Using the solid injection technique with gas chromatography and mass spectrometry to identify cuticular hydrocarbons in several thrips insect species

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This thesis is submitted for the degree of Master of Philosophy

October 2023

Keele University

Abstract

Thrips are a group of insects included in the order Thysanoptera. So far, over 6000 species have been described, among which over 100 are known as crop pests There is a plethora of studies on various species of thrips, yet the CHC composition is known in less than 10 species. By considering the fact that quite a high number of the thrips species are major pests and that some of the detected hydrocarbons are known to act as pheromones, the interest in investigating their chemistry is both for chemotaxonomy reasons and discovering sustainable measures for their control.

In this research study, the CHC content was described in five species of thrips using a new extraction technique based on solid injection. Principal Component Analysis was used to compare the CHC profile across the five species of thrips and the results showed that each species of thrips is characterized by the presence of a different class of hydrocarbons. Therefore, this indicates that the CHC profile can be used as a tool in thrips identification.

The efficiency of the solid injection method was tested on a cultured thrips species, western flower thrips, *Frankliniella occidentalis*. A proof-of-concept experiment was conducted and the CHC dataset obtained through whole-body analysis was compared with the one obtained through hexane extraction which is currently the most commonly used extraction method. Unpaired *t* - test was used for the statistical comparison of the two techniques and it showed no difference between the two hydrocarbon patterns, proving the efficiency of the solid injection method. Additionally, alkenes monoenes have been detected for the first time in western flower thrips with the aid of the solid injection method, through direct analysis of the whole body, but not through solvent extraction.

Moreover, in this work, it is reported for the first time, the CHC content in three species of thrips (*Franklinella borinquen, Frankliniella schultzei* and *Hydatothrips adlofifriderici*). Also, based on the data processing and the overall findings, it can be confirmed that with the aid of solid injection method specific CHC patterns can be detected and associated with a particular species of thrips, highlighting their role in thrips chemotaxonomy.

Table of Contents

List of tables

List of figures

Acknowledgements

I would like to start by saying that I dedicate this project to the most important person in my life, my partner, Andrei Stefan. Without your love and support I would not have seen this thesis through. I love you so much!

I am deeply grateful to my supervisor Dr Falko Drijfhout for the training and support he has given me during my studentship. I appreciate a lot all those early days and late afternoons you spent training me in operating the GC-MS, and the long online meetings to teach me how to identify hydrocarbons based on their mass spectra. You did an amazing job because I came a long way considering that I had absolutely no prior knowledge about the use of the GC-MS and compounds identification. I have always admired your dedication and commitment in your teaching role! Now, I am a teacher too and I hope to be able to have the same positive impact on my students, as well! Thank you so much!

There are several persons that have helped me a lot in seeing this project through, and I would like to express my gratitude to them:

- To Dr Akynyemy Adenyemi and Dr David Mfuti Kupesa for collecting the insects from the field, preparing the samples in the laboratory according to the instructions and for delivering them from Kenya to UK. I would have no results without your help, I appreciate a lot your effort!
- A special thank you to Dr David Buss for his friendship, encouragement during the short coffee breaks! But most of all for taking the time to read my thesis and making sure of an accurate English I appreciate a lot your help and effort!

- I am grateful to Prof. Dr William Kirk, it was your advice I followed to pursue an MPhil in Chemistry and highly recommended Falko as my supervisor based on their fruitful collaboration.
- Special thanks to my office colleagues, in Lennard Jones, especially Dr Sue Shemilt for those occasion when I needed assistance in operating the GC-MS in Falko's absence. It helped a lot to know I can always rely on your assistance!

Over time, the Thrips Research Group I am member in, has been visited by a series of international students I became friends with and their encouragement meant a lot to me. I would like to mention Rene Pereira, now a Chemistry graduate in Brazil, for his advice and short chemistry revision-lessons at the beginning of my studentship. Also, a special thank you to Dr Lekia Kumbe who has been a good friend and who inspired me that is never too late to pursue a course. Thank you so much for being there for me!

In the end I would like to say a massive thank you to my family, especially to my parents (Gabriela and Gigel) for their moral support and all the goodies they sent me from Romania, to help me cook faster and healthier so I can focus on my thesis! Thank you and I love you all very much!

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

The order Thysanoptera comprises insects commonly known as thrips. Over 6000 species of this group have been described and a number of them are major pests[1–3]. The order name indicates that in the adult both pair of wings are constituted of a marginal fringe of long cilia (thysano = fringed, pteron = wing). These insects are minute in size, most of them are 1 mm long with a narrow body[4]. A particular morphological feature is that thrips mouth parts are asymmetric, the left mandible is fully developed whereas the right one is resorbed within the embryonic stage[5].The mouth parts form a mouth cone underneath the head and enable the thrips to feed by piercing and sucking the cells' content[6]. The tarsi, the end part of their legs, is provided with an arolium, a small bladder that improves thrips ability to walk upside down and eases surface adhesion[5].

Thrips species are widely distributed across the world from tropical to temperate regions[7]. The food source varies and it is an important criterion to distinguish the economically important species. Based on their diet, there are thrips species that feed on fungi (hyphae and spores)[8] and other species which act as beneficial insects particularly as active pollinators[6,9,10]. Some thrips feed on other arthropods, being natural predators, therefore they play an important role in biocontrol strategies[11]. There are also the polyphagous and opportunistic species such as western flower thrips

(WFT) which feeds on a wide range of plants including crops causing serious damage that leads to significant economic losses[3,12].

Within the order Thysanoptera two main sub-orders are distinguished: Terebrantia and Tubulifera[2]. Terebrantian thrips have a life cycle that consists of four stages: egg, two active feeding larva instars, two non-feeding instars (propupa and pupa) and the adult (Fig. 1.1). In comparison, the Tubulifera species have a second pupa stage, thus six stages altogether[4].

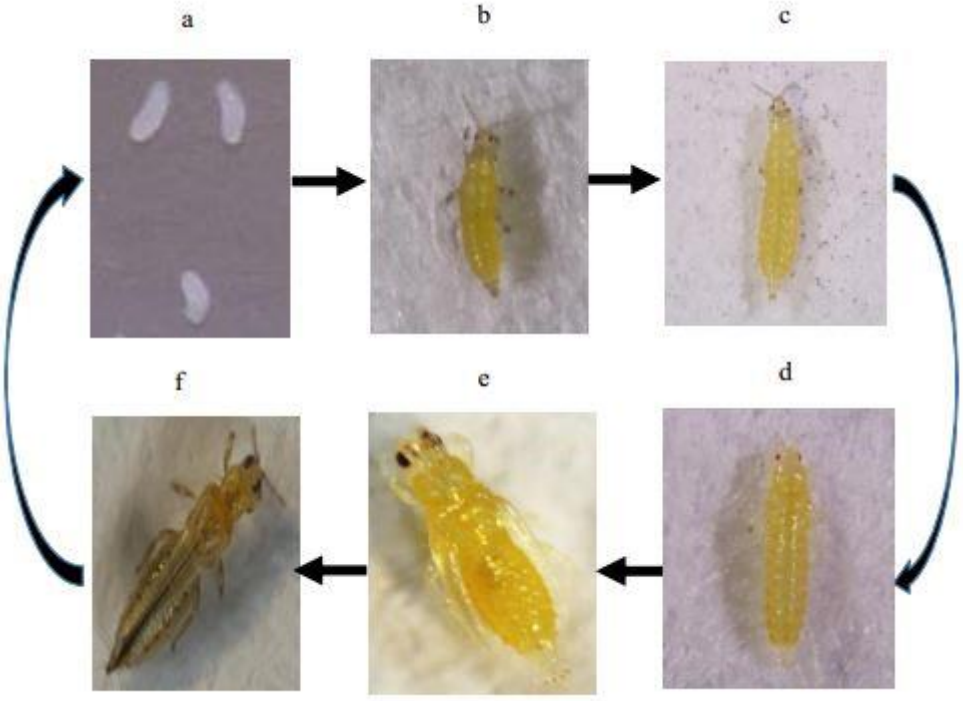


Fig. 1.1 Life cycle from egg to adult of the Terebrantian western flower thrips (from Akinyemi, 2018- with permission) a – eggs; b – first instar larva; c – second instar larva; d – propupa; e – pupa; f – adult female

