	7.6	173.7	15.0	13.8	12.8	323.8	23.5
43[θ]	17.18	α-copaene	1354	31.9	26.2	21.6	12.2	17.2	19.0	17.4	16.0	3.9	30.4

44 θ	17.61	α-Cedrene	1385	4.8	4.5	4.3	6.3	9.6	10.1	9.4	8.8	4.2	15.1
45 θ	17.69	β-Longipinene	1391	5.4	5.0	4.7	7.5	14.5	14.3	13.2	12.3	6.6	22.4
46*	17.71	2-epi-α-Funebrene	1392	11.4	9.8	8.5	7.0	8.3	8.8	8.3	7.8	3.8	12.9
47 θ	17.79	(*E*)-Caryophyllene	1398	4.3	4.1	4.0	21.1	30.8	34.6	31.4	28.6	4.1	57.5
48 θ	17.99	α-Guaiene	1413	5.2	4.8	4.6	9.5	13.8	14.1	13.0	12.1	5.6	22.0
49 θ	18.23	α-Humulene	1432	8.8	7.7	6.9	11.1	16.6	18.3	16.8	15.5	3.9	29.3
50 θ	18.47	Germacrene D	1450	4.6	4.3	4.1	7.0	13.8	15.2	14.1	13.0	3.6	24.0
51 θ	18.57	β-Cubebene	1458	3.9	3.8	3.7	9.7	19.7	22.1	20.2	18.5	3.6	35.8
52*	18.76	Butyl 6,9,12,15-octadecatetraenoate	1472	4.0	3.9	3.8	12.2	35.7	13.2	12.2	11.3	51.0	20.4
53 θ	18.77	α-Farnesene	1473	122.6	98.7	79.7	8.3	8.1	7.9	7.4	7.0	4.9	11.2
54 θ	18.85	δ-guaiene	1479	7.2	6.4	5.8	19.0	10.7	11.6	10.8	10.0	3.8	17.6
55 θ	18.86	β-Selinene	1480	4.3	4.1	4.0	10.1	10.1	10.8	10.0	9.4	3.9	16.2
56 θ	19.05	δ-Cadinene	1495	4.7	4.4	4.2	14.3	5.2	5.1	4.9	4.7	4.1	6.3
57*	19.77	Ethyl dodecanoate	1554	5.2	4.8	4.5	8.0	7.8	8.3	7.8	7.3	3.7	11.8
58*	21.99	Ethyl tetradecanoate	1763	4.2	4.0	3.9	4.1	8.9	9.8	9.1	8.6	3.8	14.5

R_t Retention time (min), the reliability of identification proposal is indicated by the following: θ mass spectrum and Kovat index agreed with standards, * mass spectrum and Kovat index agreed with literature. RI retention index

6.3.2.2 Chemical composition of apple mango VOCs infested with *Bactrocera dorsalis* larvae

The retention time (min), chemical identity and concentration (ng/h) of the VOCs headspace analysis of non-infested apple mango fruit are highlighted in Figure 6.2 and Table 6.3 while Table 6.7 shows the structures and major fragments for the VOCs observed. Sixty-two VOCs were detected in the first 3 days of trapping with the number dropping to 40 on day 4 to day 10. One monoterpene and an organic acid, 3 alcohols, 3 sesquiterpenes and 14 esters were among the compounds not detected from day 4 onwards.

Cumulatively for the entire 10 days, α-pinene was the most abundant compound 18995.3 ng/h followed by β-myrcene 3630 ng/h, while the least was isobutyl acetate 10.6 ng/hr. Out of the total 62 VOCs identified in the first 3 days, 3.2, 3.2, 8.1, 22.5, 29.0, and 32.3 % represented hydrocarbons (straight chains, branched and cyclic), organic acids, alcohols, sesquiterpenes monoterpenes, and esters respectively.

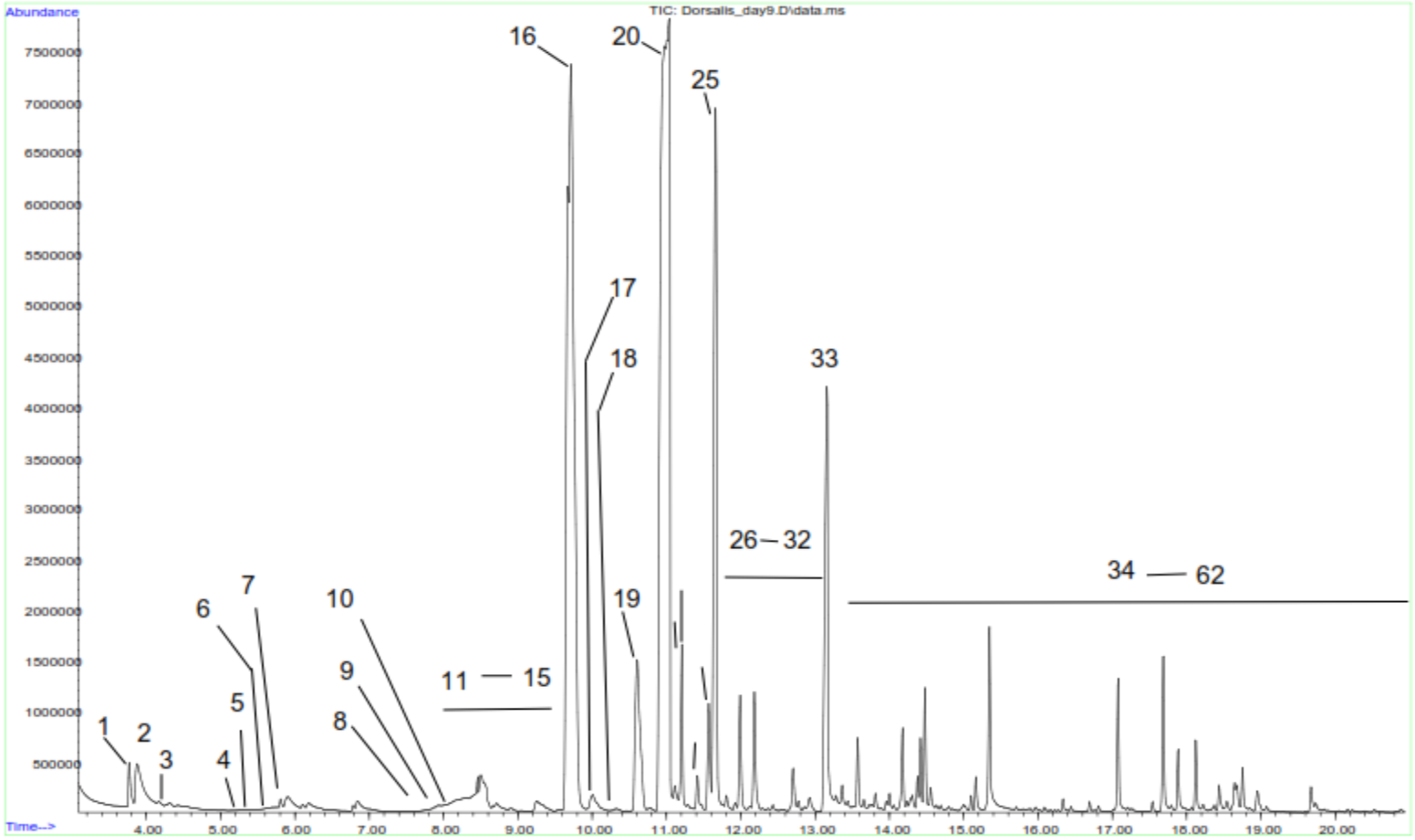


Figure 6.2: Representative total ion chromatogram showing VOCs detected in apple mango infested with *B. **dorsalis*** **larvae**. Peaks 1-62 indicate the VOCs shown in Table 6.3

Table 6.3: Concentration of VOCs (ng/hr) in mango infested with *Bactrocera dorsalis* larvae

No	RT (min)	Compound name	RI	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1*	3.85	Acetoin	640	13.0	9.0	5.9	24.9	47.5	177.0	47.5	70.1	92.8	86.3
2*	4.36	3-methyl-1-butanol	712	71.3	8.4	4.8							
3 θ	4.44	1-Heptanol	715	208.0	101.8	2.3							
4*	5.17	2-methyl-ethyl propanoate	741	4.8	5.4	2.6							
5*	5.27	(*E*) methyl-2-butenoate	745	6.9	7.9	2.7							
6*	5.64	Isobutyl acetate	759	3.1	3.3	4.3							
7*	5.83	2,3-Butanediol	765	16.4	19.3	2.9	47.5	92.8	206.4	92.8	138.1	183.4	25.2
8*	6.43	Ethyl butanoate	787	243.6	6.7	2.9							
9*	7.66	(*Z*)-ethyl-2-butenoate	834	141.4	17.3	32.3							
10*	7.72	3-methyl-butanoic acid	837	7.0	24.5	10.3	57.7	113.2	269.6	113.3	168.7	224.3	47.5
11*	7.91	(*E*)-3-methyl-1,3-Pentadiene	844	18.0	17.9	2.6							
12*	8.08	2-methylbutanoic acid	851	6.0	8.5	4.8							
13*	8.12	5-ethyl-2-heptanol	852	6.9	6.1	9.9							
14*	8.49	Isopentyl acetate	867	49.4	65.7	6.4	34.1	66.0	143.0	66.0	97.8	129.7	15.4
15*	8.72	Styrene	875	10.4	28.8	6.3	10.1	17.9	43.2	18.0	25.7	33.6	11.8
16θ	9.69	α-Pinene	915	1713.6	1536.2	23.1	823.1	1644.0	4572.1	1644.0	2464.9	3285.8	1288.5
17*	9.91	Ethyl tiglate	925	19.1	6.8	2.6							
18θ	10.00	Camphene	929	92.0	57.7	14.9	18.7	35.3	95.1	35.3	51.8	68.4	28.9
19θ	10.60	β Pinene	956	404.0	276.9	11.1	95.4	188.7	526.9	188.7	281.9	375.1	154.0

20 θ	10.97	β-Myrcene	973	1644.6	1973.1	12.4							
21 *	11.11	Ethyl hexanoate	979	100.7	35.8	69.4	27.1	52.0	105.5	52.0	76.8	101.8	6.0
22 θ	11.18	α-Phellandrene	982	105.6	80.4	6.3	72.4	142.7	376.9	142.7	212.9	283.2	96.0
23 *	11.25	(*Z*)-3-Hexenyl iso-butyrate	985	21.1	24.9	47.5							
24 θ	11.41	δ-2-Carene	993	44.6	53.0	5.5	28.6	55.0	136.9	55.1	81.4	107.9	31.3
25 θ	11.65	β-Phellandrene	1004	101.4	64.5	124.6	109.5	214.5	541.2	214.6	319.5	424.7	121.0
26 θ	11.81	(*Z*)-β-Ocimene	1014	17.2	20.2	2.7	18.3	34.3	83.0	34.3	50.3	66.4	18.8
27 *	11.93	ethyl-2-Hexenoate	1022	7.2	8.1	14.1	8.0	13.8	48.2	13.8	19.6	25.4	25.0
28 θ	11.99	(*E*)-β-Ocimene	1025	34.0	24.7	47.3	56.1	110.0	272.5	110.0	163.8	217.7	56.9
29 θ	12.18	ɣ-Terpinene	1037	50.7	32.3	62.3	54.7	107.3	270.6	107.3	159.8	212.3	60.5
30 *	12.34	*p*-Menth-8-en-1-ol	1046	5.8	6.5	10.7	24.3	46.4	136.9	46.4	68.5	90.6	48.5
31 θ	12.71	Terpinolene	1069	16.7	19.6	37.0	3.1	4.0	28.9	4.0	4.9	5.8	25.3
32 *	12.86	Ethyl heptanoate	1078	5.1	5.6	9.0	4.9	7.6	17.5	7.6	10.2	12.9	6.7
33 *	13.14	Phenyl ethyl alcohol	1095	12.8	5.4	8.6	166.5	330.8	915.0	330.8	495.1	659.4	257.9
34 *	13.29	Methyl octanoate	1104	10.3	4.6	6.9							
35 θ	13.38	allo-Ocimene	1109	9.7	9.1	16.0	15.8	29.4	75.0	29.5	43.1	56.7	20.5
36	13.96	Pinocarvone	114	5.6	6.1	10.6	6.8	11.3	52.3	11.4	15.9	20.5	34.0

*			3										
37	14.00	(+)-Borneol	114	6.9	8.1	4.7							
*			5										
38	14.06	Ethyl benzoate	114	6.0	5.0	13.9							
*			9										
39	14.18	Terpinen-4-ol	115	21.8	7.0	11.8	27.8	53.4	138.7	53.4	79.0	104.6	36.3
θ			6										
40	14.31	(Z)-Ethyl-4-Octenoate	116	18.5	5.4	8.6	10.1	18.0	121.4	18.1	25.9	33.9	89.7
*			4										
41	14.43	Ethyl octanoate	117	290.3	69.9	137.7	20.2	38.1	87.6	38.2	56.1	74.1	15.7
*			1										
42	14.70	Verbenone	118	19.8	6.7	11.2	10.2	18.1	38.8	18.2	26.1	34.1	7.0
*			6										
43	15.16	Ethyl (2*E*) octenoate	121	14.3	16.5	29.6	6.8	10.2	21.3	10.3	13.7	17.2	7.5
*			5										
44	15.26	tetradecyl-hexanoate	122	5.7	6.1	8.9	10.9	18.4	47.8	18.5	26.0	33.5	17.6
*			2										
45	16.71	α-Cubebene	132	4.8	5.1	6.8	4.5	5.6	11.2	5.6	6.7	7.8	6.7
θ			0										
46	16.82	Myrtenoic acid, ethyl	132	4.6	5.8	7.0							
θ		ester	8										
47	17.02	Ethyl-(4*E*)-decenoate	134	11.5	3.9	4.4							
*			2										
48	17.09	α-Copaene	134	30.2	9.0	14.6	16.5	29.7	107.0	29.7	42.8	56.0	54.4
θ			7										
49	17.22	Ethyl decanoate	135	9.1	10.2	17.0							
*			7										
50	17.55	α-Gurjunene	138	6.2	3.5	3.6	4.3	5.3	11.1	5.4	6.3	7.3	7.2
θ			1										
51	17.61	α-2-epi-funebrene	138	4.7	5.0	6.6							
*			5										
52	17.69	(*E*)-Caryophyllene	139	28.3	7.2	11.0	21.8	40.3	136.2	40.4	58.8	77.3	62.3
θ			1										

53θ	17.80	(*Z*)-muurola-4(14),5-diene	1399	5.8	6.3	9.3						
54θ	17.89	α-Guaiene	1405	19.0	5.3	7.2	10.4	17.3	55.4	17.4	24.3	31.3	27.5
55θ	18.07	Dauca-5,8-diene	1419	6.5	7.1	10.8	8.6	13.9	26.7	14.0	19.2	24.5	5.6
56θ	18.13	α-Humulene	1424	14.9	5.0	6.6	12.3	21.3	65.9	21.3	30.2	39.2	30.0
57θ	18.55	β-Selinene	1456	8.8	9.9	16.5	5.8	8.3	20.2	8.4	10.8	13.3	10.3
58θ	18.65	α-Selinene	1464	11.0	12.5	21.6	7.1	10.7	30.3	10.8	14.4	18.2	15.6
59θ	18.76	α-bulnesene	1472	25.9	6.1	8.8	8.6	13.9	43.3	13.9	19.2	24.5	22.2
60θ	18.95	δ-Cadinene	1487	10.5	4.2	5.0						
61θ	19.08	Ylangene	1497	6.7	7.4	11.4	7.7	12.0	35.0	12.1	16.3	20.7	17.6
62*	19.68	Ethyl dodecanoate	1546	30.5	35.9	68.4	4.8	6.3	13.7	6.4	7.8	9.3	7.8

R_t Retention time (min), the reliability of identification proposal is indicated by the following: $^{\theta}$ mass spectrum and Kovat index agreed with standards, * mass spectrum and Kovat index agreed with literature. RI retention index

6.3.2.3 Chemical composition of apple mango VOCs infested with *Ceratitis cosyra* larvae

The retention time (min), chemical identity and concentration (ng/h) of the VOCs headspace analysis of apple mango fruit infested with *C. cosyra* are indicated in Figure 6.3 and Table 6.4 while Table 6.7 shows the structures and major fragments for the VOCs observed. A total of leading 66 VOCs was detected in day 3 of trapping. Day 1 and 2 each recorded 64 VOCs while the least number of compounds was detected starting day 4 up to day 10 with 29 different compounds recorded daily for this entire period.

Cumulatively for the entire 10 days, myrcene was the most abundant compound 22665.9 ng/h followed by β-pinene and α-pinene with 15632.5 and 15463.1 ng/h respectively, while the least was 3-methylbutanol 12.4 ng/h. In terms of frequencies, from the 69 VOCs identified, sesquiterpenes represented 36.2 % followed by monoterpene 24.6 % and esters 11.6 %, the remaining 27.6 % comprised of aldehydes, ketones, non-terpenoid hydrocarbons, alcohols and organic acids.