Most of the thrips pest species (ca. 100), are in the Thripidae family in the Terebrantia sub-order. The thrips species in this sub-order possess a saw-like ovipositor used to lay the eggs within the plant tissue layer[5]. This oviposition behaviour represents one of the direct modes of injuring the plants. The attack symptoms of thrips on plants consist of grey or silvery spots, scarring, necrosis, distorted growth and deformed flowers[6]. If these symptoms occur in early phenological stages of the plants, they fail to reach maturity. Moreover, if uncontrolled, the presence of thrips can cause serious economic loss[1]. In the UK, thrips damage has been associated with a number of crop plants grown in different systems: on leek cultivated in the field[13], on cucumber grown in greenhouses[14] and on strawberry in open-sided polytunnels[12].

A series of factors make the control of thrips a great challenge: cryptic behaviour, high reproductive rate and low sensitivity to pesticides[15]. Moreover, their feeding and egg laying behaviour along with their virus vector status contributes to increased plant injuries which leads to downgraded or unmarketable produce and to severe yield loss[16]. For instance, thrips damage affected nearly 60-80% of French bean and up to 80% of tomato production in Africa[17], 30-50% of cotton seedling yield in the United States[18] and 10-15% of strawberry crop in the UK[12]. As thrips control cannot be based on a single method, a combination of different tactics proved to be more effecient[16], a concept also known as Integrated Pest Management (IPM).

The IPM premise involves a series of pest control approaches aimed to reduce the negative impact of the crop pests on the economy, environment and society[19]. For this purpose, a range of measures (physical, mechanical, cultural, chemical and biological) are combined with regular monitoring[20,21]. When these approaches are selected cost-benefit analyses are also considered in order to establish the Economical Injury Level (EIL) and develop an Economic Threshold (ET). The concept

of EIL indicates the lowest number of pest insects capable of causing economically damage to a crop[22,23], whereas the ET, as defined by Zalom[24] refers to pest density at which the value of resulting damage exceeds the cost of applying a control. Thereby, the ET is often used by growers as an indicator for control method decisions[22]. Unfortunately, quite frequently the chosen treatments are mainly based on chemical insecticides[21,25–28]. According to a recent Pesticides Usage Survey, in the UK, in 54% of the cases farmers used the insecticides to manage the thrips attack for the outdoor onion and leek[29]. Additionally, 48% of the edible protected crops were chemically sprayed due to thrips injuries[30].

Overall, the combination of the chemical and biological methods is often critical for the integrated control of pest insects[31]. However, in order to enable the effective and economic use of the synthetic insecticides new targeted approaches have been developed using the thrips semiochemicals[32–36]. According to Law and Regnier[37] semiochemicals are natural chemical signals that are used for intra- and interspecific communication. These compounds are widely used as management techniques for thrips monitoring and behaviour control[21,36,38] (section 1.4).

For better usage of semiochemicals a comprehensive knowledge is required regarding thrips chemistry and in-depth research to be conducted both in the field and laboratory in regards to how their behaviour is manipulated by the new identified compounds[36,39,40]. Furthermore, it is crucial to optimize and improve the analytical methods for detecting these highly volatile molecules from very small insects[41,42].

1.2 Thrips identification

Thrips identification is mainly based on their external morphology and therefore acquiring detailed information on biology and morphology of the insect is necessary

for an accurate identification. Regardless of whether the scope is a routine identification, archiving or taxonomic research, slide preparation is required. If slides are prepared for taxonomic research, then the mounted specimens need to retain their shape and colour in a condition as close as possible to the natural state. This type of slide preparation is quite time consuming as it could take up to 3 days. Also, good quality slide preparation is important in order to distinguish and locate the main anatomical traits. However, for the identification process, dichotomous and interactive keys are available[43–47], though only a limited number of species are included. One of the challenges for taxonomists is the identification of the thrips instar larvae, though Fail[48] suggested it could be overcome with the aid of molecular identification, but this tool is not available for all the species.

Identification can also be difficult when two species cannot be distinguished based on their morphological traits. This challenge is encountered in dealing with cryptic species (also known as species complex). This term is defined as two or more morphologically indistinguishable biological groups that are incapable of inter-breeding[49]. A species complex occurs within thrips species, in onion thrips, *Thrips tabaci* (Lindeman). The onion thrips mode of reproduction is both arrhenotokous (unfertilised eggs develop into male progeny, although, fertilised eggs develop into females)[50] and thelytokous (unfertilised eggs develop into female progeny)[50], thus these two types of adult females of thrips are morphological identical and co-occur in the field. They have been distinguished, though, based on their DNA sequences into three lineages: a tobacco-associated (T) and two leek-associated types (L1, L2)[48]. Additionally, Li and co-workers[51] demonstrated that there is a difference between these lineages in terms of plant host related performance, life table and demographic growth performance. Their results revealed that the performance of arrhentokous and

thelytokous *T. tabaci* was host-plant dependent, highlighting the relationship and evolution of the two reproductive modes and their population dynamics on different crops.

In terms of larvae, unfortunately, the genetic-based method was not successful in separating the progeny of these two types of females[48]. Thus, new approaches are necessary to find suitable solutions for a correct species recognition. An alternative could be the separation of the species based on their chemical profile which could be, perhaps, integrated with the genetic-based method in order to enhance the accuracy of the identification process.

In general, a significant advantage of the chemical analysis technique, for both adult and larval identification, is that it provides rapid analysis, thus laboratory rearing of the insect to obtain the adults would not be needed. In addition, this highlights its potential role in taxonomy[52]. However, in regards to species differentiation, the insect chemical analysis relies on the identification of their cuticular hydrocarbon profile (section 1.6) to generate a species-specific 'fingerprint'[53]. Hence, the difficulties regarding species separation, could be overcome with the aid of the CHC as a potential tool. CHC analysis was successfully used in distinguishing two species of ants which were morphologically very similar[52]. Moreover, it proved to be an additional tool in identification of the first instar larvae for three forensically important blowfly[53]. Based on these studies, there might be a good prospect for successfully using the CHCs in distinguishing the three lineages of the larvae in *T. tabaci.*

1.3 Plant semiochemical response in thrips

Semiochemicals are the basis of inter- and intraspecific chemical communication[42]. Interspecific communication involves a class of semiochemicals

called allelochemicals[21,54]. This class is further divided into two sub-classes: allomones and kairomones. Allomones, which are defensive chemicals emitted by species of plants against phytophagous insects, benefit the sender. On the other hand, kairomones evoke a behavioural or physiological response in the receiver of the signal that is adaptively favourable to the receiver but not the emitter[41,55]. Plants release volatiles that are perceived by insects as olfactory cues which have an important role, among others, in guiding the insects to locate their host-plants[56]. Based on such plant-insect interaction many studies have shown the attractant, feeding deterrent, repellent or toxic effects of different plant volatiles on thrips[57–62]. For instance, several previous investigations indicated that *p* - anisaldehyde, a floral scent component, strongly attracted thrips pests like: western flower thrips *Frankliniella occidentalis* Pergande[56,63], the onion thrips *T. tabaci*[61] and flower thrips *Frankliniella intonsa* Trybom[64]. Various plant substances belonging to monoterpene and sesquiterpene chemical groups tested in Y-shaped olfactometer attracted thrips at a range of concentrations, whereas the benzenoids benzaldehydes attracted the thrips at only one of the tested concentrations[56]. However, within the same research, Koschier and co-workers[56], concluded that non-floral odours, like ethyl nicotinate, attracted WFT over a range of four different concentrations. Some of these thrips' attractant odours have been tested in the field in conjunction to the sticky traps. For example, the addition of methyl isonicotinate – commercially available under the name Lurem-TR (marketed by Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands), significantly increased the number of trapped adults of *F. occidentalis*[61,65].

In contrast, a few compounds have a repellent effect on thrips[58]. For example, Abtew and co-workers[66] tested the repellent effect of 24 plant extracts against adult female thrips of *Meglurothrips sjostedti* Trybom. The compounds with the highest

repellent effect were mono and sesquiterpene hydrocarbons and were collected from *Piperum nigrum* Lindeman*, Cinnamomum zeylanicum* Blume and *C. cassia* Blume. Another study conducted on *F. occidentalis* showed that the repellent effect of the tested essential oils was enhanced by incorporating the treatment compounds (*Thymus vulgaris* L. and *Satureja montana* L.) in alginate and amethyst cellulose polymers[67].

Plant volatiles induce behavioural responses not only in pest insects but also in their natural enemies[68]. For instance, rosemary volatiles repelled both *F. occidentalis* and the predatory bug *Orius laevigatus* Fieber, whereas carvacrol attracted *O. laevigatus*, but deterred *F. occidentalis* from feeding[69]. Therefore, multi-trophic interactions must be considered when plant semiochemicals are applied.

1.4 Thrips pheromones and their use in IPM

The term pheromone is derived from the Greek *pherein* (to carry) and *horaman* (to excite or to stimulate)[70]. Within the same paper, Karlson and his co-worker[70] proposed for the first time the term pheromone and defined it as a substance which is secreted to the outside by an individual and causes a specific reaction in a receiving individual of the same species. Pheromones are species-specific semiochemicals and just like plant produced volatiles are nontoxic for the environment, hence their high potential as tools for the management of agricultural pests[41].

Different types of pheromones having numerous roles (attraction, aggression, aphrodisiac, anti-aphrodisiac, aggregation, kin recognition and alarm signalling) were elucidated in a wide variety of arthropods[71]. The attraction pheromones are mainly associated with reproductive behaviour, thus, quite often, may be referred to as sex pheromones. The chemical signals emitted by one of the conspecific insects induce

the orientation reaction of one sex to the other for courtship and mating initiation[72]. Although, in most insects the sex attractant pheromone is produced by females (for instance, as firstly seen in moths[73]), there are groups of insects in which it is produced by males. Male-produced pheromones can also be named aggregation pheromone if they attract both females and males[72,74,75]. Male pheromones that are produced to attract females, may be released in association with host plants or plant material used by females for feeding or oviposition[54,72]. Likewise, the aggregation behaviour may result in attracting a potential mate.

In arthropods, mating behaviour can also be mediated by chemical cues that have an opposite function of a sex attractant pheromone. Such pheromones, known as anti-aphrodisiacs, are transferred by males to females and mark a female's mating status[76,77]. In the literature, the function of the anti-aphrodisiac pheromone was also interpreted as male's scope to ensure paternity or to induce female monogamy[76,78]. For instance, a male that marks a female with an anti-aphrodisiac pheromone will increase his chances that the offspring carry his genes and him being the only mating partner as the female will be less disturbed by subsequent males. On top of that, this pheromone could affect females' attractiveness which also results in other males staying away from her[78]. However, if this pheromone is scented from a certain distance, a male would be able to discriminate an available female against a less willing one and therefore, he will reduce the effort of searching for a mating partner[79].

Beside the sex attractant pheromone, the contact pheromone has also an essential role in insect reproduction[80,81]. In some longhorned beetle species mate recognition is mediated by contact pheromones. Since in these species of beetles there were not identified any long-range sex attractants, the male and female are speculated to be united by the volatiles emitted by the host plant or using visual cues[82].

The behaviour studies conducted by Mutis and co-workers[80] indicated that the antennal contact between the two sexes is critical for a successful copulation rate, suggesting that important receptors are located in the antenna sensory structure. This was also highlighted within laboratory experiments which showed that males' inability to contact the females with their antennae resulted in reduction of the mating success[83]. Also, the laboratory bioassays showed that along mechano-reception, chemical cues, such as CHCs were involved. This was shown by immersing freeze-killed females in a solvent which washed off the CHCs and when males made contact with these females no attempt in copulation was seen[80,84].

When in danger, the insects defend themselves by releasing an alarm pheromone which is used to signal, for example, the presence of a natural predator. The produced reactions are usually described by increased activities such as: enhanced take-off rates and decreased landing rates[85], rapid flight in flying insects[54], stopping feeding, dispersal[86] and increased aggression towards an antagonist[87]. However, as compared to aggregation pheromone, the alarm pheromone is less species-specific, thus it can be detected by other species. For instance, the natural enemies can use the alarm pheromone as kairomone in order to detect their source of prey[71].

In the Thysanoptera order, pheromone identification is still in progress[38]. As of now, naturally produced pheromones (alarm, aggregation, contact and anti-aphrodisiac) have been identified across both Tubulifera and Terebrantia sub-orders, respectively in Thripidae and Phlaeothripidae families[88].

The larval alarm pheromone was identified, so far, in *F. occidentalis*[32,89,90]. It consisted of a mixture of decyl acetate (10AC) and dodecyl acetate (12AC) (Table 1.2) and was found in the anal droplet of larvae. The blend affects the activity of adults by

decreasing landing and increasing the take-off rates[91], and of the instar stages by causing larvae to retreat to refuges[92]. The different released ratios are perceived by the natural predators like *Amblyseius cucumeris* Oudemans and *Orius tristicolor* White which were shown to use the alarm pheromone as a kairomone to recognize the smaller larva, their main prey[93]. Additionally, the alarm pheromone was reported to enhance insecticides efficacy against *F. occidentalis* causing high mortality among larva stages[85,90]. Also, the synthetic form led to reduced oviposition rate, thus it can be used in thrips behavioural control[85].

The anti-aphrodisiac pheromone was seen as of yet only in *Echinothrips americanus* Morgan (Poinsettia thrips)[94]. Chemically, it is a blend of the dimethyl ester of hexanedioic acid (dimethyl hexanedioate or dimethyl adipate, DBE-6) (Table 1.4) and the dimethyl ester of pentanedioic acid (dimethyl pentanedioate or dimethyl glutarate, DBE-5) (Table 1.4) and both compounds were collected from the adults' head-throrax area. These substances were proven to have a role in mating strategies. Specifically, when virgin females were treated with DBE-5 or DBE-6, they were more likely to be ignored by the males[94].

An adult male contact pheromone was identified, so far, in WFT[95]. This pheromone - 7–methyltricosane (Table 1.3) represents a unique pheromone among thrips species, as well. However, as it is a CHC this compound will be discussed further within Section 1.6.