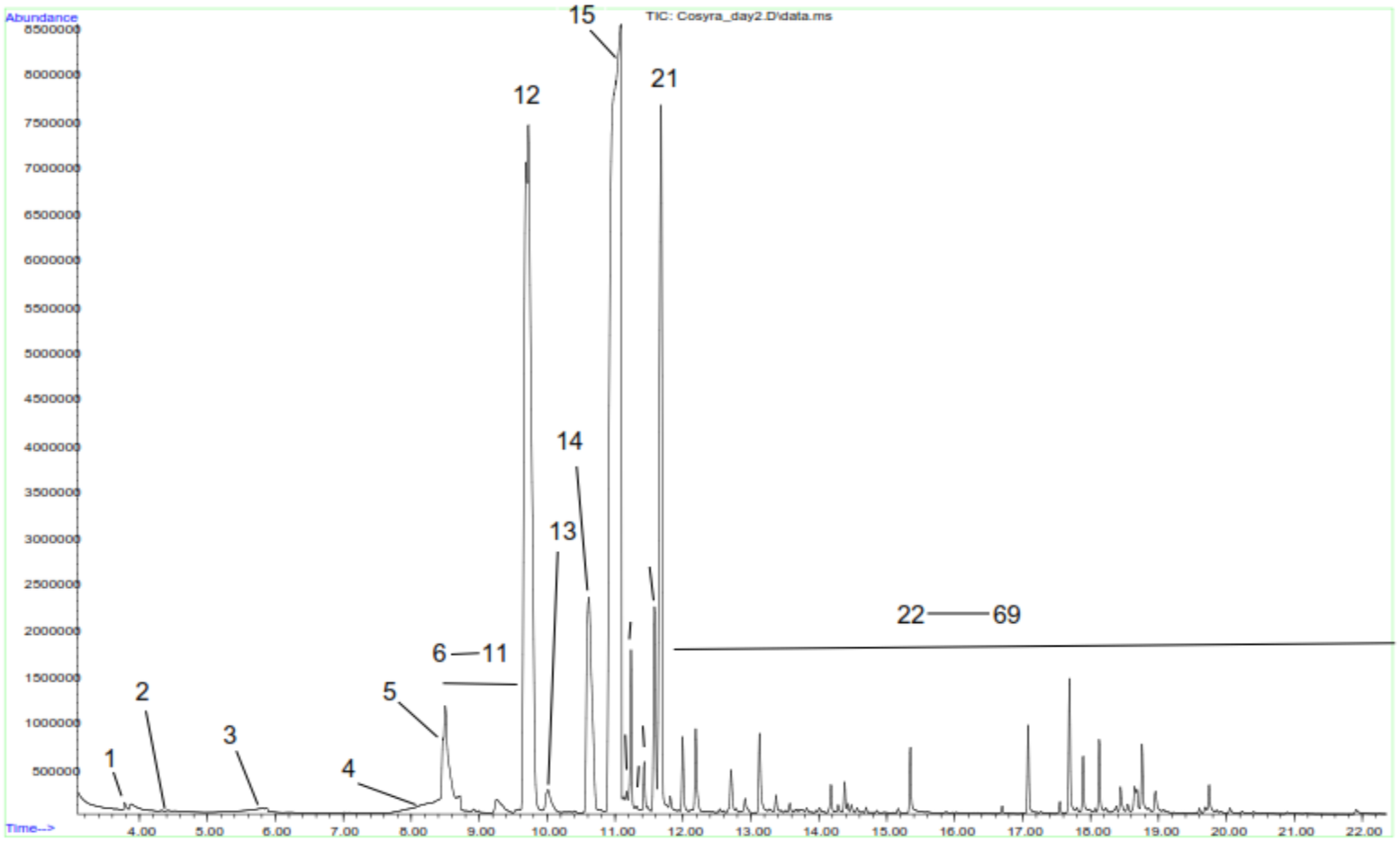


Figure 6.3: Representative total ion chromatogram showing VOCs detected in apple mango infested with *C. cosyra* larvae**.** Peaks 1-62 indicate the VOCs shown in Table 6.4

Table 6.4: Concentration of VOCs (ng/h) in mango infested with *Ceratitis cosyra* larvae

No	RT (min)	Compound name	RI	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1*	3.78	Acetoin	603	10.8	19.3	45.8	24.0	13.1	7.7	4.9	3.6	2.9	5.4
2*	4.33	3-methylbutanol	711	3.1	4.1	5.2							
3*	5.59	2-methyl-propanoic acid	757	33.9	65.6	801.2							
4*	5.92	2,3-Butanediol	769			14.2	26.2	50.2	98.2	194.2	386.2	770.1	1538.0
5*	8.04	2-methyl-butanoic acid	849				7.6	5.8	13.1	9.4	23.9	16.6	45.6
6*	8.05	3-methyl- butanoic acid	850			10.0	9.0	5.8	15.8	9.3	29.4	16.5	56.7
7*	8.15	5-methyl-2-hexanol	853				5.6	10.0	9.0	17.7	15.7	33.3	29.2
8*	8.18	3-methyl-pentanoic acid	854	37.8	73.3	7.1	5.7	10.3	9.2	18.4	16.3	34.5	30.3
9*	8.50	Isopentyl acetate	867	401.7	801.2	499.8	10.6	11.3	19.0	20.4	35.9	38.5	69.5
10*	8.69	Styrene	874	17.5	32.8	3.1	3.7	5.6	5.1	9.0	8.1	15.7	13.9
11*	8.87	4-methylene-5-hexenal	881				5.6	5.7	9.1	16.1	29.9	57.7	113.1
12θ	9.69	α- Pinene	915	2643.3	5284.4	2981.2	196.1	211.3	315.8	472.6	707.8	1060.6	1589.9
13θ	10.02	Camphene	930	81.4	160.6	97.3	9.4	9.8	17.3	32.4	62.7	123.1	244.0
14*	10.82	Pentanoic acid	966	12.7	23.2	10.5							
15θ	11.08	Myrcene	978	4317.3	8632.3	4894.3	319.2	354.2	459.8	597.0	775.5	1007.4	1309.0
17θ	11.24	α- Phellandrene	985	150.6	298.9	32.4	5.7	9.1	16.1	29.9	57.7	113.1	21.2
18θ	11.25	α-Thujene	986	9.8	17.4	175.7							
19θ	11.32	β-Phellandrene	988	18.2	34.2	2.2							
20θ	11.43	δ-2-Carene	993	52.2	102.2	61.1							

21θ	11.68	β-Pinene	1006	1037.0	2071.8	770.2	44.7	34.3	98.6	291.4	869.7	2604.8	7809.9
22*	11.93	Benzeneacetaldehyde	1022	6.7	11.2	5.5							
23θ	12.00	(*E*)-β-Ocimene	1026	80.9	159.6	96.7	3.4	4.1	7.9	19.4	53.8	156.9	466.2
24*	12.12	γ-Hexalactone	1033	7.9	13.6	9.8	3.7	4.6	9.2	23.2	65.2	191.1	569.0
25θ	12.19	γ-Terpinene	1037	96.7	191.2	118.2							
26θ	12.34	Acetophenone	1046	5.5	8.7	5.8							
27*	12.79	Ethyl sorbate	1073	8.5	14.7	9.6							
28θ	13.13	Phenyl ethyl alcohol	1094	99.1	196.0	128.3	8.2	9.9	8632.3	4894.3	319.2	9.1	31.2
29θ	13.37	allo-Ocimene	1108	22.8	43.4	27.6							
30*	13.82	(*E,Z*)-3,6-Nonadien-1-ol	1135	9.6	16.9	11.7							
31*	13.89	4-Isopropylcyclohexanone	1139	4.1	6.0	3.3							
32*	13.97	Pinocarvone	1143	5.5	8.8	6.2							
33θ	14.01	Borneol	1146	9.5	16.9	11.7							
34*	14.07	Ethyl benzoate	1149	5.6	9.1	6.5							
35θ	14.18	Terpinen-4-ol	1156	33.0	63.8	41.2	2.9	3.7	4.1	6.0	3.3	5.6	9.1
36θ	14.29	p-Cymen-8-ol	1162	13.1	24.1	16.3							
37	14.38	α-Terpineol	116	30.0	57.8	37.5	2.8	2.8	57.8	2.8	3.7	24.1	20.0

θ			8										
38 *	14.42	Ethyl octanoate	1170	11.1	20.0	13.6							
39 θ	14.48	Myrtenol	1174	12.7	23.2	10.1	9.5	16.9	11.7	63.8	41.2	6.2	5.0
40 θ	14.70	Verbenone	1186	8.4	14.6	10.1							
41 *	14.86	β-Cyclocitral	1196	6.8	11.3	6.8							
42 *	15.17	Ethyl benzeneacetate	1216	9.7	16.0	11.6							
43 *	15.35	2-Phenethyl acetate	1228	75.5	147.6	98.4							
44 *	15.97	3,4-Diethylphenol	1270	5.2	7.0	5.0							
45 θ	16.70	α-Cubebene	1319	10.3	17.2	12.3							
46 θ	17.09	α-Copaene	1347	87.5	171.7	110.0	4.8	4.8	9.7	16.0	11.6	5.0	5.2
47 θ	17.27	Germacrene A	1361	7.7	12.1	14.9							
48 θ	17.55	α-Gurjunene	1380	14.2	25.1	17.1	7.7	4.0	4.7	6.0	8.7	14.0	5.0
49 θ	17.69	(*E*)-Caryophyllene	1391	133.0	262.7	167.6	4.4	4.6	5.8	8.2	13.0	22.6	5.7
50 θ	17.80	β-Copaene	1399	12.7	22.0	14.9							
51 θ	17.89	α-Guaiene	1406	61.9	120.4	77.6	3.8	12.7	22.0	14.9	31.7	60.0	4.4
52 θ	18.07	Dauca-5,8-diene	1420	9.1	14.8	7.0							
53 θ	18.13	α-Humulene	1424	74.4	145.4	93.2	3.8	9.7	16.0	11.4	4.0	4.8	4.7

54 θ	18.23	β-Neoclovene	1431	13.8	24.3	6.5							
55 *	18.38	Sibirene	1443	17.5	31.5	5.2							
56 θ	18.44	ɣ-Gurjunene	1448	36.7	70.1	46.0	6.9	10.5	7.1	14.9	20.9	15.1	3.9
57 θ	18.55	β-Selinene	1456	16.8	30.1	19.7	12.5	21.6	15.0	17.1	12.1	4.7	4.0
58 θ	18.65	α-Selinene	1464	35.1	66.8	41.7	39.9	76.4	50.9	167.6	5.8	5.8	5.1
59 *	18.76	α-Bulnesene	1472	80.6	157.8	100.8							
60 θ	18.96	(Z)-Calamenene	1488	42.6	81.8	49.6							
61 θ	19.08	α-Ylangene	1497	13.7	24.0	15.9							
62 *	19.22	α-Calacorene	1509	6.9	10.5	7.1							
63 *	19.70	Ethyl dodecanoate	1548	12.5	21.6	15.0							
64 *	19.75	Caryophyllene oxide	1552	39.9	76.4	50.9							
65 θ	20.05	α-Humulene epoxide II	1577	12.1	20.9	15.1							
66 *	20.24	1-epi-Cubenol	1593	7.1	10.9	8.1							
67 θ	20.40	δ-Cardene	1607	8.9	14.5	8.9							
68 *	20.90	2-Pentadecanone	1650	7.6	11.8	76.4							
69 *	21.91	Ethyl tetradecanoate	1753	13.6	23.9	17.3							

R_t Retention time (min), the reliability of identification proposal is indicated by the following: $^{\theta}$ mass spectrum and Kovat index agreed with standards, * mass spectrum and Kovat index agreed with literature. RI retention index

6.3.2.4 Chemical composition of apple mango VOCs infested with *Ceratitis rosa* larvae

The retention time (min), chemical identity and concentration (ng/h) of the VOCs headspace analysis of apple mango fruit infested with *C. cosyra* are indicated in Figure 6.4 and Table 6.5 while Table 6.7 shows the structures and major fragments for the VOCs observed. Overly a total of 72 VOCs was detected for the entire 10 days of trapping. The number detected was distributed as follows: 67 VOCs in day 1 & 2, 66 VOCs in day 3 and 47 VOCs in each of the days 4-10 of trapping. Cumulatively for the entire 10 days, α- pinene was the most abundant compound 9233.7 ng/h followed by myrcene and ethyl octanoate 5508.3 and 4158.4 ng/h respectively while the least was 2-heptanone 9.6 ng/h. Out of the total 72 VOCs identified, esters represented 41.7 % followed by monoterpene 25 %, sesquiterpenes 15.3 %, while the remaining 18 % comprised of aldehydes, ketones, non-terpenoid hydrocarbons and alcohols.

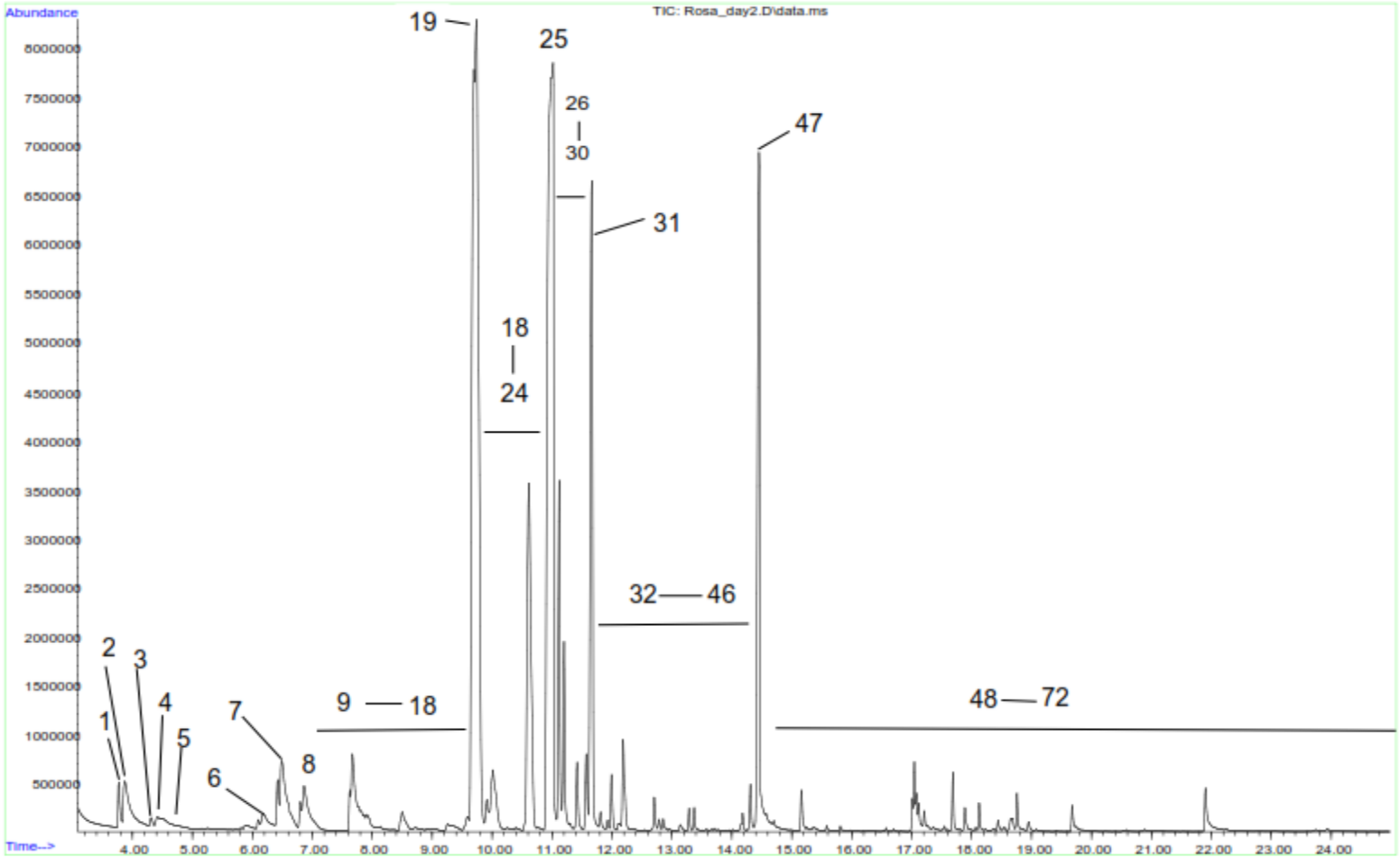


Figure 6.4: Representative total ion chromatogram showing VOCs detected in apple mango infested *C. rosa* larvae. Peaks 1-72 indicate the VOCs shown in Table 6.5

Table 6.5: Concentration of VOCs (ng/hr) in mango infested with *Ceratitis rosa* larvae

No	RT (min)	Compound name	RI	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1*	3.77	Acetoin	598	15.3	38.0	20.1	11.1	3.7	3.4	3.4	3.1	3.5	3.9
2*	3.94	Ethyl propanoate	664					8.4	7.2	7.1	6.0	7.5	9.1
3*	4.25	3-methyl-1-butanol	708	3.2	14.4	8.3							
4*	4.32	(*Z*)-methyl-2-butenoate	710					3.6	3.3	3.3	3.0	3.4	3.7
5*	4.35	Isopentyl alcohol	712	5.7	21.2	11.7							
6*	5.90	2,3-Butanediol	768	13.0	16.4	9.3	15.6	3.9	3.6	3.6	3.2	3.7	4.1
7*	6.42	Ethyl butanoate	787	193.7	45.7	24.0	2.2	342.0	274.0	266.5	206.1	292.9	379.7
8*	7.40	2-methyl-4-heptanone	824	14.1	2.3								
9*	7.63	Ethyl-(2*E*)- butanoate	833					11.6	9.7	9.5	7.8	10.2	12.6
10*	7.67	Ethyl-(2Z)-butanoate	835					65.6	52.9	51.5	40.3	56.5	72.7
11*	7.82	Ethyl 2-methylbutanoate	841	20.9	11.6	6.9		24.4	20.0	19.5	15.6	21.2	26.9
12*	7.92	Ethyl 3-methylbutanoate	844	36.8	19.5	10.9		13.8	11.5	11.2	9.2	12.1	15.1
13*	8.37	4-Heptanone	862	4.1	3.1	2.7							
14*	8.48	Isopentyl acetate	866	36.0	41.0	21.6							
15*	8.68	Styrene	874	4.1	6.9	4.6							
16*	8.78	2-Heptanone	878	3.8	3.1	2.7							
17*	8.82	5-methyl-2-hexanone	879	5.4	2.5	2.3							
18*	9.04	Ethyl pentanoate	888	3.7	71.6	36.9	123.4						
19$^{\theta}$	9.69	α- Pinene	915	460.7	750.3	376.3	950.4	1301.	1041.	1012.	781.	1113.	1445.

								2	4	6	6	6	6
20*	9.91	Ethyl tiglate	925	37.7	140.9	71.6	5.3	20.8	17.1	16.7	13.4	18.1	22.9
21θ	10.00	Camphene	929	15.1	137.6	69.9	73.0	38.3	31.1	30.3	23.9	33.1	42.3
22*	10.26	Isobutyl butyrate	941	10.4	2.7	2.5							
23*	10.40	Ethyl 2,3-epoxybutyrate	947	6.2	10.9	6.6							
24θ	10.60	β-Pinene	956	57.1	557.9	280.0	502.0	177.6	142.6	138.7	107.5	152.3	197.1
25θ	10.93	Myrcene	971	439.6	1845.7	924.0	2299.0						
26*	11.13	Ethyl hexanoate	980	245.0	123.6	62.9	28.5	133.2	107.0	104.1	80.8	114.3	147.8
27θ	11.16	α-Phellandrene	981	13.8	8.0	5.1	12.1						
28*	11.30	Ethyl 3-Hexenoate	988	11.4	6.8	4.5							
29θ	11.41	Terpinolene	993	7.2	60.3	31.3							
30θ	11.56	*p*-Cymene	999	11.4	63.9	33.1	98.3	47.0	38.0	37.0	29.1	40.5	52.0
31θ	11.63	β-phellandrene	1003	59.7	3.1	2.7	80.9	283.1	226.9	220.6	170.7	242.5	314.3
32θ	11.81	(*Z*)-β-Ocimene	1014	6.8	20.8	11.5	23.3						
33*	11.93	Ethyl (2*E*)-hexenoate	1022	10.4	2.7	2.5	2.2	6.0	5.3	5.2	4.5	5.5	6.5
34θ	11.99	(E)-β-Ocimene	1025	6.3	44.7	23.5	33.3	91.5	73.7	71.7	55.8	78.6	101.5
35θ	12.18	ɣ-Terpinene	1037	7.4	90.5	46.4	45.5	82.1	66.1	64.4	50.2	70.6	91.0

36*	12.71	iso-Sylvestrene	1069	4.2	3.1	2.7							
37θ	12.72	δ-2- Carene	1069	28.6	3.2	2.7							
38*	12.79	Ethyl sorbate	1074	12.1	3.2	2.7	20.7						
39*	12.86	Ethyl heptanoate	1078	12.0	4.1	3.1	3.0	7.2	6.2	6.1	5.2	6.5	7.8
40θ	13.14	Phenyl ethyl alcohol	1095	10.6	4.5	3.4	82.4	9.2	7.8	7.6	6.4	8.2	10.0
41*	13.29	Methyl octanoate	1104	17.2	3.4	2.8	2.2	19.9	16.4	16.0	12.8	17.4	21.9
42θ	13.38	allo-Ocimene	1109	3.7	19.9	11.1	20.6	15.2	12.6	12.3	10.0	13.4	16.7
43*	13.97	Pinocarvone	1144	3.1	18.4	10.3	10.7	4.0	3.7	3.6	3.3	3.8	4.2
44*	14.07	Ethyl benzoate	1149	3.2	2.7	2.5	9.4						
45θ	14.19	Terpinen-4-ol	1157	2.7	2.5	2.3	18.4	20.2	16.6	16.2	13.0	17.6	22.2
46*	14.31	(*Z*)-ethyl 4-octenoate	1164	36.9	19.5	10.9	2.2	62.9	50.8	49.4	38.6	54.1	69.6
47*	14.45	Ethyl octanoate	1172	636.0	319.1	160.7	30.1	585.2	468.6	455.7	352.0	501.0	650.0
48*	14.71	Verbenone	1187	20.1	3.1	2.7	6.6	25.2	20.6	20.1	16.0	21.9	27.8
49*	15.16	Ethyl (2*E*)-octenoate	1215					20.2	16.8	16.4	13.4	17.7	22.0
50*	15.25	Isopentyl hexanoate	1221	7.8	12.7	8.0	5.3						
51*	15.86	Ethyl nonanoate	1262	5.0	4.6	4.0	6.9	5.3	4.9	4.9	4.5	5.0	5.5
52	15.97	Carvacrol	1269	5.2	4.4	3.9	5.3	17.1	14.3	14.0	11.6	15.1	18.6

* 53 θ	16.70	α-Cubebene	1319	5.0	5.1	4.3	13.4	5.2	4.8	4.8	4.4	4.9	5.4
54 *	17.02	Ethyl (4*E*)-decenoate	1342	19.3	44.0	23.7	5.6	16.1	13.5	13.3	11.0	14.2	17.5
55 θ	17.09	α-Copaene	1347	4.1	25.2	14.3	127.5	19.6	16.3	16.0	13.1	17.2	21.4
56 *	17.22	Ethyl decanoate	1357	19.8	11.0	7.2	16.7	10.7	9.3	9.1	7.8	9.7	11.6
57 θ	17.55	α-Gurjunene	1381	8.9	6.4	4.9	20.7	9.3	8.2	8.0	7.0	8.5	10.0
58 θ	17.69	(*E*)-Caryophyllene	1391	4.1	41.7	22.6	169.9	37.5	30.7	29.9	23.8	32.5	41.3
59 θ	17.89	α-Guaiene	1405	3.6	25.6	14.5	84.5	15.1	12.8	12.5	10.4	13.4	16.4
60 *	18.08	(*E*)-Muurola-3,5-diene	1420	6.3	5.0	4.2	8.5	8.3	7.3	7.2	6.3	7.6	8.9
61 θ	18.13	α-Humulene	1424	23.3	5.2	4.3	87.3	10.1	8.8	8.6	7.4	9.1	10.9
62 θ	18.44	Germacrene B	1448	12.7	4.6	4.0	11.2	16.4	13.8	13.5	11.2	14.5	17.8
63 *	18.55	β-Selinene	1456	8.6	6.7	5.0	21.1	23.8	19.7	19.3	15.6	20.9	26.1
64 *	18.65	α-Selinene	1464	12.8	4.2	3.8	42.9	14.1	11.9	11.7	9.8	12.5	15.3
65 *	18.76	α-Bulnesene	1472	3.7	30.3	16.8	12.4	12.3	10.6	10.4	8.8	11.1	13.3
66 θ	18.95	δ- Cadinene	1487	13.6	3.9	3.6	50.1						
67 *	19.68	Ethyl dodecanoate	1546	23.0	5.7	4.5	6.4	8.7	7.6	7.5	6.6	7.9	9.3
68 *	20.58	(Z)-11-Hexadecen-1-ol, acetate	1622	5.3	4.4	3.9	3.4	3.4	3.4	3.4	3.4	3.4	3.4

69 *	20.89	2-Pentadecanone	1649	6.2	4.8	4.1	6.2	11.7	10.0	9.8	8.4	10.5	12.6
70 *	21.16	Methyl tetradecanoate	1673	4.7	4.1	3.7	4.2	8.7	7.6	7.5	6.6	7.9	9.3
71 *	21.90	Ethyl tetradecanoate	1752	63.8	33.6	18.5	5.6						
72 *	23.93	Ethyl hexadecanoate	1961	7.7	5.5	4.5	10.2						

R_t Retention time (min), the reliability of identification proposal is indicated by the following: $^{\theta}$ mass spectrum and Kovat index agreed with standards, * mass spectrum and Kovat index agreed with literature. RI retention index

6.3.2.5 Chemical composition of apple mango VOCs infested with *Ceratitis fasciventris* larvae

The retention time (min), chemical identity and concentration (ng/h) of the VOCs headspace analysis of apple mango fruit infested with *C. cosyra* are indicated in Figure 6.5 and Table 6.6 while Table 6.7 shows the structures and major fragments for the VOCs observed. Overly a total of 72 VOCs was detected for the entire 10 days of trapping. The number detected was distributed as follows: 67 VOCs in day 1 & 2, 66 VOCs in day 3 and 47 VOCs in each of the days 4-10 of trapping. Cumulatively for the entire 10 days, α-pinene was the most abundant compound 13181.5 ng/h followed by two close compounds myrcene and ethyl octanoate with 7865.1 and 5034.7 ng/h respectively while the least was 2-heptanone 10.8 ng/h. Out of the total 72 VOCs identified, esters represented 41.7 % % followed by monoterpene 25 %, sesquiterpenes 15.3 %, while the remaining 18 % comprised of aldehydes, ketones, non-terpenoid hydrocarbons and alcohols.

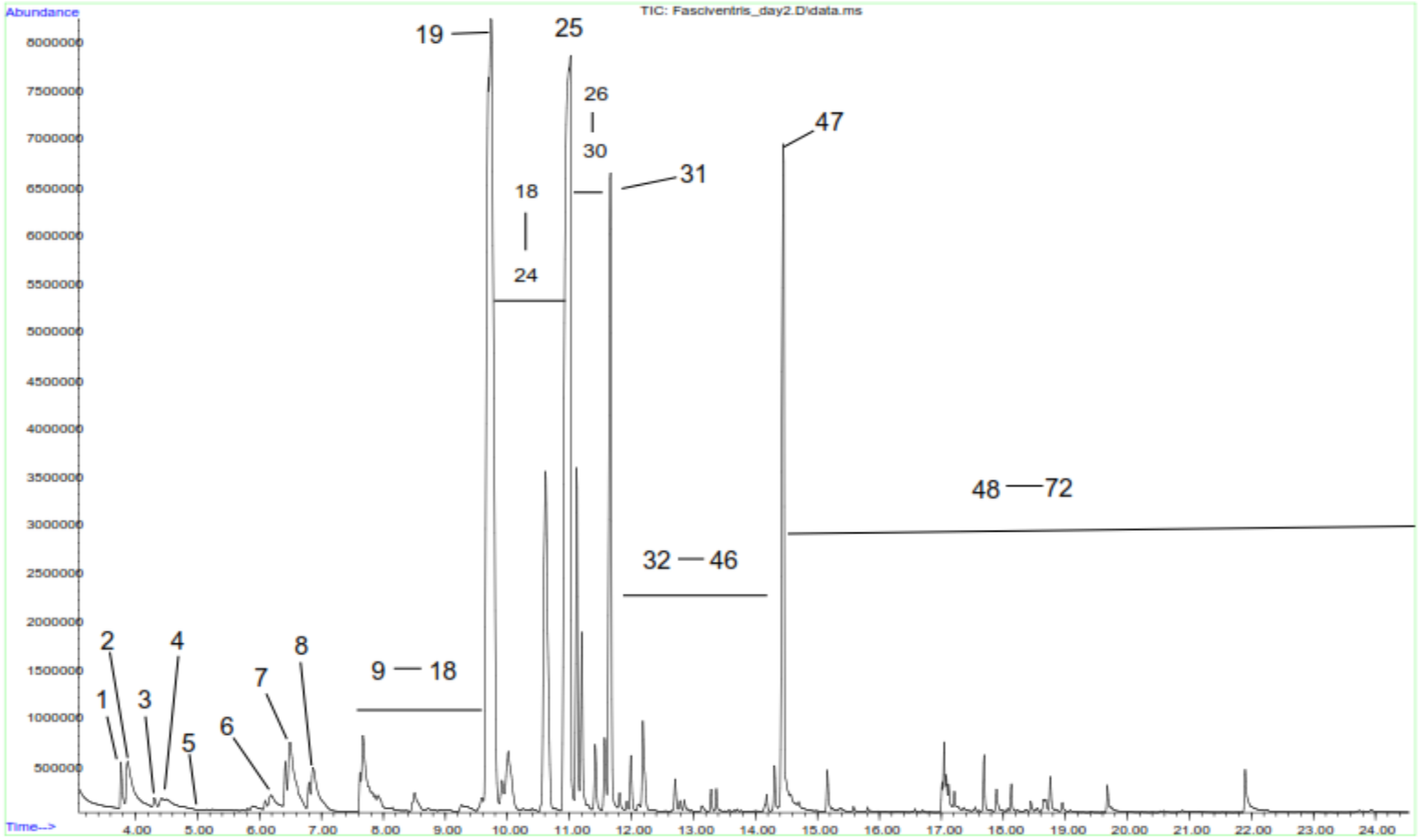


Figure 6.5: Representative total ion chromatogram showing VOCs detected in apple mango infested *C. fasciventris* larvae. Peaks 1-72 indicate the VOCs shown in Table 6.6

Table 6.6: Concentration of VOCs (ng/h) in mango infested with *Ceratitis fasciventris* larvae

No	RT (min)	Compound name	RI	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1*	3.77	Acetoin	597	20.8	53.3	27.8	14.9	4.3	3.9	3.9	3.5	4.0	4.6
2*	3.94	Ethyl propanoate	664					11.1	9.3	9.1	7.6	9.8	12.1
3*	4.25	3-methyl-1-butanol	708	3.6	19.6	10.9							
4*	4.32	(*Z*)-methyl-2-Butenoate	710					4.1	3.8	3.7	3.4	3.9	4.4
5*	4.35	Isopentyl alcohol	712	7.2	29.3	15.8							
6*	5.90	2,3-Butanediol	768	17.6	22.4	12.3	21.4	4.7	4.2	4.1	3.7	4.3	4.9
7*	6.42	Ethyl butanoate	787	275.8	64.3	33.3	2.2	487.6	390.5	379.7	293.4	417.5	541.5
8*	7.40	2-methyl-4-heptanone	824	19.2	2.3								
9*	7.63	Ethyl (2*E*)-Butenoate	833					15.6	12.9	12.6	10.2	13.6	17.1
10*	7.67	Ethyl (2Z)-Butenoate	835					92.8	74.7	72.7	56.6	79.7	102.9
11*	7.82	Ethyl 2-methylbutanoate	841	29.0	15.6	8.9		34.0	27.6	26.9	21.3	29.4	37.5
12*	7.92	Ethyl 3-methylbutanoate	844	51.6	26.9	14.6		18.8	15.5	15.1	12.2	16.4	20.6
13*	8.37	4-Heptanone	862	4.8	3.5	2.9							
14*	8.48	Isopentyl acetate	866	50.5	57.7	29.9							
15*	8.68	Styrene	874	4.9	8.9	5.6							
16*	8.78	2-Heptanone	878	4.5	3.5	2.9							
17*	8.82	5-methyl-2-hexanone	879	6.8	2.6	2.3							
18*	9.04	Ethyl pentanoate	888	4.3	101.3	51.8	175.3						
19$^{\theta}$	9.69	α- Pinene	915	657.2	1070.9	536.6	1356.7	1858.0	1486.8	1445.6	1115.7	1589.9	2064.2

20*	9.91	Ethyl tiglate	925	52.9	200.4	101.3	6.7	28.8	23.5	22.9	18.2	25.0	31.7
21θ	10.00	Camphene	929	20.6	195.6	98.9	103.3	53.8	43.5	42.3	33.2	46.3	59.5
22*	10.26	2-methylpropyl butanoate	941	13.9	2.9	2.6							
23*	10.40	Ethyl 2,3-epoxybutyrate	947	8.0	14.6	8.4							
24θ	10.60	β-Pinene	956	80.6	796.0	399.1	716.2	252.8	202.7	197.1	152.6	216.6	280.7
25θ	10.93	Myrcene	971	627.1	2635.8	1319.0	3283.3						
26*	11.13	Ethyl hexanoate	980	349.0	175.6	88.9	39.8	189.4	151.9	147.8	114.5	162.3	210.2
27θ	11.16	α-Phellandrene	981	18.8	10.5	6.4	16.3						
28*	11.30	Ethyl 3-Hexenoate	988	15.4	8.8	5.5							
29θ	11.41	Terpinolene	993	9.4	85.2	43.7							
30θ	11.56	*p*-Cymene	999	15.3	90.3	46.3	139.4	66.2	53.4	52.0	40.6	56.9	73.3
31θ	11.63	β-phellandrene	1003	84.4	3.5	2.9	114.5	403.4	323.2	314.3	242.9	345.5	448.0
32θ	11.81	(*Z*)-β-Ocimene	1014	8.7	28.8	15.5	32.4						
33*	11.93	Ethyl (2*E*)-hexenoate	1022	13.9	2.9	2.6	10.5	7.7	6.6	6.5	5.5	6.9	8.3
34θ	11.99	(*E*)-β-Ocimene	1025	8.1	62.9	32.6	46.6	129.8	104.3	101.5	78.8	111.4	144.0
35θ	12.18	ɣ-Terpinene	1037	9.7	128.4	65.3	64.1	116.4	93.5	91.0	70.7	99.9	129.0
36	12.71	iso-Sylvestrene	1069	5.1	3.5	2.9							