An aggregation pheromone was found in several species of thrips (Table 1.1). The chemical composition in most of the insects in which the aggregation pheromone was found comprises of a blend of a minor and a major compound. However, except the melon thrips *Thrips palmi* Karni for which the aggregation pheromone contains a single compound, the major compound[96]. The blend found in *F. occidentalis*[33]

consisted of (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate. This mixture, qualitatively similar but quantitatively different, was also found in *F. intonsa*[97]. A pheromone with a comparable structure and similar function was discovered in *M. sjostedti*[36]. In this thrips species the blend consists of (R)-lavandulyl 3-methylbutanoate as the major compound and (R)-lavandulol as the minor compound[36].

In all species, the major compound of the aggregation pheromone is an enantiomer of the esters of a monoterpene alcohol and a five-carbon acid[36]. The role of the minor compound in the bean flower thrips is still unclear[36], although, in WFT it was suggested it might act as a mating pheromone[95]. Noteworthy is that, in these molecules a chiral centre is present, thus the identification is challenging due to required separation of the enantiomers on a specific chiral column by Gas Chromatography (GC) and due to the required time needed for synthetizing and matching the enantiomers[36,38,96].

In terms of the body release area, it is assumed that the aggregation pheromone is produced by the abdominal sternal glands, though it has not been proved yet, but all the male thrips species that release this pheromone were shown to possess sternal glands along their abdomen[38].

Laboratory bioassays revealed that the aggregation pheromone elicited a positive behavioural response in all thrips species, specifically, both females and males were attracted to the adult male's odour[33,36,96,97]. Additionally, in the field, the neryl (S)-2-methylbutanoate extracted from WFT and (R)-lavandulyl 3-methyl-3-butenoate collected from the melon thrips have both been tested with the aid of sticky coloured traps and proved their function as sexual aggregation pheromones by trapping both females and males[12,33,96,98]. One of the WFT male-produced aggregation pheromone is commercially available as a synthetic analogue: Thripline AMS -

marketed by Bioline AgroSciences Clacton, UK) and ThriPher - marketed by Biobest, Westerlo, Belgium[21]. The synthetic analogue was successfully used along sticky traps in monitoring and mass trapping. According to Broughton and co-workers[99], trials conducted on fruit trees in Australia revealed that the number of insects caught on the baited traps was 3x higher in comparison with not baited ones. In the UK, on semi-protected strawberry the addition of the aggregation pheromone to blue roller traps led to doubling the number of thrips caught on the traps[98]. Furthermore, experiments performed in Romania on cucumber[100] and in Spain on protected sweet pepper and tomatoes[101] also concluded that the aggregation pheromone can be used as a thrips control measure highlighting its role in pest management.

1.5 Thrips pheromones chemical analysis

All of the above thrips' pheromones were separated, detected and identified with the aid of a widely used analytical technique known as Gas Chromatography – Mass Spectrometry (GC-MS). This technique is a combination of two techniques aimed to separate and analyse the components of a complex mixture[102].

Gas-chromatography, which is responsible for the separation of the mixture (according to their boiling points), involves two phases, stationary and mobile. The stationary phase is contained in the column and is a liquid or solid chemical that can selectively attract components in the sample mixture[103]. The mobile phase is a carrier gas, an inert gas such as helium, introduced to the column through a high-pressure regulator from a cylinder of compressed gas[102,103]. The major components of a GC, beside the column are the injector, the oven and the detector. The injector port is used to introduce the samples into the GC system. Because the injector port is heated, in here, the sample is vaporized without decomposition. The vaporized state enables the

sample to be transferred on to the column by the carrier gas. The oven holds the column and keeps the column temperature constant. The oven temperature increases gradually and this contributes to a better separation of compounds. The compounds interaction with the column is subject to their affinity for the stationary phase. Hence, high volatile molecules will travel quicker through the column as compared to low volatile molecules[102]. The eluted molecules will be further analysed with the aid of the mass-spectrometer which is coupled to the gas-chromatogram.

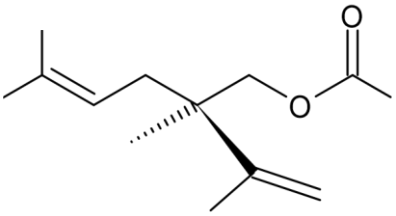
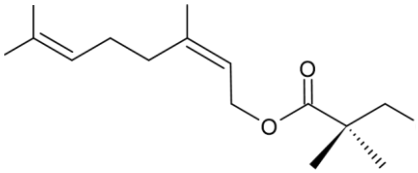
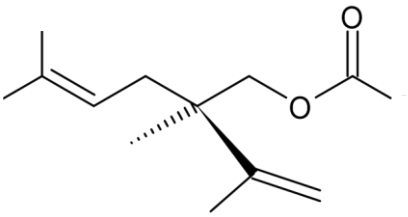
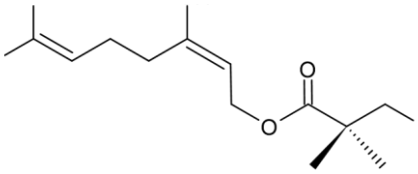
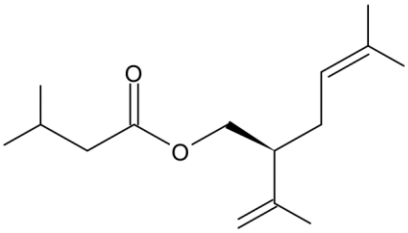
Mass-spectrometry includes three main stages: ionisation, analysis and detection. The ionisation process takes place in a vacuum, where the eluted molecules are fragmented into a characteristic group of ions. The ions are then separated based on their mass-to-charge ratio (*m/z*). The signal produced by these ions, based on their relative abundance, is detected by a highly sensitive mass analyser and then plotted by the computer connected to the GC-MS system as a mass spectrum. The displayed fragmentation pattern, within a mass spectrum, is characteristic to each compound and provides valuable information regarding its structural composition[104].

For ionisation operation, different methods are available, such as: Electrospray Ionisation (ESI), Atmospheric Pressure Chemical Ionisation (APCI), Chemical Ionisation (CI) and Electron Ionisation (EI). The most commonly used in gas-chromatography mass-spectrometry are chemical ionisation and electron ionisation. When one of these techniques is selected, sample characteristics are taken into consideration. In this research, electron ionisation will be used. Throughout electron ionisation process the sample's molecules are broken into charged fragments due to loss of an electron or simply become charged without fragmenting. The charged fragments provide structural information about the sample's molecules[102–104].

After passing through the ionisation source, the ions are guided towards the mass analyser, which produces the mass spectrum. Mass analysers explore different characteristics of the parent or fragmented ions. The main function of a mass analyser is to separate the ions according to their *m/z* ratio basically by their behaviour in electric or magnetic fields[105]. Common mass analysers used in mass spectrometry are Quadrupole Ion Trap, Time-of-Flight and linear Quadrupole. The later mass analyser is widely used in GC-GCMS systems. A quadrupole consists of four parallel metal rods between which oscillating electric field is created due to Radio Frequency (RF) voltage applied across them. The ions that pass through this system must have a certain *m/z* ratio. However, quadrupole enables a rapid scanning of a wide range of ions. All ions are sent to the detector where their abundance and mass is measured and displayed in the shape of a mass spectrum[104,105].

Gas-chromatography mass-spectrometry analysis supports new research in diverse fields because it has proved to be applicable to the separation of various mixtures of volatile materials[104]. Therefore, GC-MS represents a tool of choice for scientists researching the insects' volatiles. It is also, its sensitivity and effectiveness that makes it suitable to investigate highly volatile compounds like pheromones.

Table 1.1 Adult-male-produced aggregation pheromones in thrips

Species	Pheromone name	Chemical structure	Reference
F. occidentalis	(R)-lavandulyl acetate		33
	Neryl (S)-2-methylbutanoate		
F. intonsa	(R)-lavandulyl acetate		97
	Neryl (S)-2-methylbutanoate		
M. sjostedti	(R)-lavandulyl-3-methylbutanoate		36

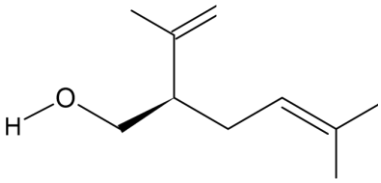
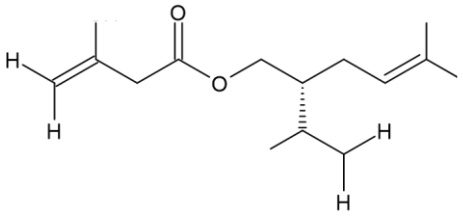
	(R)-lavandulol		
T. palmi	(R)-lavandulyl 3-methyl-3-butenoate		96

Table 1.2 Larval alarm pheromone in thrips

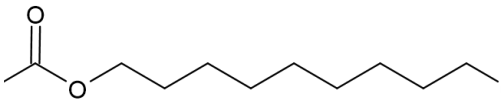
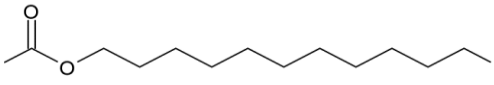
Species	Pheromone name	Chemical structure	Reference
F. occidentalis	Decyl acetate		89–91
	Dodecyl acetate		

Table 1.3 Adult-male-produced contact pheromone in thrips

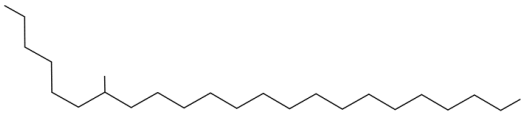
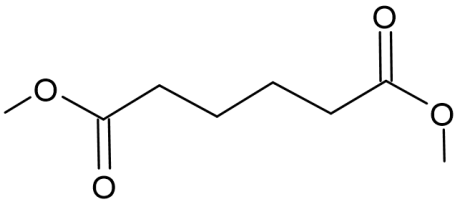
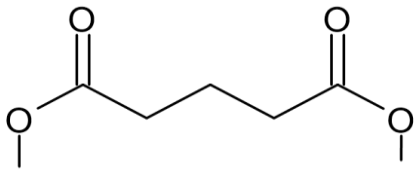
Species	Pheromone name	Chemical structure	Reference
F. occidentalis	7-methyl tricosane		[34]

Table 1.4 Adult-male-produced anti-aphrodisiac pheromone in thrips

Species	Pheromone name	Chemical structure	Reference
E. americanus	DBE-6		[94]
	DBE-5		

1.6 Cuticular hydrocarbons in thrips

Insect exoskeletons consist of multiple layers. The outmost layer, the epicuticle, is coated with a fine film of wax whose role amongst others is to produce chemical signals[106]. The most important compounds in the wax layer are the hydrocarbons, often referred to as cuticular hydrocarbons (CHC). As deduced from the name, these compounds' structure consists of chains of carbon and hydrogen. The CHCs can be produced by different stages of arthropods and are active in fulfilling different roles. From a chemical point of view, they can be saturated and unsaturated, thus they may include *n*-alkanes, *n*-alkenes and methyl-branched hydrocarbons[107]. The *n*-alkanes are mainly involved in trans-cuticular water movement while the *n*-alkenes and the methyl-branched hydrocarbons serve as communication signals[108–110].

In arthropods CHCs play diverse physiological functions serving as waterproof agents[107,108,111] and acting as a barrier for microorganisms[112], chemicals and toxins. Furthermore, the CHCs profiles have been determined in different groups of insects for their role in taxonomy[107,108,113], forensic entomology[114–116] and evolutionary biology[109,117,118].

So far, the CHC profile of Thysanoptera has been analysed in six species of thrips. The first study on CHC in thrips was reported in 2007 on adults and larvae of *F. occidentalis*[119] with no mention of adults' gender. Subsequent studies on CHC had also focused on *F. occidentalis*. For instance, in 2011 Zhao and collaborators[120] tested a new technique of extracting the CHC in *F. occidentalis* whereas in 2013 Olaniran *et al*.[34] focused their work on testing the pheromone role of CHC in this species. It was in 2018 when the CHC studies targeted a different species of thrips (other than WFT), more specifically on virgin females of *T. tabaci*[40]. More recently, in 2020, Chen and co-workers[121] demonstrated the importance of the CHC to identify different species of

thrips. As per these studies, *F. occidentalis* CHC profile contains a mixture of linear alkanes (*n*-C_{25} - *n*-C_{30}) and branched monomethyl and dimethyl alkanes. These results are slightly different from Olaniran and co-workers[34] who in addition also found the 7-methyl tricosane. In their work, Olaniran and co-workers[34] reported that this male-produced cuticular substance attracted both male and female *F. occidentalis* in close contact in contrast to the aggregation pheromone neryl (S)-2-methylbutanoate which attracts male and female *F. occidentalis* over a distance[33]. However, this compound's functions are not clear yet, but it has been suggested that it has the potential to act as an anti-aphrodisiac pheromone[40].

The first records of CHC analysis for *T. tabaci* have been reported by Akinyemi[40]. Within this thesis the author revealed that the chemical composition of the cuticular layer of this species is similar to that of WFT, but some methyl branched compounds like 9,X-diMeC_{25}, 5,X-diMeC_{27}, 3-MeC_{25} and 4-MeC_{26} were absent in *T. tabaci* virgin females whereas the ketones pentacosanone and heptacosanone were absent in WFT and only found in the onion thrips. Moreover, he speculated that any of the above methyl branched alkane present in *F. occidentalis* but absent in *T. tabaci* may be used by the adult males in species recognition.

In general, analysis of CHCs has its challenging stages (like insect handling) especially if extracted from small insects like thrips. Thus, due to their minute size, a large number of thrips is used to collect the chemicals. For example, the research group in Poland used 250 adults (females and males altogether) and 330 larvae[119], whereas in the UK 100 females of each of the tested species were used[40]. Additionally, in Kenya for the studies conducted on bean flower thrips 1000 females and 300 males were used for each replicate (Drijfhout, personal communication). Such a high number of individuals is subject to the density of the population, especially if directly collected

from infested plants in the field. Moreover, males are less abundant in the population which creates further difficulties in ensuring the required amount of material. However, in 2011, a research group in China[120], used only one thrips per sample as the extraction was performed without a solvent using a modified syringe, for a rapid species identification tool in thrips based on its CHC profile, for quarantine purposes. Another research group from China[121] also used a single thrips per sample and a solvent-free extraction. In this case, the researchers used commercially available equipment known as a Thermal Separation Probe (TSP). A similar method, based on direct injection of the biological sample was described by Morgan and Wadhams[122] who used a customised solid injector. Morgan and Wadhams[122] described that the samples (parts of the insect body or the whole body) were sealed in glass capillary tubes which were directly inserted into the gas-chromatogram inlet port. It is not clear, though, whether Zhao and co-workers[120] used the same technique as Morgan and Wadhams[122], as there is no reference to this method in their paper and also no mention of glass capillaries or how the samples were prepared and directly injected. The most important advantage of this method is that a smaller number of insects per analysis should be sufficient for the detection of their CHC.

1.7 Aims and objectives

The above findings highlighted that the CHCs have great potential to provide valuable information regarding thrips chemotaxonomy which contributes to a better understanding of their interspecific interactions and most importantly can be included into management programmes. Thus, in-depth research should be conducted in this regard in various thrips species.