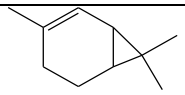
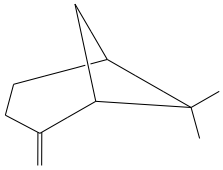
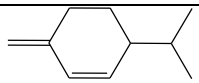
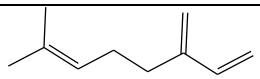
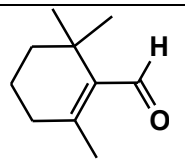
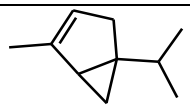
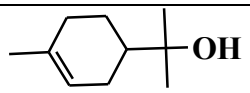
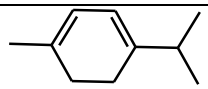
* 37 θ	12.72	δ-2- Carene	1069	40.0	3.6	2.9						
38 *	12.79	Ethyl sorbate	1074	16.4	3.6	2.9	28.6					
39 *	12.86	Ethyl heptanoate	1078	16.1	4.8	3.5	3.4	9.3	7.9	7.8	6.5	8.3	10.1
40 *	13.14	Phenyl ethyl alcohol	1095	14.2	5.5	3.9	116.7	12.2	10.2	10.0	8.2	10.7	13.3
41 *	13.29	Methyl octanoate	1104	23.7	3.9	3.1	2.9	27.5	22.5	21.9	17.4	23.9	30.3
42 θ	13.38	allo-Ocimene	1109	4.3	27.5	14.8	28.5	20.8	17.1	16.7	13.4	18.1	22.9
43 *	13.97	Pinocarvone	1144	3.5	25.3	13.8	14.4	4.8	4.3	4.2	3.8	4.4	5.1
44 *	14.07	Benzoic acid, ethyl ester	1149	3.6	2.9	2.6	12.5						
45 θ	14.19	Terpinen-4-ol	1157	2.9	2.6	2.4	25.3	27.9	22.7	22.2	17.6	24.2	30.7
46 *	14.31	(*Z*)-ethyl-4-Octenoate	1164	51.7	27.0	14.6	2.2	88.9	71.6	69.6	54.2	76.4	98.5
47 *	14.45	Ethyl octanoate	1172	11.3	455.0	228.6	42.0	835.1	668.5	650.0	501.9	714.8	927.6
48 *	14.71	Verbenone	1187	27.7	3.5	2.8	8.5	35.1	28.5	27.8	21.9	30.3	38.7
49 *	15.16	Ethyl (2*E*)-octenoate	1215					27.4	22.6	22.0	17.8	23.9	30.0
50 *	15.25	Isopentyl hexanoate	1221	9.7	16.7	10.0	6.2						
51 *	15.86	Ethyl nonanoate	1262	5.7	5.1	4.2	8.4	6.1	5.6	5.5	5.0	5.7	6.4
52 *	15.97	Carvacrol	1269	6.0	4.8	4.1	6.2	23.0	19.0	18.6	15.1	20.1	25.1

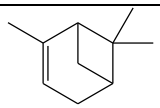
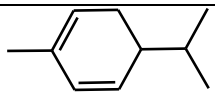
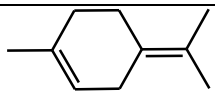
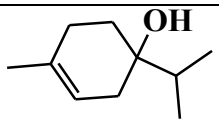
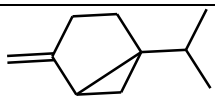
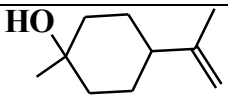
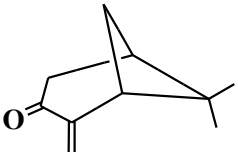
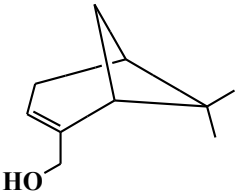
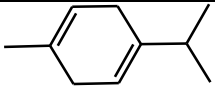
53θ	16.70	α-Cubebene	1319	5.7	5.9	4.6	17.7	5.9	5.4	5.4	4.9	5.6	6.2
54*	17.02	Ethyl-(4*E*)-decenoate	1342	26.1	61.4	32.4	6.5	21.5	17.9	17.5	14.3	18.9	23.5
55θ	17.09	α-Copaene	1347	4.4	34.6	19.0	180.8	26.5	21.9	21.4	17.3	23.2	29.1
56*	17.22	Ethyl decanoate	1357	26.8	14.2	8.8	22.4	13.9	11.8	11.6	9.7	12.4	15.1
57θ	17.55	α-Gurjunene	1381	11.3	7.7	5.6	28.1	11.9	10.2	10.0	8.5	10.7	12.9
58θ	17.69	(*E*)-Caryophyllene	1391	4.4	58.2	30.8	241.3	52.1	42.3	41.3	32.6	45.1	57.5
59θ	17.89	α-Guaiene	1405	3.7	35.1	19.2	119.2	20.1	16.8	16.4	13.4	17.7	22.0
60*	18.08	(*E*)-Muurola-3,5-diene	1420	7.5	5.7	4.6	10.7	10.4	9.0	8.9	7.6	9.4	11.2
61θ	18.13	α-Humulene	1424	31.9	6.0	4.7	123.3	13.0	11.1	10.9	9.2	11.6	14.1
62θ	18.44	Germacrene B	1448	16.7	5.1	4.2	14.5	21.9	18.2	17.8	14.5	19.2	24.0
63*	18.55	β-Selinene	1456	10.9	8.1	5.7	28.7	32.6	26.7	26.1	20.9	28.3	35.8
64*	18.65	α-Selinene	1464	16.8	4.5	3.9	59.8	18.7	15.6	15.3	12.6	16.5	20.4
65*	18.76	α-Bulnesene	1472	3.9	41.8	22.6	16.2	16.2	13.6	13.3	11.1	14.3	17.6
66θ	18.95	δ- Cadinene	1487	17.9	4.1	3.7	70.1						
67*	19.68	Ethyl dodecanoate	1546	31.4	6.7	5.0	7.8	11.0	9.5	9.3	7.9	9.9	11.8
68*	20.58	(*Z*)-11-Hexadecen-1-ol, acetate	1522	6.2	4.8	4.1							
69	20.89	2-Pentadecanone	1549	7.4	5.4	4.4	7.4	15.2	12.9	12.6	10.5	13.5	16.6

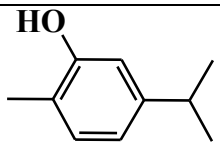
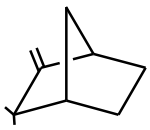
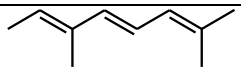
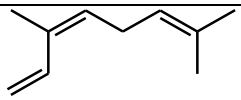
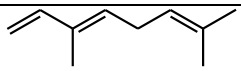
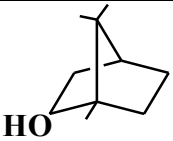
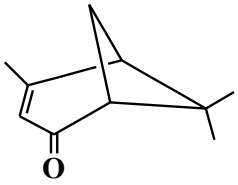
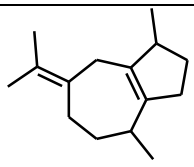
70*	21.16	Methyl tetradecanoate	1573	5.3	4.3	3.9	4.6	10.9	9.4	9.3	7.9	9.8	11.8
71*	21.90	Ethyl tetradecanoate	1652	89.7	46.5	25.0	6.5						
72*	23.93	Ethyl hexadecanoate	2052	9.6	6.5	4.9	13.1						

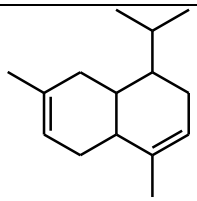
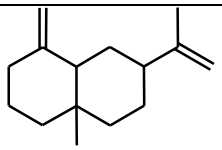
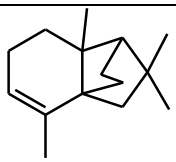
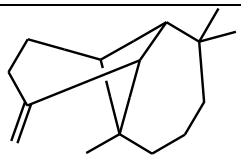
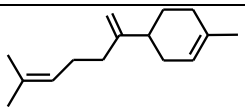
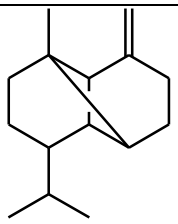
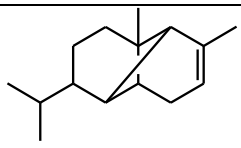
R_t Retention time (min), the reliability of identification proposal is indicated by the following: $^{\theta}$ mass spectrum and Kovat index agreed with standards, * mass spectrum and Kovat index agreed with literature. RI retention index

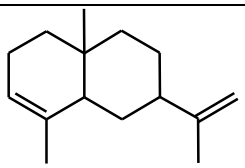
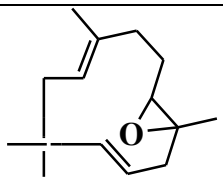
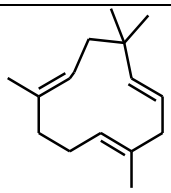
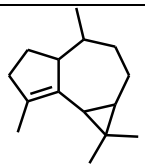
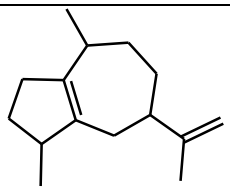
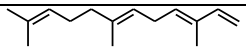
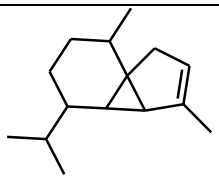
Table 6.7: Summary of VOCs detected from the various treatments

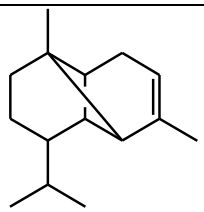
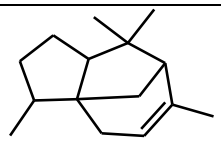
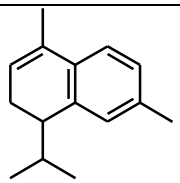
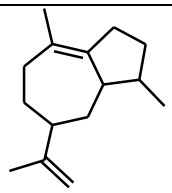
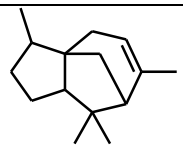
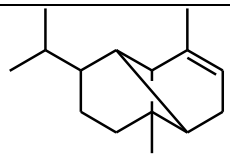
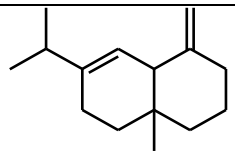
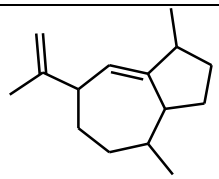
Monoterpenes			
No.	**Compound Name**	**structures**	**Major fragments**
1	δ-2- Carene		m/z 136 [55%, M^+], 93 [100 %, M- 43], 121 [90 %, M- 15], 79 [40 %, M-57]
2	β-Pinene		m/z 136 [10 %, M^+], 93 [100 %, M- 43], 121 [15 %, M- 15], 77 [27 %, M-59]
3	β-Phellandrene		m/z 136 [10 %, M^+], 93 [100 %, M- 43], 121 [15 %, M- 15], 77 [27 %, M-59]
4	β-Myrcene		m/z 136 [15 %, M^+], 93 [100 %, M- 43], 121 [5 %, M- 15], 77 [27 %, M-59]
5	β-Cyclocitral		m/z 152 [15 %, M^+], 93 [80 %, M- 59], 121 [5 %, M- 31], 81 [99 %, M-71]
6	α-Thujene		m/z 136 [15 %, M^+], 93 [100 %, M- 43], 121 [5 %, M- 15], 77 [27 %, M-59]
7	α-Terpineol		m/z 136 [47 %, M^+], 93 [68 %, M- 43], 121 [58 %, M- 15], 81 [35 %, M-55], 59 [17%, M-77]
8	α-Terpinene		m/z 136 [35 %, M^+], 93 [100 %, M- 43], 121 [30 %, M- 15], 77 [28 %, M-59]

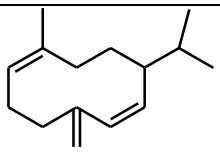
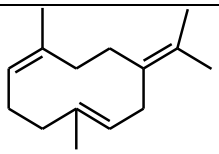
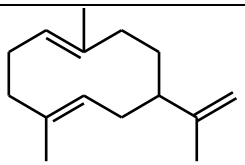
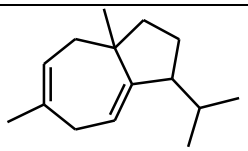
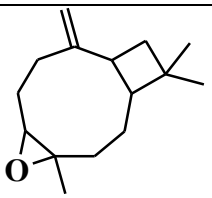
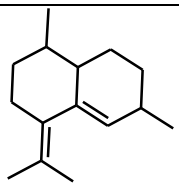
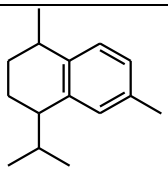
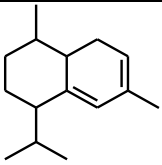
9	α-Pinene		m/z 136 [5 %, M^+], 93 [100 %, M- 43], 121 [30 %, M- 15], 77 [26%, M-59]
10	α-Phellandrene		m/z 136 [10 %, M^+], 93 [100 %, M- 43], 121 [3 %, M- 15], 77 [27 %, M-59]
11	Terpinolene		m/z 136 [55 %, M^+], 93 [99 %, M- 43], 121 [78 %, M- 15], 79 [27 %, M-57]
12	Terpinen-4-ol		m/z 154 [20 %, M^+], 93 [38 %, M- 61], 111 [60 %, M- 94], 71 [100 %, M-83]
13	Sabinene		m/z 136 [10 %, M^+], 93 [100 %, M- 43], 121 [3 %, M- 15], 77 [27 %, M-59]
14	*p*-Menth-8-en-1-ol		m/z 154 [20 %, M^+], 93 [38 %, M- 61], 111 [60 %, M- 94], 71 [100 %, M-83]
15	Pinocarvone		m/z 150 [30 %, M^+], 135 [25 %, M- 15], 108 [60 %, M- 42], 81 [100 %, M-69]
16	Myrtenol		m/z 152 [1 %, M^+], 134 [2 %, M- 18], 108 [30 %, M- 44], 79 [100 %, M-52]
17	ɣ-Terpinene		m/z 136 [35 %, M^+], 93 [100 %, M- 43], 121 [30 %, M- 15], 77 [28 %, M-59]

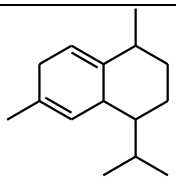
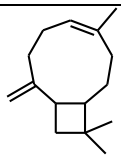
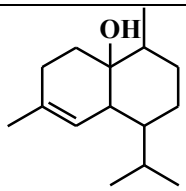
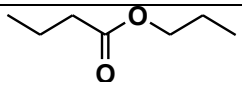
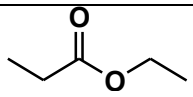
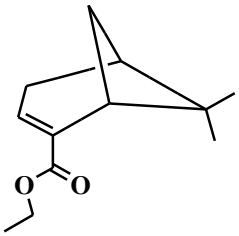
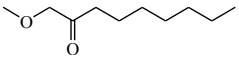
18	Carvacrol		m/z 150 [30 %, M^+], 135 [100 %, M-15], 107 [60 %, M- 43],
19	Camphene		m/z 136 [5 %, M^+], 93 [100 %, M- 43], 121 [30 %, M- 15], 107 [25%, M-29], 77 [26%, M-59]
20	allo-Ocimene		m/z 136 [51 %, M^+], 121 [100 %, M- 15], 105 [28, M-31], 93 [18 %, M- 43], 79 [10%, M-57]
21	(*Z*)-β Ocimene		m/z 136 [5 %, M^+], 93 [100 %, M- 43], 121 [15 %, M- 15], 105 [25%, M-31], 79 [31 %, M-57]
22	(*E*)-β Ocimene		m/z 136 [1 %, M^+], 93 [100 %, M- 43], 121 [15 %, M- 15], 105 [10 %, M-31], 79 [31 %, M-57]
23	Borneol		m/z 154 [2 %, M^+], 95 [38 %, M- 59], 110 [60 %, M- 44]
24	Verbenone		m/z 150 [60 %, M^+], 135 [80 %, M- 15], 107 [100 %, M- 43], 91[58% M-59], 80 [57 %, M-70]
Sesquiterpenes			
25	δ-guaiene		m/z 204 [77 %, M^+], 189 [41 %, M- 23], 161 [100 %, M- 43], 119[58% M-85], 105 [95 %, M-99]

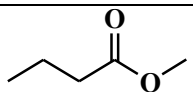
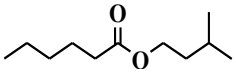
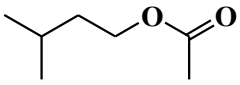
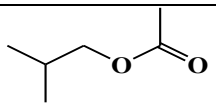
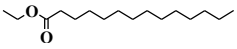
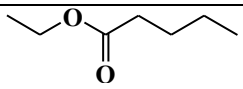
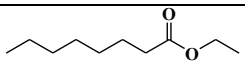
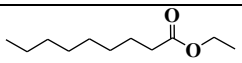
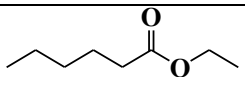
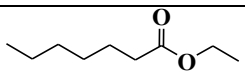
26	δ-Cadinene		m/z 204 [68 %, M^+], 189 [76 %, M- 23], 161 [100 %, M- 43], 119[31 % M-85], 105 [52 %, M-99]
27	β-Selinene		m/z 204 [88 %, M^+], 189 [50 %, M- 23], 161 [60 %, M- 43], 105 [90 %, M-99], 41 [100 %, M-163]
28	β-Neoclovene		m/z 204 [62 %, M^+], 189 [100 %, M- 23], 161 [75 %, M- 43], 119 [31 % M-85], 105 [50 %, M-99]
29	β-Longipinene		m/z 204 [62 %, M^+], 189 [65 %, M- 23], 161 [70 %, M- 43], 119 [73 % M-85], 105 [100 %, M-99]
30	β-Cubebene		m/z 204 [25 %, M^+], 189 [5 %, M- 23], 161 [100 %, M- 43], 119 [93 % M-85], 105 [90 %, M-99]
31	β-Copaene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
32	α-Ylangene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]