In here we hypothesis that the solid injection method can be used to detect and identify the CHCs in thrips insects. Therefore, the overall aim of this thesis is to conduct CHC analysis of various thrips pest species. Initially, it will investigate the optimum number of thrips required to collect the CHC compounds from, then it will test the efficacy of CHC extraction using the solid injection method which will be compared to solvent extraction technique.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter describes the methods related to the experiments conducted in the present study. Specific details related to a certain experiment are also reported. The main sections in this chapter will cover details regarding the insects (rearing, collection and handling), sample preparation (whole-body and liquid extraction) and optimisation, solid-sampler injector, GC-MS analysis and statistical analysis.

2.2 Thrips insects

In total, in this study, five species of thrips belonging to the Terebrantia sub-order (Table 2.1) were used to analyse their CHC profile using the solid injection method: *F. occidentalis*, *Frankliniella borinquen* (Hood), *Frankliniella schultzei* (Trybom), *Hydathothrips adolfifridericí* (Karni) and *T. tabaci*. With the exception of *F. occidentalis* which was collected from a laboratory culture (section 2.2.1) reared at Keele University (UK), the rest of the thrips species were collected directly from their host plants, in Nairobi (Kenya), in September 2019. These plants were tapped five times gently with the palm of the hand, on to a white barber tray (25 x 45 cm) held underneath the selected plant[124]. The collection site in Kenya, for most of the thrips species, was the International Centre of Insect Physiology and Ecology

(ICIPE), apart from *F. borinquen* which was collected outside ICIPE premises. At the time of collection (2019) the institute had a separate thrips project in collaboration with Keele University, which facilitated the delivery of the samples. The field work and subsequent sample preparation were conducted by Dr Ankinyemi Adenyemi (Keele University post doctorate representative) and Dr David Mfuti Kupesa (researcher collaborator and employee of the institute), respectively.

Table 2.1 Thrips species used for CHC analysis performed with the solid injection technique

Thrips Species	Source	Host Plant
Frankliniella occidentalis	Keele	Chrysanthemum
Frankliniella borinquen	Kenya	*Tithonia diversifolia*
Frankliniella schultzei	Kenya	*Ipomea setosa*
Hydathothrips adolfifriderici	Kenya	*Phaseolus vulgaris*
Thrips tabaci	Kenya	*Brassica oleracaea* var. *acephala*

2.2.1 Laboratory thrips colony

Frankliniella occidentalis has been cultured at Keele University for over 20 years in a temperature controlled-room. In the rearing room, temperature is maintained using an in-built heating system linked to a thermostat regulator. Insect are reared on whole plants using pot chrysanthemums placed separately in rearing cages inside the cage, the temperature and humidity are kept at 25°C ± 2°C and 73 ± 2%, respectively. These parameters are monitored using higro-thermometers hung inside the cage on the back wall. The cages are provided with a fan on the back panel which continuously

circulates the air inside and prevents condensation. The cage floor is covered with a double layer capillary matting (Vattex Black Berrycroft Stores Ltd., UK) cut to fit the plastic tray in which each cage is set. The top side of the cage is made of UVA-transmissible plastic sheets; thus, the light is provided from the top. Four neon tubes (58 W Sylvania Activa 172 professional, 1500 m length and 26 mm diameter, 240 V AC; Sylvania Lighting International, West Yorkshire, UK) in 4 strips are horizontally set up at 78 cm above the bench. The light-dark regime L16-D8 is constantly used because it mimics the natural environment conditions during the summer time. The light is programmed to switch on at 5.00 am and then off at 21.00 pm. The chrysanthemums were acquired from the local Sainsbury's supermarket.

2.3 Thrips collection, handling and identification

Working with small and active insects like thrips species could be challenging, therefore for some experiments, pre-trials were performed to practice their effective handling.

In Kenya the insects were collected directly from their host plant by gently beating the infested plants over a white tray. The fallen specimens were picked up using a mouth pooter, thus the insects were transferred straight into the collection vial. The insects were transferred to the laboratory and stored in the freezer until used for sample preparation. Collection from the laboratory culture, was also done similarly, using the tapping method, except the insects were not stored, but immediately used for preparing the glass capillary samples (section 2.5).

When the plants were tapped specimens of all stages fell in the collection pot. Larvae and pupae stages of the tested species, have distinct morphological features as compared to adults, so they could be clearly separated. Regarding the thrips adults,

the genders were separated based of the known fact that the males of most Terebrantia are smaller than females[123]. Therefore, winged darker and larger individuals were identified as female adults and the narrow, smaller and light-coloured ones as males.

In terms of species identification, the colony species was confirmed to be *F. occidentalis* by mounting specimens on a microscope slide and checking the main identification features (antennae with eight clear antennal segments, post-ocular setae about three times as long as the other ocular setae, the forewing with two complete rows of setae).

In Kenya, the field operators were able to recognise the thrips species based on their previous research experience, still they were confirmed by Dr Laurence Mound, a specialist on the biology and systematics of Thysanoptera (Akinyemi, personal communication).

2.4 Glass-capillary preparation

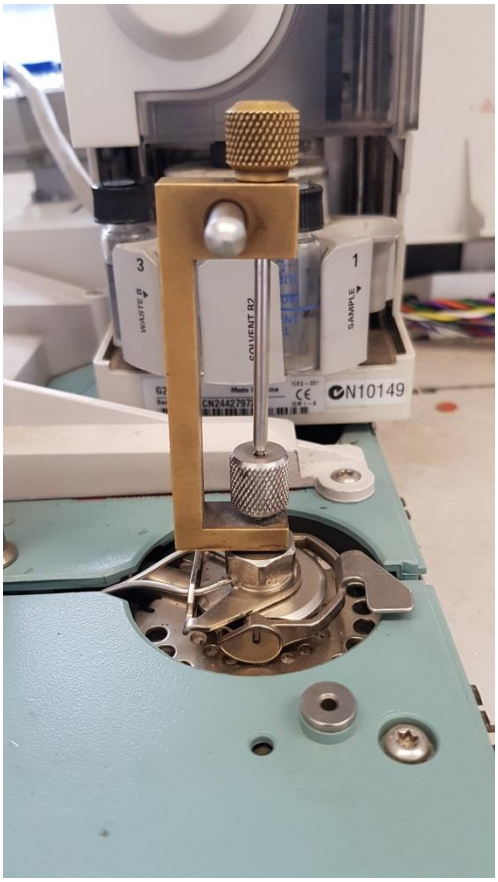
For the solid injection analysis, the samples must be sealed in micro-capillary glass tubes, because the amount of effort required to be crushed is minimum due to their small diameter (OD 1-2 mm) and very thin walls. The glass tubes were purchased (Scientific Glass Laboratories, Ltd, Stoke-on-Trent, UK) as 5m long tubes, thus they were sized to the desired length (10 mm). After cutting, they were cleaned in hexane for 20-30 min, drained and baked overnight in an oven set at 80°C. The next day, they were sealed at one end using a small kitchen blow torch. To preserve them sterile, they were kept in sterile screw top vials.

2.5 Solid-sample injector

A solid-sample injector[122] was used to introduce glass tube samples to the inlet port of the GC. This device was custom-made. The usage of the sample injector, has the advantage that the analysis does not involve any solvents, hence any trace contaminants in the solvent are avoided. It is therefore likely to be more sensitive for small biological samples.