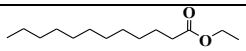
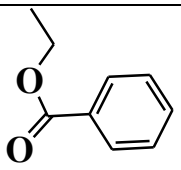
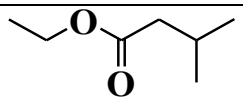
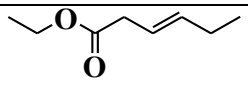
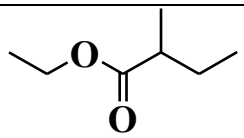
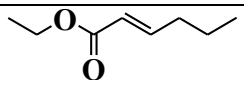
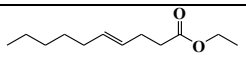
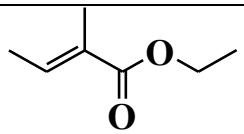
33	α-Selinene		m/z 204 [88 %, M^+], 189 [50 %, M- 23], 161 [60 %, M- 43], 105 [90 %, M-99], 41 [100 %, M-163]
34	α-Humulene epoxide II		m/z 220 [10 %, M^+], 189 [30 %, M- 23], 161 [3 %, M- 43], 107 [90 %, M-97], 93 [100 %, M-111]
35	α-Humulene		m/z 204 [3 %, M^+], 189 [1 %, M- 23], 161 [3 %, M- 43], 107 [90 %, M-97], 93 [100 %, M-111]
36	α-Gurjunene		m/z 204 [77 %, M^+], 189 [41 %, M- 23], 161 [100 %, M- 43], 119[58% M-85], 105 [95 %, M-99]
37	α-Guaiene		m/z 204 [76 %, M^+], 189 [70 %, M- 23], 161 [100 %, M- 43], 105 [94 %, M-99], 91 [60 %, M-113]
38	α-Farnesene		m/z 204 [1 %, M^+], 119 [30 %, M- 85], 107 [45 %, M-94], 93 [88 %, M-116], 41 [100 %, M-163]
39	α-Cubebene		m/z 204 [25 %, M^+], 189 [5 %, M- 23], 161 [100 %, M- 43], 119 [93 % M-85], 105 [90 %, M-99]

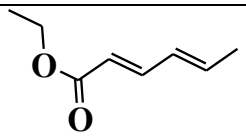
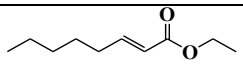
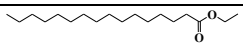
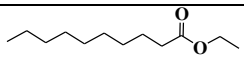
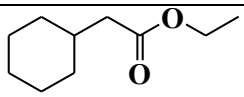
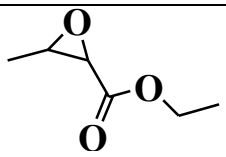
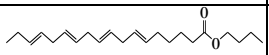
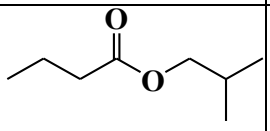
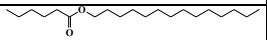
40	α-Copaene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
41	α-Cedrene		m/z 204 [62 %, M^+], 189 [5 %, M- 23], 161 [70 %, M- 43], 119 [100 %, M-85]
42	α-Calacorene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
43	α-Bulnesene		m/z 204 [77 %, M^+], 189 [41 %, M- 23], 161 [100 %, M- 43], 119[58% M-85], 105 [95 %, M-99]
44	α-2-epi-funebrene		m/z 204 [62 %, M^+], 189 [5 %, M- 23], 161 [70 %, M- 43], 119 [100 %, M-85]
45	Ylangene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
46	Sibirene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
47	ɣ-Gurjunene		m/z 204 [77 %, M^+], 189 [41 %, M- 23], 161 [100 %, M- 43], 119[58% M-85], 105 [95 %, M-

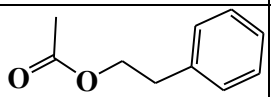
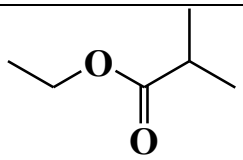
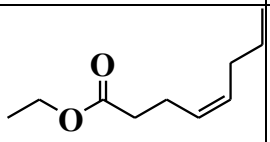
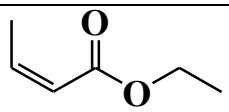
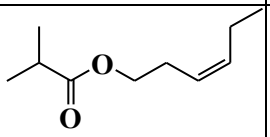
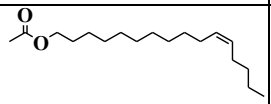
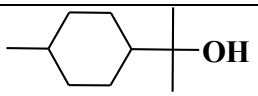
			99]
48	Germacrene D		m/z 204 [30 %, M^+], 161 [100 %, M- 43], 119[60 % M-85], 105 [77 %, M-99]
49	Germacrene B		m/z 204 [15 %, M^+], 161 [21 %, M- 43], 121 [100 % M-83], 107 [43 %, M-97]
50	Germacrene A		m/z 204 [15 %, M^+], 161 [21 %, M- 43], 121 [100 % M-83], 107 [80 %, M-97]
51	Dauca-5,8-diene		m/z 204 [62 %, M^+], 189 [5 %, M- 23], 161 [70 %, M- 43], 119 [100 %, M-85]
52	Caryophyllene oxide		m/z 204 [2 %, M^+], 109 [5 %, M- 95], 93 [70 %, M- 111], 79 [89 %, M-125], 43 [100 %, M-161]
53	(*Z*)-muurola-4(14),5-diene		m/z 204 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
54	(*Z*)-Calamenene		m/z 202 [55 %, M^+], 189 [75 %, M- 23], 161 [90 %, M- 43], 119 [90 % M-85], 105 [100 %, M-99]
55	(*E*)-Muurola-3,5-diene		m/z 204 [77 %, M^+], 189 [41 %, M- 23], 161 [100 %, M- 43], 119[58% M-85], 105 [95 %, M-99]

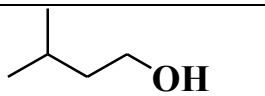
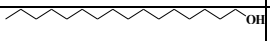
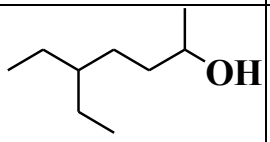
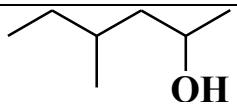
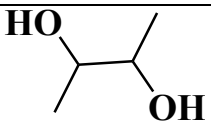
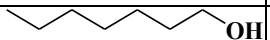
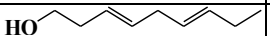
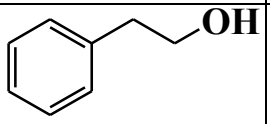
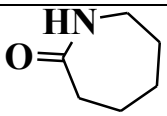
56	(*E*)-Cadina-1,4-diene		m/z 204 [70 %, M^+], 189 [10 %, M- 23], 161 [100 %, M- 43], 119[58% M-85], 105 [48 %, M-99]
57	(*E*)-Caryophyllene		m/z 204 [5 %, M^+], 189 [10 %, M- 23], 161 [50 %, M- 43], 133 [58% M-71], 105 [70 %, M-99], 93 [100 %, M-111]
58	1-epi-Cubenol		m/z 204 [70 %, M^+], 189 [10 %, M-23], 161 [90 %, M- 43], 119[100 % M-85], 105 [70 %, M-99]
Esters			
59	Propyl butanoate		m/z 130 [1 %, M^+], 89 [50 %, M-41], 71 [93 %, M-59], 43 [100 %, M-87]
60	Propyl propanoate		m/z 116 [1 %, M^+], 87 [50 %, M-29], 75 [93 %, M-41], 57 [100 %, M-59]
61	Myrtenoic acid, ethyl ester		m/z 194 [1 %, M^+], 150 [50 %, M-44], 122 [93 %, M-72], 79 [100 %, M-115]
62	Methyl octanoate		m/z 158 [5 %, M^+], 127 [30 %, M-31], 115 [93 %, M-43], 87 [42 %, M-71], 74 [100 %, M-84]
63	Methyl butanoate		m/z 102 [5 %, M^+], 87 [30 %, M-15], 74 [77 %, M-28], 43

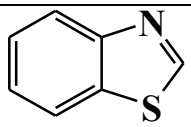
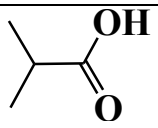
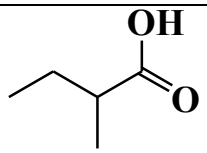
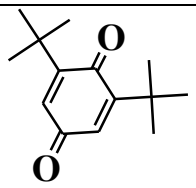
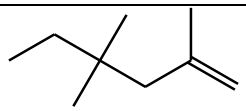
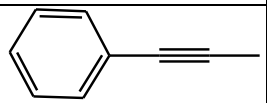
			[100 %, M-59]
64	Isopentyl hexanoate		m/z 102 [5 %, M^+], 87 [30 %, M-15], 74 [77 %, M-28], 43 [100 %, M-59]
65	Isopentyl acetate		m/z 186 [1 %, M^+], 99 [44 %, M-87], 70 [100 %, M-116], 43 [100 %, M-143]
66	Isobutyl acetate		m/z 116 [1 %, M^+], 73 [14 %, M-43], 56 [30 %, M-60], 43 [100 %, M-73]
67	Ethyl tetradecanoate		m/z 256 [10 %, M^+], 211 [9 %, M-45], 101 [50 %, M-155], 88 [100 %, M-68]
68	Ethyl pentanoate		m/z 130 [1 %, M^+], 101 [20 %, M-29], 85 [50 %, M-45], 57 [100 %, M-73], 29 [100 %, M-101]
69	Ethyl octanoate		m/z 172 [1 %, M^+], 127 [20 %, M-45], 101 [70 %, M-71], 88 [100 %, M-84]
70	Ethyl nonanoate		m/z 186 [1 %, M^+], 157 [20 %, M-29], 101 [70 %, M-85], 88 [100 %, M-98]
71	Ethyl hexanoate		m/z 144 [1 %, M^+], 99 [70 %, M-45], 88 [70 %, M-85], 88 [100 %, M-56], 43 [86% M-101]
72	Ethyl heptanoate		m/z 158 [1 %, M^+], 113 [70 %,

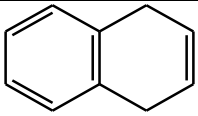
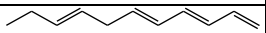
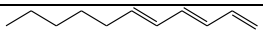
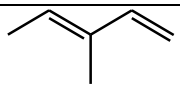
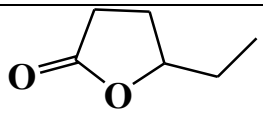
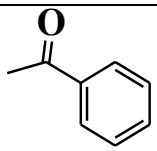
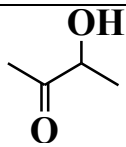
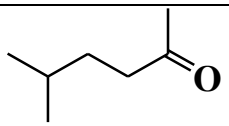
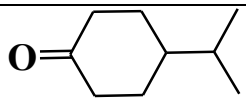
			M-45], 88 [70 %, M-85], 88 [100 %, M-56], 43 [86% M-101]
73	Ethyl dodecanoate		m/z 228 [1 %, M^+], 183 [15 %, M-85], 101 [47 %, M-127], 88 [100 %, M-140], 73 [16% M-155]
74	Ethyl benzoate		m/z 150 [22 %, M^+], 122 [55 %, M-28], 105 [100 %, M-45], 77 [39 %, M-73]
75	Ethyl 3-methylbutanoate		m/z 130 [2 %, M^+], 88 [100 %, M-42], 57 [91 %, M-73]
76	Ethyl 3-hexenoate		m/z 142 [15 %, M^+], 88 [10 %, M-54], 69 [91 %, M-73], 41 [100 %, M-101]
77	Ethyl 2-methylbutanoate		m/z 130 [5 %, M^+], 115 [10 %, M-15], 102 [64 %, M-28], 57 [100 %, M-73]
78	Ethyl 2-hexenoate		m/z 142 [1 %, M^+], 114 [10 %, M-28], 97 [98 %, M-44], 55 [100 %, M-87]
79	Ethyl (4*E*)-decenoate		m/z 198 [10 %, M^+], 152 [10 %, M-46], 110 [98 %, M-88], 88 [100 %, M-110], 69 [96%, M-129]
80	Ethyl tiglate		m/z 128 [50 %, M^+], 113 [40 %, M-15], 100 [34 %, M-28], 83 [100 %, M-45], 55 [96%, M-73]

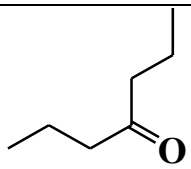
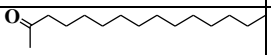
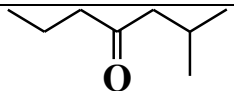
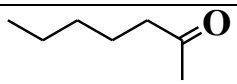
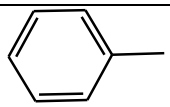
83	Ethyl sorbate		m/z 140 [30 %, M^+], 125 [20 %, M-15], 95 [72 %, M-45], 83 [100 %, M-45], 55 [96%, M-73]
84	Ethyl oct-(2E)-enoate		m/z 170 [30 %, M^+], 125 [20 %, M-45], 95 [72 %, M-75], 88 [100 %, M-82], 55 [96%, M-115]
85	Ethyl hexadecanoate		m/z 284 [10 %, M^+], 239 [10 %, M-45], 157 [98 %, M-127], 101 [60%, M-183], 88 [100 %, M-110]
86	Ethyl decanoate		m/z 200 [1 %, M^+], 115 [14 %, M-85], 101 [41%, M-99], 88 [100 %, M-112]
87	Ethyl benzeneacetate		m/z 164 [15 %, M^+], 91 [100 %, M-64], 65 [20%, M-99]
88	Ethyl 2,3-epoxybutyrate		m/z 130 [1 %, M^+], 115 [5 %, M-15], 102 [71%, M-28], 74 [100 %, M-56], 45 [100 %, M-85]
89	Butyl 6,9,12,15-octadecatetraenoate		m/z 332 [1 %, M^+], 161 [65 %, M-171], 108 [71%, M-224], 79 [100 %, M-253], 67 [55 %, M-265]
90	2-methylpropyl butanoate		m/z 144 [5 %, M^+], 114 [10 %, M-30], 102 [64 %, M-28], 57 [100 %, M-73]
91	Tetradecyl hexanoate		m/z 312 [1 %, M^+], 196 [55 %, M-116], 117 [98%, M-195], 99

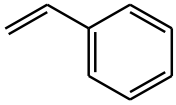
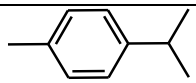
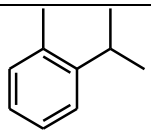
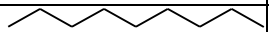
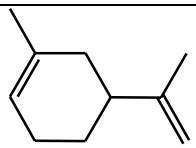
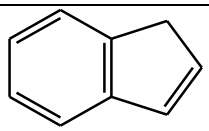
			[55 %, M-213], 43 [100 %, M-269],
92	2-Phenethyl acetate		m/z 164 [1 %, M^+], 104 [100 %, M-64], 91 [18%, M-73], 43 [100 %, M-121],
93	Ethyl 2-methylpropanoate		m/z 116 [1 %, M^+], 88 [5 %, M-28], 71 [98%, M-45], 43 [100 %, M-73],
94	Ethyl-(4*Z*)-octenoate		m/z 170 [30 %, M^+], 125 [20 %, M-45], 95 [72 %, M-75], 83 [100 %, M-87], 55 [96%, M-115], 43 [100 %, M-127],
95	Ethyl-(2*Z*)-butenoate		m/z 114 [1 %, M^+], 73 [14 %, M-41], 56 [30 %, M-58], 43 [100 %, M-71]
96	(3*Z*)-hexenyl iso-butyrate		m/z 170 [30 %, M^+], 125 [20 %, M-45], 95 [72 %, M-75], 83 [100 %, M-87], 55 [80 %, M-115], 43 [100 %, M-127],
97	(11*Z*)-Hexadecen-1-ol, acetate		m/z 282 [1 %, M^+], 222 [20 %, M-60], 95 [22 %, M-75], 110 [30 %, M-172], 55 [100 %, M-227], 43 [100 %, M-239]
Others			
98	*p*-Cymen-8-ol		m/z 150 [1 %, M^+], 135 [53 %, M-15], 117 [22 %, M-33], 43 [100 %, M-107]

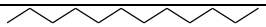
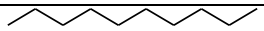
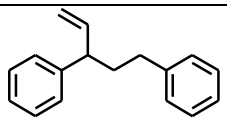
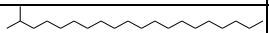
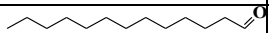
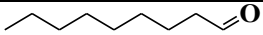
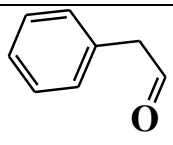
99	Isopentyl alcohol		m/z 88 [1 %, M^+], 70 [71 %, M-18], 55 [100 %, M-33], 43 [100 %, M-46]
100	Hexadecanol		m/z 242 [1 %, M^+], 111 [30 %, M-131], 55 [100 %, M-187], 41 [100 %, M-201]
101	5-ethyl-2-heptanol		m/z 242 [1 %, M^+], 111 [30 %, M-131], 55 [100 %, M-187], 41 [100 %, M-201]
102	4-Methyl-2-hexanol		m/z 116 [1 %, M^+], 98 [30 %, M-18], 59[100 %, M-57], 45 [100 %, M-71]
103	2,3-Butanediol		m/z 90[1 %, M^+], 75[3 %, M-15], 57[10 %, M-33], 45 [100 %, M-45]
104	1-Heptanol		m/z 116 [1 %, M^+], 98 [30 %, M-18], 70[100 %, M-46], 56 [100 %, M-60]
105	(*E,Z*)-3,6-Nonadien-1-ol		m/z 140 [10 %, M^+], 122 [30 %, M-18], 93[50 %, M-47], 67[100 %, M-73]
106	Phenyl ethyl alcohol		m/z 122 [25 %, M^+], 104 [30 %, M-18], 91[100 %, M-31], 65[100 %, M-57]
107	Caprolactam		m/z 113 [55 %, M^+], 84 [30 %, M-29], 55[100 %, M-58], 30[100 %, M-83]