For the analysis of CHC in thrips species, an important advantage is that a reduced density of insect is required for the detection of their cuticular volatiles. Also, the solid injection method is able to detect any variance across the replicates within a sample. If the insects used were mixed age and mixed reproductive status (virgin females or males that mated once or multiple times) this could explain the variance. However, retention time (t_r) was seen to vary slightly between replicates, possibly due to a requirement to manually start each run. The main parts of the solid-sample injector are the plunger, the holder and the hollow tube. The barrel (3 mm OD, 2 mm ID) section is introduced into the instrument's injector port at a high temperature (250°C); hence it is entirely metallic (Fig. 2.1a). Towards the end, the barrel is provided with 4 small holes (1 mm diam.) which allow the compounds from a crushed sample to be released into the instrument. Along the plunger there is a septum accommodated inside a screw nut that helps to keep a constant gas pressure into the injector port. When the plunger is lifted up there is access to the hollow tube and a glass capillary containing the sample can be dropped into the tube (Fig. 2.1b). The plunger is then lowered to stop just above the glass capillary, and the system is sealed by tightening a nut housing the O-ring. After three minutes, the plunger is pushed down to crush the glass capillary and this releases all the compounds from the sample in the capillary.

The carrier gas or the mobile phase that flows through the GC system will push the components of the sample onto the GC column.

a 

b 

Fig. 2.1 The solid-sample injector in the GC inlet: (a) with plunger inserted; (b) with glass capillary being dropped into the tube

2.6 Sample preparation

Preparing the samples for solid injection analysis involves inserting the insects into the micro-capillaries. The minute size of the thrips makes their transfer into the narrow glass tubes fairly easy, by naked eye or under the microscope. This technique requires a steady hand, but it could be achieved quite fast, once a few samples have been prepared. Prior to the transfer into the glass capillaries, the insects were freeze-killed and then with a fine artist's paint brush, they were picked up and inserted into

the micro-capillary. After that, the glass tube was sealed at the top end. An extra precaution was taken at this step, because compounds of interest can be partly or entirely lost if the thrips are too close to the flame. To overcome this, after the transfer, a gentle shake of the hand holding the tweezers with the glass tube was sufficient to move thrips away from the flame. Once prepared, the sample is set into the barrel of the solid injector and the plunger is pressed to crush the sample.

Mixed age adult individuals belonging to different species of thrips (Table 2.1) were used to prepare the glass tube samples as described in section 2.5. The micro-capillary samples containing *F. occidentalis* were prepared at Keele University, whereas the glass tubes containing the species collected in Kenya, were prepared in Nairobi and delivered to Keele University for GC-MS analysis. For *F. occidentalis* there were 18 replicates prepared altogether. They were included in the sample density optimisation (section 2.8). But some of them were also used to test the efficiency of the solid injection method (section 2.9). However, the number of replicates of the sample containing the thrips species from Kenya, varied (Table 2.2) due to low field populations, possibly related to low September temperatures. To ensure consistency in sample preparation, clean glass capillaries (10 mm long) were sent to Kenya along with a detailed sample preparation protocol.

Table 2.2 Number of replicates corresponding to the thrips species delivered from Kenya

	Number of replicates	
Thrips species	**Female**	**Male**
Frankliniella borinquen	9	10
Frankliniella schultzei	11	10
Hydathothrips adolfifridericí	8	10
Thrips tabaci	11	3

2.7 Sample density optimisation

In order to determine the optimum number of thrips in a capillary tube, different densities were investigated. For this purpose, glass tubes containing 1, 2 or 3 insects in a micro-vial, were tested. For each density, six replicates were prepared and analysed on the GC-MS (section 2.10) with the solid-sample device. The tapping-the-flower method was used to collect these insects from the colony (section 2.3) and the glass tubes with the various densities of thrips were prepared as detailed in section 2.6.

For this experiment laboratory reared *F. occidentalis* was chosen as the study insect. This experiment was conducted in July 2019 and based on the results acquired in this trial, two insects per microcapillary (section 3.1) was the density size that was set for the thrips sample delivered from Kenya, in October 2019. Western flower thrips was selected as model species mainly because Keele's culture was a reliable source of insect material. In fact, the poor availability of the insects in the field, was the main reason optimisation pre-trials were not run individually for each species of thrips included in this research.

In the end, the experiment not only revealed information regarding the optimum number or thrips per glass capillary, but also provided the CHC profile of the whole-body analysis for each individual density. The detected hydrocarbons were identified as described in section 2.11. It is worth mentioning that *F. occidentalis* was taken into account in the principal component analysis (section 2.12) as the fifth species, along with the thrips species delivered from Kenya, and for this dataset from the samples with two insects per micro-capillary were considered.

2.8 Solid injection efficiency

To demonstrate the efficiency of the solid injection technique, a proof-of-concept trial was set up. It involved a comparison of adult female *F. occidentalis* CHC composition identified by whole-body analysis using the solid injector and the more conventional liquid extraction analysis. For this purpose, some of the data obtained from the experiment described in section 2.7 were further used to test the solid injection efficiency. Basically, the hydrocarbon profile detected through liquid extraction of 100 insects (section 2.9) was compared with the one detected through whole-body analysis of two insects. Because the optimum density size was set for two adults per micro-capillary (section 3.1), it was reasonable to select this density for comparison of the CHC profile. A liquid extraction protocol was conducted as detailed below in section 2.9.

2.9 Liquid extraction

Prior detailing the steps of this trial, it is important to mention that the liquid extraction protocol requires precision in handling the insects and the syringe, therefore pre-trial runs were carried out to gain dexterity. Firstly, all the materials were

thoroughly washed with hexane and two pots filled with crushed ice were prepared. The CHCs were extracted in *n*-hexane between 10-12am at room temperature (26°C ± 1°C). *F. occidentalis* females (n = 102) were collected in a white plastic pot which was immediately set on top of one of the pots filled with ice, because short exposure to cold immobilizes the insects and eases the handling. A fine brush was used to delicately sweep the insects off from the pot and transfer them into a 1.5 ml microcentrifuge plastic tube. Five plastic tubes were used altogether, each containing 20 females, apart from one tube that contained 22 (two extra insects in case of escaping or dying). The reason the insects were separated in sets of 20 was that water on the bottom of the pot condensed and when the insects were picked up small amounts of water would be taken on the brush, too. To remove the insect from the brush, it was slightly wiped of the internal wall of the plastic tube, thus water was also transferred into the tube. With a higher number of insects, the water would accumulate and would make thrips to stick to the tube's walls and that would make their further transfer difficult. Moreover, the insects regained their active behaviour shortly after being transferred into the warm centrifuge tube, thus, the higher the number of insects, the higher the chances to escape when a new individual was inserted. However, to avoid losing insects, the index finger of the hand holding the tube was set on the tube opening and quickly removed only to add a new individual. Also, before inserting a new thrips, the tube was gently tapped so the insects would be at its lower side and not run out. Once 102 adult females were counted and transferred in separate centrifuge tubes, all the insects were brought together into a single plastic tube. Then the thrips were further transferred into a 2 ml GC glass vial (Fisher Scientific, UK). Beforehand, the GC vial was set upright in ice (with the lid on to keep out condensation) to cool down so the insects remained still. Immediately after the

transfer, the lid was closed to keep the insects enclosed and opened when 1 ml of hexane was added. This volume was added in two separate loads using a Hamilton syringe for 500 µl with a flat needle tip (Fisher Scientific, UK).