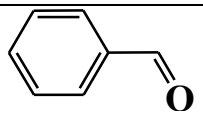
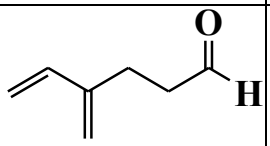
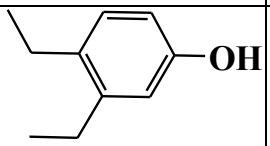
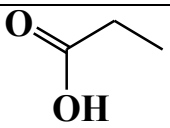
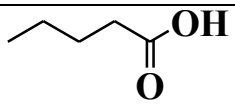
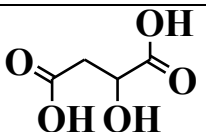
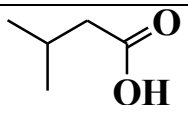
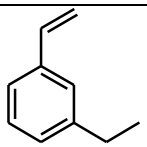
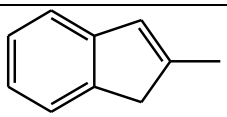
108	Benzothiazole		m/z 135 [100 %, M^+], 108 [50 %, M-27], 69[10 %, M-66]
109	2-methyl-propanoic acid		m/z 88 [10 %, M^+], 73 [30 %, M-15], 43[50 %, M-45]
110	2-methyl-butanoic acid		m/z 102 [1 %, M^+], 87 [50 %, M-15], 74[100 %, M-28], 57[70 %, M-45]
111	2,6-Di-tert-butylquinone		m/z 202 [81 %, M^+], 205 [50 %, M-15], 117[100 %, M-85], 135[70 %, M-85]
112	2,4,4-Trimethyl-1-hexene		m/z 126 [1 %, M^+], 97 [50 %, M-29], 71[100 %, M-55]
113	1-propynyl-benzene		m/z 116 [100 %, M^+], 89 [20 %, M-27]
114	1-Pentadecene		m/z 210 [31 %, M^+], 182 [50 %, M-28], 97[50 %, M-113], 83[70 %, M-127], 41[100 %, M-161]
115	1-Octadecene		m/z 252 [31 %, M^+], 111 [100 %, M-141], 97[50 %, M-155], 83[70 %, M-169], 55[100 %, M-197] 43[100 %, M-209]
116	1-Eicosene		m/z 280 [31 %, M^+], 111 [70 %, M-169], 97[100 %, M-183], 83[70 %, M-197], 69[80 %, M-211] 43[100 %, M-237]
117	1-Docosene		m/z 308 [31 %, M^+], 111 [60 %, M-197], 97[50 %, M-211],

			83[70 %, M-197], 57[100 %, M-223]
118	1,4-Dihydronaphthalene		m/z 130 [100 %, M^+], 115 [50 %, M-15]
119	1,3,5,8-Undecatetraene		m/z 148 [30 %, M^+], 119 [30 %, M-29], 91[50 %, M-50], 79[100 %, M-69]
120	(*E,E*)-1,3,5-Heptatriene		m/z 150 [30 %, M^+], 91 [30 %, M-51], 79[100 %, M-71], 67[80 %, M-83]
121	(*E*)-5-Eicosene		m/z 280 [31 %, M^+], 111 [70 %, M-169], 97[50 %, M-183], 83[70 %, M-197], 69[80 %, M-211] 43[100 %, M-237]
122	(*E*)-3-methyl-1,3-Pentadiene		m/z 82 [50 %, M^+], 67 [100 %, M-15], 53[20 %, M-29]
123	ɣ-Hexalactone		m/z 114 [90 %, M^+], 99 [100 %, M-15], 96[80 %, M-18]
124	Acetophenone		m/z 120 [30 %, M^+], 105 [100 %, M-15], 77[80 %, M-43]
125	Acetoin		m/z 88 [30 %, M^+], 45 [100 %, M-43]
126	5-methyl-2-hexanone		m/z 114 [1 %, M^+], 58 [40 %, M-56], 43[100 %, M-71]
127	4-Isopropylcyclohexanone		m/z 140 [60 %, M^+], 107 [40 %, M-33], 84[75 %, M-56], 69[100

			%, M-71]
128	4-Heptanone		m/z 114 [10 %, M^+], 71 [90 %, M-43], 43[100 %, M-43]
129	2-Pentadecanone		m/z 226 [10 %, M^+], 71 [50 %, M-155], 58[100 %, M-168]
130	2-methyl-4-heptanone		m/z 128 [30 %, M^+], 85 [80 %, M-43], 71 [80 %, M-57], 57[100 %, M-71]
131	2-Heptanone		m/z 114 [10 %, M^+], 71 [20 %, M-43], 58[28 %, M-56], 43[100 %, M-43]
132	Undecane		m/z 156 [1 %, M^+], 85 [25 %, M-71], 57[100 %, M-99], 43[89 %, M-113]
133	Tridecane		m/z 184 [1 %, M^+], 85 [25 %, M-99], 57[100 %, M-127], 43[89 %, M-141]
134	Tricosane		m/z 324 [10 %, M^+], 85 [25 %, M-239], 57[100 %, M-267], 43[89 %, M-281]
135	Toluene		m/z 92[77 %, M^+], 91 [100 %, M-1], 65[10 %, M-27]
136	Tetradecane		m/z 198 [1 %, M^+], 85 [50 %, M-113], 57[100 %, M-141], 43[80 %, M-155]
137	Tetracosane		m/z 338 [1 %, M^+], 85 [50 %, M-253], 57[100 %, M-281], 43[60 %, M-295]

138	Styrene		m/z 104[100%, M^+], 78 [100 %, M-26], 51[10 %, M-53]
139	Pentadecane		m/z 212 [1 %, M^+], 85 [40 %, M-127], 57[100 %, M-155], 43[80 %, M-169]
140	*p*-Cymene		m/z 134[25%, M^+], 119 [100 %, M-15], 91[10 %, M-43]
141	o-Cymene		m/z 134[25%, M^+], 119 [100 %, M-15], 91[50 %, M-43]
142	Octadecane		m/z 254 [1 %, M^+], 85 [40 %, M-169], 57[100 %, M-197], 43[80 %, M-211]
143	Nonadecane		m/z 268 [30 %, M^+], 85 [40 %, M-183], 57[100 %, M-211], 43[80 %, M-225]
144	nonane		m/z 128 [3 %, M^+], 85 [30 %, M-43], 57[60 %, M-71], 43[100 %, M-85]
145	iso-Sylvestrene		m/z 136[25%, M^+], 121 [25 %, M-15], 93[100 %, M-43], 68[80 %, M-68]
146	Indene		m/z 136[25%, M^+], 121 [25 %, M-15], 93[100 %, M-43], 68[80 %, M-68]
147	Hexadecane		m/z 226 [3 %, M^+], 85 [40 %, M-141], 57[100 %, M-169], 43[80 %, M-183]
148	Heptadecane		m/z 240 [3 %, M^+], 85 [40 %,

			M-155], 57[100 %, M-83], 43[70 %, M-197]
149	Heneicosane		m/z 240 [3 %, M^+], 85 [40 %, M-155], 57[100 %, M-83], 43[70 %, M-197]
150	Dodecane		m/z 170 [1 %, M^+], 85 [40 %, M-85], 57[100 %, M-113], 43[70 %, M-127]
151	Docosane		m/z 310 [10 %, M^+], 85 [40 %, M-225], 57[100 %, M-253], 43[70 %, M-267]
152	Decane		m/z 142 [3 %, M^+], 57 [90 %, M-85], 43[100 %, M-99]
153	3,5-Diphenyl-1-pentene		m/z 222 [3 %, M^+], 117 [100 %, M-105], 91[30 %, M-131]
154	2-methyl-eicosane		m/z 296 [1 %, M^+], 253 [20 %, M-43], 57[100 %, M-239], 43[70 %, M-253]
155	Tridecanal		m/z 198 [1 %, M^+], 154 [20 %, M-44], 82 [80 %, M-116], 57[100 %, M-141], 43[93 %, M-155]
156	nonanal		m/z 141 [1 %, M^+], 98 [20 %, M-43], 82 [80 %, M-59], 57[100 %, M-85], 43[89 %, M-99]
157	Benzeneacetaldehyde		m/z 120 [1 %, M^+], 91 [100 %, M-30], 65 [80 %, M-55]

158	Benzaldehyde		m/z 106 [94 %, M^+], 105 [94 %, M-1], 77 [100 %, M-29]
159	4-methylene-5-hexenal		m/z 110 [4 %, M^+], 67 [100 %, M-43], 53 [100 %, M-57]
160	3,4-Diethylphenol		m/z 150 [54 %, M^+], 135 [100%, M-15], 121 [15 %, M-29]
161	Propanoic acid		m/z 74 [100 %, M^+], 57 [50 %, M-17], 45 [90 %, M-29]
162	Pentanoic acid		m/z 102 [1 %, M^+], 73 [40 %, M-29], 60 [100 %, M-42],
163	Malic Acid		m/z 134 [1 %, M^+], 89 [88 %, M-45], 71 [88 %, M-63], 43 [100 %, M-91],
164	3-methyl-butanoic acid		m/z 102 [1 %, M^+],87 [88 %, M-15], 60 [100 %, M-42]
165	α-Methylstyrene		m/z 112 [50 %, M^+],117 [100 %, M-15], 91 [20 %, M-21]
166	2-Methylindene		m/z 130 [100 %, M^+],115 [90 %, M-15]

6.4 Discussion

The ultimate goal of this objective was to provide some insight regarding the volatile chemistry of the ripening apple mango fruit when exposed to topical application of *C. cosyra* HMP, GSH and when larva of *B. dorsalis, C. cosyra, C. rosa* and *C. fasciventris* are allowed to develop inside the fruit. From our results, topical application of glutathione, a *C. cosyra* HMP, did not alter qualitatively the profile of the VOCs detected across the various treatment compared to the intact mango. This observation may be ascribed to cryptic nature of the ovipositing fruit fly in that, HMP only serves to provide conspecific females with the means of identifying hosts that they have already attacked (Nufio *et al*., 2004) and not eavesdropped by parasitoids and predators. Our result is in agreement with that of Bezerra-silva *et al*., (2012) who reported that fruit flies lay their eggs inside the fruit, provoking a small visible damage hence no evidence of variability in the emission of volatiles when the plant is infested only with the eggs of the fly . This may also be extrapolated to cover the HMPs because they are deposited immediately following egg laying (Edmunds *et al*., 2010; Papa *et al*., 1993; Papaj *et al*., 1992). This results contradicts (Spinelli *et al*., 2011) who showed that there is variation in the compounds (VOCs) released by the plant or oviposition substrate following damaged by insects through oviposition or when immature or adults insects develop inside the fruit.

Differential detection of acetoin, a common volatile product of aerobic fermentation and also pheromone compound of two scarab beetles: the dynast *Scapanes autralis* and the chafer *Amphimallon solstitiale* (Chouaia *et al*., 2014) in spiked mango and spiked mango + HMP might be due to contamination introduced by pricking which accelerated the rate of decomposition.

For the second category of mango VOCs from larvae infested experiment. VOCs trapped from intact mango which also served as control, 60 compounds were identified similar to those reported by Pino *et al*., (2005) who documented large number of compounds including, terpenoids like monoterpenes, sesquiterpenes and irregular terpenes of low molecular weight. With regard to a fairly large number of aroma compounds in fruits

mango included, the question arises as to what is the significance of these compounds to fruits. To answer this question, (Rodrıguez *et al*., 2013)classified fruits into four categories on the basis of their flavor volatiles: a) fruits whose aroma is largely given by one compound, i.e. a single character impact compound, b) fruits in which a small number of compounds are involved, c) fruits where a large number of compounds are required to reproduce an aroma, and d) fruits whose aroma cannot be reproduced even by a large number of compounds. Examples of fruits whose aroma is largely given by a single compound or a group of compounds with similar structure such as methyl anthranilate in grapes, ethyl ester of (2*E*,4*Z*)-decadienoic acid in 'Bartlett' pear (Lalel *et al*., 2003; Rodrıguez & Alquezar & Pena, 2013) and ethyl 2-methylbutyrate in 'Golden delicious' apple (Rodrıguez *et al*., 2013).

In other fruits, aroma character relates to a small group of compounds such as (*E*)-2-hexenal, ethyl 2-methylbutyrate and ethyl 3-methylbutyrate in bilberry and linalool, (2*E*)-hexenal and (2*E*)-hexenol in blueberry(Rodrıguez *et al*., 2013). In others, such as mango, peach, pineapple and apricot large numbers of components which have been isolated are obviously more important from the point of view of imparting flavor notes, but no claims have been made for any individual or a specific set of compounds which give a recognizable aroma. Those compounds can only be considered as 'contributory to flavor compounds' not as 'character impact components' (Rodrıguez *et al*., 2013). The intact mango VOCs identified has been reported to serve as attractant to seed disseminators because of their unique scent and thus to ensure reproductive and evolutionary success (Rodrıguez *et al*., 2013) as most frugivorous mammals rely on olfactory stimuli to detect ripe fruits (Dominy, 2004).

While continuously trapping the VOCs from intact mango between day 1-10, six new metabolites of VOCs were detected (after day 4), while 6 VOCs which were present in day 1- 4, were not detected starting from between day 4 and 5. The new metabolites detected included: acetoin, 2,3-butanediol, propionic acid, propyl butanoate, ethyl 2,3-epoxybutyrate and phenylethyl alcohol. The alcohol, 2,3-butanediol, is likely to have been biosynthesized from acetoin, a fermentation product of the rotting mango fruit, using

acetoin reductase (Siemerink *et al*., 2011). Propionic acid is likely to be formed by action of bacteria on lactic acid (Boyaval and Corre, 1995) while propyl butanoate, ethyl 2,3-epoxybutyrate and phenylethyl alcohol are VOCs associated with rotting mango (Moalemiyan *et al*., 2006). The 6 VOCs which were not detected included: 5-methyl-2-hexanol, 4-methyl-2-hexanol, benzaldehyde, α-methyl styrene, myrcene, α-phellandrene, *β*-phellandrene. Their absence might be due to rotting of the mango fruit resulting to closure of the major biosynthetic pathways or it could also be as a result of the limited sample size use.

Additionally, when the larvae of the selected fruit flies; *B. dorsalis*, *C. cosyra C. rosa* and *C. fasciventris* were allowed to develop inside the mango and VOCs analyzed for a period of up to 10 days, the compounds detected varied qualitatively compared to the VOCs of intact mango. *B. dorsalis*, *C. cosyra C. rosa* and *C. fasciventris* infested fruit recorded a total of, 62, 69, 72 and 72 VOCs respectively with some of the new metabolites being detected while others were lost during the entire period of trapping owing to the change of the physiological state of the fruit. Two compounds worth noting are 2,3-butanediol and acetoin which were present in all the larvae- infested fruit starting from day 1 to 10 contrary to their appearance in day 5 in intact mango VOCs. It is recommended herein that further investigation be carried out with a view to pinpointing their role in the mango-larvae interactions.

In conclusion, we managed to identify a vast number of VOCs in this study, some of which can be directly associated to fruit fly larval infestation such as 2,3-butanediol and acetoin. In the context of agriculture these results constitute a significant contribution towards understanding the chemical ecology of the fruit fly-oviposition substrate interaction matrix which can be exploited in the optimization of HMPs as an integrated management tool for some species of fruit flies.

CHAPTER SEVEN

CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The current study reports the chemical identity of HMP of *C. cosyra* as glutathione (GSH) and for the two related fruit flies *C. fasciventris* and *C. rosa* as glutamic acid (GA) pending field evaluation This study isolated and identified GSH and GA from the mature females' feces of. *cosyra, C. fasciventris* and *C. rosa.* The HMP of *C. cosyra* GSH, was able to deter conspecifics and heterospecifics *C. fasciventris, C. rosa, C. capitata* and *Z. cucurbitae* from ovipositing and was also elicit an arresting behavior in the egg parasitoid *F. arisanus*. In contrast, GSH had no effect on oviposition responses of the invasive oriental fruit fly species *B. dorsalis* and *C. anonae.* These two fruit flies do not exhibit host marking behavior. Additionally, GSH did not alter the volatile composition of the mango which was used as an oviposition substrate. These results open an avenue towards potential of the host- marking technique/pheromone(s) in the Integrated Pest Management of fruit flies.

7.2 Recommendations of the study

The HMP of *C. cosyra, C. fasciventris* and *C. rosa* has been identified and shown to work in the laboratory and semi-field conditions. For the HMPs to be adopted as an Integrated Pest Management (IPM) tool, field evaluation has to be conducted more so using glutathione as the current results showed it to have a broad-spectrum oviposition deterrence to both conspecifics and heterospecifics.

The government and policy makers should implement stringent control measures for use of pesticides for control of fruit flies in order to meet international market standards on strict maximum residue levels (MRLs). This can be achieved through spot spraying and adoption of eco-friendly techniques such as the use of HMP

More research is required to establish the biosynthetic pathway and site of production of the identified host marking pheromone in order to fully understand the HMP Chemistry.

Environmental impact assessment for the use of HMPs needs to be evaluated more so its mode of detection and long-term effects on the fruit fly parasitoids

The results from this study showed varied oviposition deterrence by conspecifics and heterospecifics of *C. fasciventris* and *C. rosa* compared to their fecal matter extract suggesting additional minor components in the fecal matter that were not characterized.

The identified volatile organic compounds from the mango fruits should be exploited in the development of fruit fly odor baited traps.

7.3 Recommendations for further research

This study should be expounded to identify host marking pheromones in other insect pests that exhibit host marking behavior following the protocol developed here.

REFERENCES

Adams, R. P. (2007). *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry* (4th ed.). Allured Publishing Corporation, Carol Stream, pp 45-89.

Addesso, K. M., McAuslane, H. J., Stansly, P. A., & Schuster, D. J. (2007). Host-marking by female pepper weevils, *Anthonomus eugenii*. *Entomologia Experimentalis et Applicata*, *125*, 269–276.

Alfaro, C., Vacas, S., Zarzo, M., Navarro-Llopis, V., & Primo, J. (2011). Solid phase microextraction of volatile emissions of *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae): Influence of fly sex, age, and mating status. *Journal of Agricultural and Food Chemistry*, *59*, 298–306.

AliNiazee, M. T., & Long, L. E. (1996). *Biology and Control of the Cherry Fruit Flies: A Worldwide Perspective*. *Proceedings of the International Cherry Fruit Fly Symposium*, pp 5-10.

Aluja, M, & Boller, E. F. (1992). Host marking pheromone of *Rhagoletis cerasi*: field deployment of synthetic pheromone as a novel cherry fruit fly management strategy. *Entomologia Experimentalis et Applicata*, *65*, 141–147.

Aluja, M, Diaz-Fleisher, F., Edmundus, A. F., & Hagmann, L. (2003). Isolation, structural determination, synthesis, biological activity and application as control agent of the host marking pheromone (and derivatives thereof) of fruit flies of the type *Anastrepha* (Diptera: Tephritidae). U.S Patent 6,555,120 B1, 2003.

Aluja, Martin, & Boller, E. F. (1992). Host marking pheromone of *Rhagoletis cerasi*: Foraging behavior in response to synthetic pheromonal isomers. *Journal of Chemical Ecology*, *18*, 1299–1311.

Aluja, Martin, & Díaz-Fleischer, F. (2006). Foraging behavior of *Anastrepha Ludens*, *A. obliqua*, and *A. serpentina* in response to feces extracts containing host marking pheromone. *Journal of Chemical Ecology*, *32*, 367–389.

Aluja, Martñ, Díaz-Fleischer, F., Boller, E. F., Hurter, J., Edmunds, A. F., Hagmann, L., Patrian, B.; Reyes, J. (2009). Application of feces extracts and synthetic analogues of the host marking pheromone of *Anastrepha ludens* significantly reduces fruit infestation by *A. obliqua* in Tropical plum and mango backyard orchards. *Journal of Economic Entomology*, *102*, 2268–2278.

Arredondo, J., & Díaz-Fleischer, F. (2006). Oviposition deterrents for the Mediterranean fruit fly, *Ceratitis capitata* (Diptera : Tephritidae) from fly faeces extracts. *Bulletin of Entomological Research*, *96*, 35–42.

Asquith, A., & Messing, R. H. (1992). Attraction of Hawaiian ground litter invertebrates to protein hydrolysate Bait. *Environmental Entomology*, *21*, 1022–1028.

Australia, P. H. (2011). *The Australian Handbook for the Identification of Fruit Flies. Version 1.0*, pp 10-69.

Barr, N. B., & McPheron, B. A. (2006). Molecular phylogenetics of the genus *Ceratitis* (Diptera: Tephritidae). *Molecular Phylogenetics and Evolution*, *38*, 216–230.

Barrera, J. F., Gomez, J., & Alauzet, C. (1994). Evidence for a marking pheromone in host discrimination by *Cephalonomia Stephanoderis* (Hym.: Bethylidae). *Entomophaga*, *39*, 363–366.

Birke, A., Aluja, M., Greany, P., Bigurra, E., Pérez-Staples, D., & McDonald, R. (2006). Long aculeus and behavior of *Anastrepha ludens* render gibberellic acid ineffective as an agent to reduce “ruby red” grapefruit susceptibility to the attack of this pestiferous fruit fly in commercial groves. *Journal of Economic Entomology*, *99*, 1184–1193.

Boller, E. F., Hippe, C., Prokopy, R. J., Enkerlin, W., Katsoyannos, B. I., Morgante, J. S., Zapater, M. (1994). Response of wild and laboratory-reared *Ceratitis capitata* Wied. (Dipt., Tephritidae) flies from different geographic origins to a standard host marking pheromone solution. *Journal of Applied Entomology*, *118*, 84–91.

Boyaval P, & Corre C. (1995). Production of propionic acid. *Lait*, *75*, 453–461.

Bradbury, J. W., & Vehrencamp, S. L. (1999). *Principles of Animal Communicationin The design of animal communication*. (D. M. Hausser & K. Mark, Eds.). Sunderland. Bradbury: Sinauer Associates, Inc., pp 451-461.

Bray, D. P., & Hamilton, J. G. C. (2007). Host odor synergizes attraction of virgin female *Lutzomyia longipalpis* (Diptera: Psychodidae). *Journal of Medical Entomology*, *44*, 779–787.

Broillet, M.C., Randin, O., & Chatton, J.Y. (2001). Photoactivation and calcium sensitivity of the fluorescent NO indicator 4,5-diaminofluorescein (DAF-2): implications for cellular NO imaging. *FEBS Letters*, *491*, 227–232.

Bruce, T. J. A., & Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects--finding the right mix. *Phytochemistry*, *72*, 1605–11.

Butenandt, A., Beckmann, R., Stamm, D., & Hecker, E. (1959). On the sex attractant of the silk moth *Bombyx mori*. Purification and structure determination. (Ueber den Sexuallockstoff des Seidenspinners Bombyx mori. Reindarstellung und Konstitutionsermittlung). *Z. Naturforsch*, *14b*, 283–284.

Campbell, N. A., & Reece, J. B. (2005). *Biology* (7th Edition). San Francisco, California: Pearson Education, Inc., pp 10-72.

Cheseto, X., Kachigamba, D. L., Ekesi, S., Ndung'u, M., Teal, P. E. A., Beck, J. J., & Torto, B. (2017). Identification of the ubiquitous antioxidant tripeptide glutathione as a fruit fly semiochemical. *Journal of Agricultural and Food Chemistry*, *65*, 8560–8568.

Chouaia, B., Gaiarsa, S., Crotti, E., Comandatore, F., Esposti, M. D., Ricci, I., Daffonchio, D. (2014). Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts. *Genome Biology and Evolution*, *6*, 912–920.

Clarke, A. R., Armstrong, K. F., Carmichael, A. E., Milne, J. R., Raghu, S., Roderick, G. K., & Yeates, D. K. (2005). Invasive phytophagous pests arising through a recent tropical evolutionary radiation: the *Bactrocera dorsalis* complex of fruit flies. *Annual Review of Entomology*, *50*, 293–319.

Copeland, R. S., & Wharton, R. A. (2006). Year-round production of pest *Ceratitis* species (Diptera : Tephritidae) in fruit of the invasive species S*olanum mauritianum* in Kenya. *Annals Of The Entomological Society Of America*, *99*, 530–535.

Copeland, R. S., Wharton, R., Luke, Q., De Meyer, M., Lux, S., Zenz, N., Okumu, M. (2006). Geographic distribution, host fruit, and parasitoids of African fruit fly pests *Ceratitis anonae*, *Ceratitis cosyra*, *Ceratitis fasciventris*, and *Ceratitis rosa* (Diptera : Tephritidae) in Kenya. *Annals of the Entomological Society of America*, *99*, 261–278.

Cross, J. H., Byler, R. C., Silverstein, R. M., Greenblatt, R. E., Gorman, J. E., & Burkholder, W. E. (1977). Sex pheromone components and calling behavior of the female dermestid beetle, *Trogoderma variabile* Ballion (Coleoptera: Dermestidae). *Journal of Chemical Ecology*, *3*, 115–125.

de Graaf, J. (2009). Host status of avocado ('Hass') to *Ceratitis capitata*, *Ceratitis rosa*, and *Ceratitis cosyra* (Diptera: Tephritidae) in South Africa. *Journal of Economic Entomology*, *102*, 1448–1459.

De Meyer, M., & Freidberg, A. (2006). Revision of the subgenus *Ceratitis* (Pterandrus) Bezzi (Diptera: Tephritidae). *Israel Journal of Entomology*, *35*, 197–315.

De Meyer, M., Robertson, M. P., Peterson, A. T., & Mansell, M. W. (2008). Ecological niches and potential geographical distributions of Mediterranean fruit fly (*Ceratitis capitata*) and Natal fruit fly (*Ceratitis rosa*). *Journal of Biogeography*, *35*, 270–281.

Depetris-Chauvin, A., Galagovsky, D., & Grosjean, Y. (2015). Chemicals and chemoreceptors: ecologically relevant signals driving behavior in Drosophila. *Frontiers in Ecology and Evolution*, *3*, 1–21.

Diamantidis, A. D., Papadopoulos, N. T., & Carey, J. R. (2008). Medfly populations differ in diel and age patterns of sexual signalling. *Entomologia Experimentalis et Applicata*, *128*, 389–397.

Díaz-Fleischer, F, Papaj, D. R., Prokopy, R. J., Norrbom, A. L., & Aluja, M. (2000). *Evolution of fruit fly oviposition behavior. In: Aluja M, Norrbom AL, editors. Fruit Flies (Tephritidae): Phylogeny and Evolution of Behavior. Boca Raton*. CRC Press, pp 811- 841.

Díaz-Fleischer, Francisco, & Aluja, M. (2001). Behavior of Tephritid Flies: A Historical Perspective. *Fruit Flies (Tephritida) Phylogeny and Evolution of Behavior*, pp 39–69.

Dimbi, S., Maniania, N. K., & Ekesi, S. (2009). Effect of *Metarhizium anisopliae* inoculation on the mating behavior of three species of African Tephritid fruit flies, *Ceratitis capitata*, *Ceratitis cosyra* and *Ceratitis fasciventris*. *Biological Control*, *50*, 111–116.

Dominy, N. J. (2004). Fruits, Fingers, and Fermentation: The Sensory Cues Available to Foraging Primates. *Integrative and Comparative Biology*, *44*, 295–303.

Drew, R. A. I. and, & Romig, M. C. (2007). Records of dacine fruit flies and new species of *Dacus* (Diptera: Tephritidae) in Bhutan. *Raffles Bulletin of Zoology*, *55*, 1–21.

Droney, D. C. (1994). Tests of hypotheses for lek formation in a Hawaiian Drosophila. *Animal Behaviour*, *47*, 351–361.

Duyck, A. P., David, P., Junod, G., Brunel, C., & Quilici, S. (2006). Importance of competition mechanisms in successive invasions by polyphagous Tephritids in La Re´ Union. *Ecology*, *87*, 1770–1780.

Duyck, P.F., David, P., & Quilici, S. (2004). A reveiw of relationshipos between interspecific competition and invasions in fruit flies (Diptera: Tephritidae). *Ecological Entomology*, *29*, 511–512.

Edmunds, A. J. F., Aluja, M., Diaz-Fleischer, F., Patrian, B., & Hagmann, L. (2010). Host marking pheromone (HMP) in the Mexican fruit fly *Anastrepha ludens*. *Chimia*, *64*, 37–42.

Egonyu, J. P., Ekesi, S., Kabaru, J., Irungu, L., & Torto, B. (2013). Cashew volatiles mediate short-range location responses in *Pseudotheraptus wayi* (Heteroptera: Coreidae). *Environmental Entomology*, *42*, 1400–7.

Ekesi, S, Nderitu, P. W., & Rwomushana, I. (2006). Field infestation, life history and demographic parameters of the fruit fly *Bactrocera invadens* (Diptera: Tephritidae) in Africa. *Bulletin of Entomological Research*, *96*, 379–386.

Ekesi, Sunday, Billah, M. K., Nderitu, P. W., Lux, S. A., & Rwomushana, I. (2009). Evidence for competitive displacement of *Ceratitis cosyra* by the invasive fruit fly *Bactrocera invadens* (Diptera: Tephritidae) on mango and mechanisms contributing to the displacement. *Journal of Economic Entomology*, *102*, 981–991.

Ekesi, Sunday, & Kelemu, S. (2017). Fruit fly bait production facility launched in Kenya Plant will produce icipe Fruitfly Mania. Retrieved May 16, 2017, from

Ekesi, Sunday, Mohamed, S. A., & Meyer, M. De. (2016). *Fruit Fly Research and Development in Africa - Towards a Sustainable Management Strategy to Improve Horticulture*. (Sunday Ekesi, S. A. Mohamed, & M. De Meyer, Eds.). Switzerland: Springer International Publishing, pp 10-90.

Enjin, A., & Suh, G. S. B. (2013). Neural mechanisms of alarm pheromone signaling. *Molecules and Cells*, *35*, 177–181.

Faraone, N., Hillier, N. K., & Cutler, G. C. (2016). Collection of host-marking pheromone from Rhagoletis mendax (Diptera: Tephritidae). *The Canadian Entomologist*, *148*, 552–555.

Ferguson, A. W., Solinas, M., Ziesmann, J., Isidoro, N., Williams, I. H., Scubla, P., … Wadhams, L. J. (1999). Identification of the gland secreting oviposition-deterring pheromone in the cabbage seed weevil, *Ceutorhynchus assimilis*, and the mechanism of pheromone deposition. *Journal of Insect Physiology*, *45*, 687–699.

Ferreres, F., Silva, B. M., Andrade, P. B., Seabra, R. M., & Ferreira, M. A. (2003). Approach to the study of C-glycosyl flavones by ion trap HPLC-PAD-ESI/MS/MS: application to seeds of quince (*Cydonia oblonga*). *Phytochemical Analysis*, *14*, 352–359.

Flath, R. A., Jang, E. B., Light, D. M., Mon, T. R., Carvalho, L., Binder, R. G., & John, J. O. (1993). Volatile pheromonal emissions from the male Mediterranean fruit fly: effects of fly age and time of day. *Journal of Agricultural and Food Chemistry*, *41*, 830–837.

Fletcher, B. S. and R. J. P. (1991). *Host location and oviposition in tephritid fruit flies.In: Bailey, W. J. and Ridsdill-Smith J. (Eds.). Reproductive Behaviour of lnsects: Individuals and Populations*. New York City: Chapman and Hall, pp 44-72.

Francke, W., Heemann, V., Gerken, B., Renwick, J. A. A., & Vité, J. P. (1977). 2-Ethyl-1,6-dioxaspiro[4.4]nonane, principal aggregation pheromone of *Pityogenes chalcographus* (L.). *Naturwissenschaften*, *64*, 590–591.

Grieco, F., L. Loigens, O. Krips, G. Smith, A. Spink, and P., & Zimmerman. (2010). *EthoVision XT: the next generation of video tracking software, reference manual version 8. Noldus Information Technology*. Wageningen, The Netherlands.

Griesbach, J. (2003). Description of mango cultivars. in Mango growing in Kenya. In A. M. Nyamu & T. Simons (Eds.), *World Agroforestry Centre (ICRAF)*. Nairobi, Kenya: Kul Graphics, pp. 18–78.

Haq, I., Vreysen, M. J. B., Cacéres, C., Shelly, T. E., & Hendrichs, J. (2014). Methyl eugenol aromatherapy enhances the mating competitiveness of male *Bactrocera carambolae* Drew & Hancock (Diptera: Tephritidae). *Journal of Insect Physiology*, *68*, 1–6.

Hern, A., & Dorn, S. (2002). Induction of volatile emissions from ripening apple fruits infested with *Cydia pomonella* and the attraction of adult females. *Entomologia Experimentalis et Applicata*, *102*, 145–151.

Ian M. White, M. M. E.-H. (1992). *Fruit Flies of Economic Significance: Their Identification and Bionomics*. Australian Centre for International Agricultural Research: CAB International.

ICIPE. (2007). *Development and implementation of a sustainable IPM program for major mango pests and opportunity for improving market information and processing in sub-Saharan Africa*.

Irene, M. (2017, March 30). Boost for mango farmers as Icipe launches fruit fly bait. *Daily Nation*. Retrieved from https://www.nation.co.ke/counties/muranga/Icipe-fruit-fly-bait/1183310-3870338-14182pw/index.html

Kachigamba, D. L., Ekesi, S., Ndung'u, M. W., Gitonga, L. M., Teal, P. E. A., & Torto, B. (2012). Evidence for potential of managing some African fruit fly species (Diptera: Tephritidae) using the mango fruit fly host-marking pheromone. *Journal of Economic Entomology*, *105*, 2068–75.

Kandel, E. R., & Siegelbaum, S. A. (2000). Transmitter release from insect excitatory motor nerve terminals. *Principles of Neural Science*, *227*, 253–279.

Karlson, P., & Luscher, M. (1959). Pheromones': a new term for a class of biologically active substances. *Nature*, *183*, 55–56.

Kriticos, D. J., Stephens, A. E. A., & Leriche, A. (2007). Effect of climate change on oriental fruit fly in New Zealand and the pacific. *New Zealand Plant Protection*, *60*, 271–278.

Kumaran, N., Balagawi, S., Schutze, M. K., & Clarke, A. R. (2013). Evolution of lure response in tephritid fruit flies: Phytochemicals as drivers of sexual selection. *Animal Behaviour*, *85*, 781–789.

Lalel, H. J. D., Singh, Z., & Tan, S. C. (2003). Aroma volatiles production during fruit ripening of 'Kensington Pride'mango. *Postharvest Biology and Technology*, *27*, 323–336.

Larsson, M. C. (2003). Nose talk: pheromones and animal behaviour; communication by smell and taste. *Journal of Experimental Biology*, *206*, 4382–4383.

Loy, F., Solari, P., Isola, M., Crnjar, R., & Masala, C. (2016). Morphological and electrophysiological analysis of tarsal sensilla in the medfly *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae). *Italian Journal of Zoology*, *83*, 456–468.

Lux, S. A., Copeland, R. S., White, I. M., Manrakhan, A., & Billah, M. K. (2003). A new invasive fruit fly species from the *Bactrocera dorsalis* (Hendel) group detected in East Africa. *International Journal of Tropical Insect Science*, *23*, 355–361.

Lux, S. A., Ekesi, S., Dimbi, S., Mohamed, S., & Maxwell, B. (2002). *Mango-infesting fruit flies in Africa: perspectives and limitations of biological approaches to their management.* Nairobi, Kenya.

Mack, R. N., Simberloff, D., Lonsdale, W. M., Evans, H., Clout, M., & Bazzaz, F. A. (2005). Biotic invasions: causes, epidemiology, global consequences, and control. *Bulletin of the Ecological Society of America*, *86*, 249–250.

Marc, D. M., Copeland, R. S., Lux, S. A., Mansell, M., Quilici, S., Wharton, R., Zenz, N. (2002). Annotated check list of host plants for Afrotropical fruit flies (Diptera: Tephritidae) of the genus *Ceratitis*. *Documentation Zoologique, Musée Royal de l'Afrique Centrale*. Tervuren, Belgium: Royal Museum for Central Africa, pp 271-299

Meister, A., & Anderson, M. E. (1983). Glutathione. *Annual Review of Biochemistry*, *52*, 711–760.

Midland, M. M., & Nhan H. Nguyen. (1981). Asymmetric synthesis of .gamma.-lactones. A facile synthesis of the sex pheromone of the Japanese beetle. *The Journal of Organic Chemistry*, *46*, 4107–4108.

Mitra, S. K. (2016). Mango production in the world – present situation and future prospecto Title. In *XXIX International Horticultural Congress on Horticulture: Sustaining Lives, Livelihoods and Landscapes (IHC2014): IV International Symposium on Papaya, VIII International Pineapple Symposium, and International Symposium on Mango .Acta Horticulturae* (Vol. 1111, pp. 287–296).

Moalemiyan, M., Vikram, A., Kushalappa, A. C., & Yaylayan, V. (2006). Volatile metabolite profiling to detect and discriminate stem-end rot and anthracnose diseases of mango fruits. *Plant Pathology*, *55*, 792–802.

Montoya, P., Liedo, P., Benrey, B., Cancino, J., Barrera, J. F., Sivinski, J., & Aluja, M. (2000). Biological Control of Anastrepha spp. (Diptera: Tephritidae) in Mango Orchards through Augmentative Releases of *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae). *Biological Control*, *18*, 216–224.

Moreno, D. S., Martinez, A. J., & Sanchez Riviello, M. (1994). Cyromazine Effects on the Reproduction of Anastrephaludens (Diptera: Tephritidae) in the Laboratory and in the Field. *Journal of Economic Entomology*, *87*, 202–211.

Mwatawala, M., Virgilio, M., Joseph, J., & De Meyer, M. (2015). Niche partitioning among two *Ceratitis rosa* morphotypes and other *Ceratitis* pest species (Diptera, tephritidae) along an altitudinal transect in central Tanzania. *ZooKeys*, *2015*, 429–442.

Mwatawala, M., White, I. ., Maerere, A., Senkondo, F. ., & Meyer, M. (2004). A new invasive *Bactrocera species* (Diptera: Tephritidae) in Tanzania. *African Entomology*, *12*, 154–158.

Noctor, G., Queval, G., Mhamdi, A., Chaouch, S., & Foyer, C. H. (2011). Glutathione. *The Arabidopsis Book / American Society of Plant Biologists*, *9*, e0142.

Nufio, C. R., & Papaj, D. R. (2001). Host marking behavior in phytophagous insects and parasitoids. *Entomologia Experimentalis et Applicata, 99*, 273-293.

Nufio, C. R., & Papaj, D. R. (2004). Host-marking behaviour as a quantitative signal of competition in the walnut fly *Rhagoletis juglandis*. *Ecological Entomology*, *29*, 336–344.

Ojeda-Chi, M. M., Rodriguez-Vivas, R. I., Galindo-Velasco, E., & Lezama-Gutirrrez, R. (2010). Laboratory and field evaluation of *Metarhizium anisopliae* (Deuteromycotina: Hyphomycetes) for the control of *Rhipicephalus microplus* (Acari: Ixodidae) in the Mexican tropics. *Veterinary Parasitology*, *170*, 348–354.

Papa, J. D. R., & Aluja, M. (1993). Temporal dynamics of host-marking in the tropical tephritid fly,. *Physiological Entomology*, *18*, 279–284.

Papadopoulos, N. T., Katsoyannos, B. I., Nikos, K. A., & Hendrichs, J. (2003). Effect of orange peel substances on mating competitiveness of male *Ceratitis capitata*. *Entomologia Experimentalis et Applicata*, *99*, 253–261.

Papaj, D. R., Averill, A. L., Prokopy, R. J., & Wong, T. T. Y. (1992). Host-marking pheromone and use of previously established oviposition sites by the mediterranean fruit fly (Diptera: Tephritidae). *Journal of Insect Behavior*, *5*, 583–598.

Pare, P. W., & Tumlinson, J. H. (1999). Update on plant-insect interactions plant volatiles as a defense against insect herbivores by releasing greater amounts of a variety. *Plant Physiology*, *121*, 325–331.

Picker, M., Griffiths, C., & Weaving, A. (2002). *Field guide to insects of South Africa*. *African Zoology* (Vol. 38). Struik.

Pickett, S. T. A., & Cadenasso, M. L. (2002). The ecosystem as a multidimensional concept: meaning, model, and metaphor. *Ecosystems*, *5*, 1–10.

Pino, J. A., Mesa, J., Muñoz, Y., Pilar Martí, M., & Marbot, R. (2005). Volatile components from Mango (Mangifera indica L.) cultivars. *Journal of Agricultural and Food Chemistry*, *53*, 2213–2223.

Ploetz, R. C. (1994). Distribution and prevalence of *Fusarium subglutinans* in mango trees affected by malformation. *Canadian Journal of Botany*, *72*, 7–9.

Prokopy, R., Aluja, M., & Wong, T. (1989). Foraging behavior of laboratory cultured Mediterranean fruit flies on field-caged host trees. *Hawaiian Entomological Society*, *29*, 103–109.

Qu, J., Chen, W., Luo, G., Wang, Y., Xiao, S., Ling, Z., & Chen, G. (2002). Rapid determination of underivatized pyroglutamic acid, glutamic acid, glutamine and other relevant amino acids in fermentation media by LC-MS-MS. *The Analyst*, *127*, 66–69.

Quilici, S., Schmitt, C., Vidal, J., Franck, A., & Deguine, J. P. (2013). Adult diet and exposure to semiochemicals influence male mating success in *Ceratitis rosa* (Diptera: Tephritidae). *Journal of Applied Entomology*, *137*, 142–153.

Quilici, Serge, & Rousse, P. (2012). Location of host and host habitat by fruit fly parasitoids. *Insects*, *3*, 1220–1235.

R Development Core Team. (2012). A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. Retrieved from http://www.r-project.org

Ramanathan, R., Cao, K., Cavalieri, E., & Gross, M. L. (1998). Mass spectrometric methods for distinguishing structural isomers of glutathione conjugates of estrone and estradiol. *Journal of the American Society for Mass Spectrometry*, *9*, 612–619.

Ranson, H., & Hemingway, J. (2005). Mosquito Glutathione Transferases. In *Methods in enzymology*, 401, 226-241.

Rodrıguez, A., & Berta Alquezar and Leandro Pena. (2013). Fruit aromas in mature fleshy fruits as signals of readiness for predation and seed dispersal. *New Phytologist*, *197*, 36–48.

Roitberg, B. D., & Lalonde, R. G. (1991). Host marking enhances parasitism risk for a fruit-infesting fly *Rhagoletis basiola*. *Oikos*, *61*, 389–393.

Rosi, M. C., Isidoro, N., Colazza, S., & Bin, F. (2001). Source of the host marking pheromone in the egg parasitoid *Trissolcus basalis* (Hymenoptera: Scelionidae). *Journal of Insect Physiology*, *47*, 989–995.

Rwomushana, I., Ekesi, S., Gordon, I., & Ogol, C. K. P. O. (2008). Host plants and host plant preference studies for *Bactrocera invadens* (Diptera: Tephritidae) in Kenya, a new invasive fruit fly Species in Africa. *Annals of the Entomological Society of America*, *101*, 331–340.

Rwomushana, I., Ekesi, S., Ogol, C. K. P. O., & Gordon, I. (2009). Mechanisms contributing to the competitive success of the invasive fruit fly *Bactrocera invadens* over the indigenous mango fruit fly, *Ceratitis cosyra*: The role of temperature and resource pre-emption. *Entomologia Experimentalis et Applicata*, *133*, 27–37.

Sarin, S., & Dukas, R. (2009). Social learning about egg-laying substrates in fruitflies. *Proceedings of the Royal Society B: Biological Sciences*, *276*, 4323–4328.

Schoonhoven, L. M. (1990). Host-marking pheromones in lepidoptera, with special reference to two *Pieris* spp. *Journal of Chemical Ecology*, *16*, 3043–3052.

Sekimizu, K., Larranaga, J., Hamamoto, H., Sekine, M., Furuchi, T., Katane, M., Matsuki, N. (2005). D-Glutamic acid-induced muscle contraction in the silkworm, *Bombyx mori*. *Journal of Biochemistry*, *137*, 199–203.

Shelly, T. E. (2004). Scent marking by males of the mediterranean fruit fly, *Ceratitis capitata* (Diptera: Tephritidae). *Journal of Insect Behavior*, *17*, 709–722.

Shelly, T. E., Edu, J., Pahio, E., & Nishimoto, J. (2007). Scented males and choosy females: Does male odor influence female mate choice in the Mediterranean fruit fly? *Journal of Chemical Ecology*, *33*, 2308–2324.

Shelly, T. E., & Kaneshiro, K. Y. (1991). Lek behavior of the oriental fruit fly, *Dacus dorsalis*, in Hawaii (Diptera: Tephritidae). *Journal of Insect Behavior*, *4*, 235–241.

Siebert, J. B., & Cooper, T. (1995). If medfly infestation triggered a trade ban: Embargo on California produce would cause revenue, job loss. *California Agriculture*, *49*, 7–12.

Sielezniew, M., & Stankiewicz-Fiedurek, A. M. (2013). Behavioural evidence for a putative oviposition-deterring pheromone in the butterfly, *Phengaris* (Maculinea) *Teleius* (Lepidoptera: Lycaenidae). *European Journal of Entomology*, *110*, 71–80.

Siemerink, M. A. J., Kuit, W., Contreras, A. M. L., Eggink, G., van der Oost, J., & Kengen, S. W. M. (2011). D-2,3-butanediol production due to heterologous expression of an acetoin reductase in *Clostridium acetobutylicum*. *Applied and Environmental Microbiology*, *77*, 2582–2588.

Silva, M. A., Bezerra-silva, G. C. D., & Mastrangelo, T. (2012). The Host Marking Pheromone application on the management of fruit flies - A Review. *Brazilian Archives of Biology and Technology*, *55*, 835–842.

Simon, J. Y. (1996). Insect glutathione S-transferases. *Zoological Studies*, *35*, 9–19.

Sivinski, J. M., Epsky, N., & Heath, R. R. (1994). Pheromone deposition on leaf territories by male Caribbean fruit flies, *Anastrepha suspensa* (loew) (Diptera: Tephritidae). *Journal of Insect Behavior*, *7*, 43–51.

Spinelli, F., Cellini, A., Marchetti, L., Nagesh, K. M., & Piovene, C. (2011). Emission and function of volatile organic compounds in response to abiotic stress. *Abiotic Stress in Plants - Mechanisms and Adaptations*, *16*, 367–394.

Stadler, E., Ernest, B., Hurter, J., Boller, E., & Kozlowski, M. (1992). Tarsal contact chemoreceptors of the cherry fruit fly, *Rhagoletis cerasi*: specificity, correlation with oviposition behaviour, and response to the synthetic pheromone. In *Proceedings of the 8 Symposium of Insect–Plant Relationships* . Netherlands: Kluwer Academic Publishers, Dordrecht, pp. 143–145.

Städler, E., Ernst, B., Hurter, J., & Boller, E. (1994). Tarsal contact chemoreceptor for the host marking pheromone of the cherry fruit fly, *Rhagoletis cerasi*: responses to natural and synthetic compounds. *Physiological Entomology*, *19*, 139–151.

Steck, G. J. (2012). Mango Fruit Fly , *Ceratitis cosyra* (Walker) (Insecta : Diptera: Tephritidae), p.1–3.

Stelinski, L. L., Rodriguez-Saona, C., & Meyer, W. L. (2009). Recognition of foreign oviposition-marking pheromone in a multi-trophic context. *Naturwissenschaften*, *96*, 585–592.

Sullivan, B. T., Pettersson, E. M., Seltmann, K. C., & Berisford, C. W. (2000). Attraction of the bark beetle parasitoid *Roptrocerus xylophagorum* (Hymenoptera: Pteromalidae) to host-associated olfactory cues. *Environmental Entomology*, *29*, 1138–1151.

Teal, P. E. A., & Gomez-simuta, Y. (2002). Juvenile hormone :Action in regulation of sexual maturity in Caribbean fruit flies and potential use in improving efficacy of sterile insect control technique for tephritid fruit flies. *IOBC Wprs Bulletin*, *25*, 1–15.

Teal, P. E. A., Gomez-Simuta, Y., & Proveaux, A. T. (2000). Mating experience and juvenile hormone enhance sexual signaling and mating in male Caribbean fruit flies. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 3708–3712.

Thompson, S. N. (1999). Nutrition and culture of entomophagous insects. *Annual Review of Entomology*, *44*, 561–592.

Vaníčková, L., Virgilio, M., Tomčala, A., Břízová, R., Ekesi, S., Hoskovec, M., De Meyer, M. (2014). Resolution of three cryptic agricultural pests (*Ceratitis fasciventris*, *C. anonae*, *C. rosa*, Diptera: Tephritidae) using cuticular hydrocarbon profiling. *Bulletin of Entomological Research*, *104*, 631–8.

Vargas, R. I., Piñero, J. C., & Leblanc, L. (2015). An overview of pest species of *Bactrocera* fruit flies (Diptera: Tephritidae) and the integration of biopesticides with other biological approaches for their management with a focus on the pacific region. *Insects*, *6*, 297–318.

Venu, I., Durisko, Z., Xu, J., & Dukas, R. (2014). Social attraction mediated by fruit flies' microbiome. *Journal of Experimental Biology*, *217*, 1346–1352.

Virgilio, M., Backeljau, T., Barr, N., & Meyer, M. De. (2008). Molecular evaluation of nominal species in the *Ceratitis fasciventris*, *C. anonae*, *C. rosa* complex (Diptera: Tephritidae). *Molecular Phylogenetics and Evolution*, *48*, 270–280.

Wang, X.-G., Jarjees, E. A., McGraw, B. K., Bokonon-Ganta, A. H., Messing, R. H., & Johnson, M. W. (2005). Effects of spinosad-based fruit fly bait GF-120 on tephritid fruit fly and aphid parasitoids. *Biological Control*, *35*, 155–162.

Weems, Jr HV, Heppner, J., Steck, G. J., Fasulo, T., & Nation, J. (2015). *Anastrepha ludens* (Loew) (Insecta: Diptera: Tephritidae). USA: Florida Department of Agriculture, Division of Plant Industry, Entomology. Retrieved from http://entomology.ifas.ufl.edu/creatures/fruit/tropical/mexican_fruit_fly.htm

Weems, Jr H V, & Heppner, J. B. (2015). Melon Fly , *Bactrocera cucurbitae* (Coquillett) (Insecta : Diptera : Tephritidae). *Florida Department of Agriculture and Consumer Services, Division of Plant Industry, and TR Fasulo, University of Florida. University of Florida Publication EENY-199*, 1–5.

White, I. M. (2006). Taxonomy of the Dacina (Diptera: Tephritidae) of Africa and the middle East. *African Entomology*, *2006*, 1–156.

Yoshinaga, N., Ishikawa, C., Seidl-Adams, I., Bosak, E., Aboshi, T., Tumlinson, J. H., & Mori, N. (2014). N-(18-Hydroxylinolenoyl)-l-Glutamine: A newly discovered analog of volicitin in *Manduca sexta* and its elicitor activity in plants. *Journal of Chemical Ecology*, *40*, 484–490.

Zeeck, E., Müller, C. T., Beckmann, M., Hardege, J. D., Papke, U., Sinnwell, V., Francke, W. (1998). Cysteine-glutathione disulfide, the sperm-release pheromone of the marine polychaete Nereis succinea (Annelida: Polychaeta). *Chemoecology*, *8*, 33–38.