During the pre-trial runs it was noticed that by the time the first syringe load was complete, thrips became active, and thus the vial was set back in ice for half a minute until they settled. Also, it was observed that some of the thrips could be inside the lid and not in the hexane. Therefore, in the actual experiment it was specifically checked that all the insects were submerged in solvent. The extraction time was exactly five minutes, as contaminants from the internal body would be also extracted (Drijfhout, personal communication). The solvent was removed using the same syringe and transferred into a second GC vial kept at room temperature. For the solvent removal special attention was paid to not puncture the thrips. Additionally, to get the last bit of hexane exposed to thrips, the vial was angled at about 45° so that the thrips settled in the angle of the base of the tube, then the blunt tip of the needle was gently pressed on the flat base of the tube just above the level of the thrips. The vial containing the solvent with the CHC extract was labelled and stored in the freezer for subsequent GC analysis. The vials with insects were left open and next day when the solvent on thrips' body was dry, they were removed into a Petri dish and counted under the microscope and checked if any individuals were lost during the transfer process.

To set a standard volume and to increase the concentration before the GC analysis, the extract sample was set in the fume hood to evaporate completely and then reconstituted by adding 10 µl of hexane. As the CHCs are less volatile compounds they would not be lost during the evaporation process. After, that 1 µl of the extract was transferred into a glass capillary, sealed at the top end and then analysed with the solid injection method.

2.10 GC-MS analysis

The chemical analysis was performed on an HP6890 GC instrument, modified to equip the solid-sample injector, linked to an HP5973 Network Mass Selective Detector (Agilent). The capillary column was coated with DB-5MS (30 m x 0.25 mm internal diameter, 0.25 µm film thickness). Helium (1 ml min^{-1}) was used as the carrier gas. The temperature of the inlet port, in which the solid-sample injector was inserted was set at 250°C and the injection was splitless. After placing the sealed capillary in the solid-sample injector, it was left in the inlet for 3 minutes for desorption of the compounds. After that, the plunger was pushed down to crush the glass capillary and release the desorbed CHCs. Compounds were then transferred by the carrier gas to the column. The temperature of the oven was held at 50°C for 2 min, before increasing to 200°C at 25°C min^{-1}, then to 260°C at 3°C min^{-1} and then to 320°C at 20°C min^{-1} and held for 5 min. The mass spectrometer was operated in the EI mode at 70 eV, scanning from 40 to 800 atomic mass units (i.e., ion mass). An *n*-alkane standard solution (40 ng/µL) ranging from heneicosane (*n*-C_{21}) to tetracosane (*n*-C_{40}) (Fluka, Germany) was prepared and run on the GC-MS with the solid injection under the same conditions as described above.

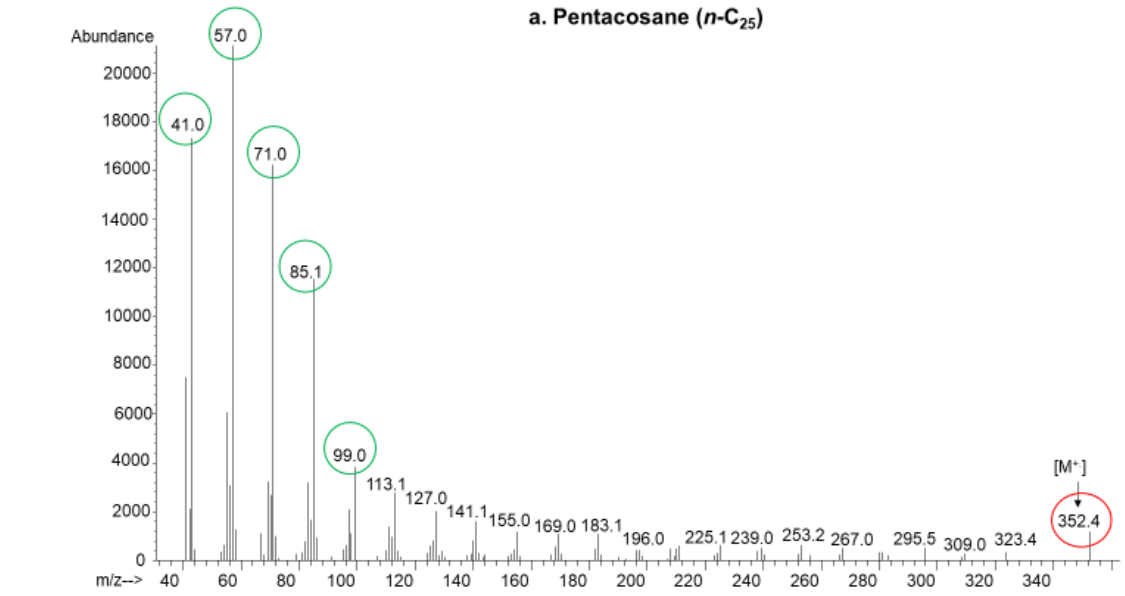
The glass fragments that resulted from crushing the sample were removed from the barrel after each run. After three samples were analysed, a blank sample was run to check for any carrier over compounds. The last crushed sample, was run again without clearing the barrel. Furthermore, to avoid overloading the column with any high boiling contaminants, the blank run was followed by a bake out run (the oven was set at 320°C for 30 minutes).

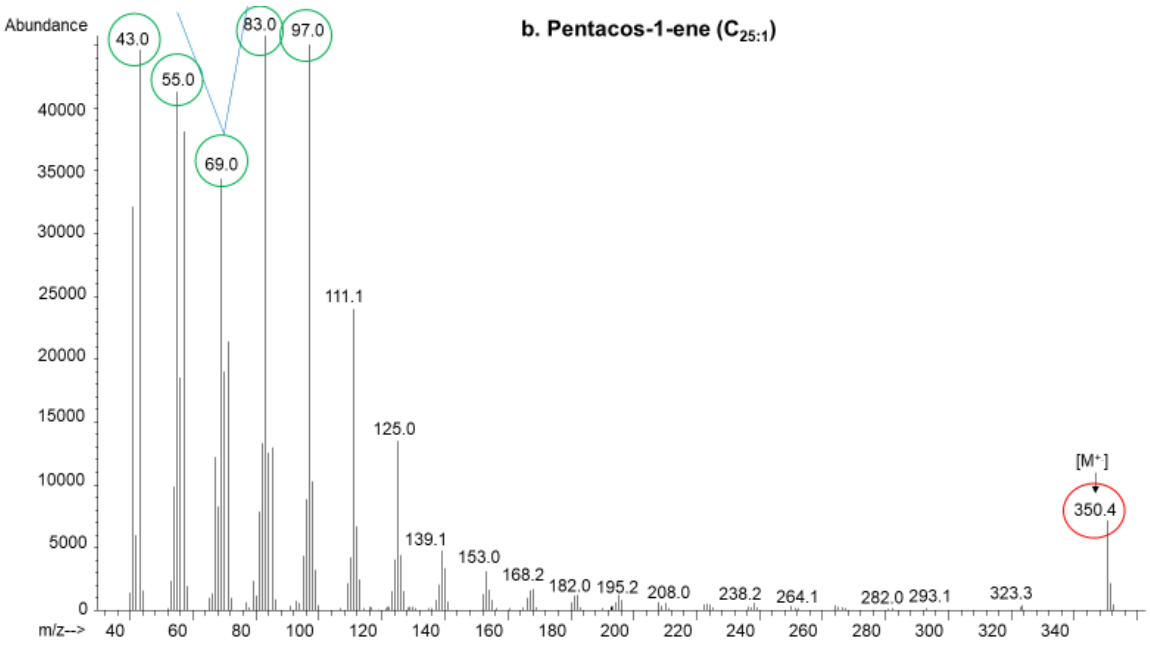
2.11 Identification of cuticular hydrocarbons

The complex mixture of molecules extracted from the cuticular layer of the various species of thrips were separated out with the aid of the GC instrument, as described above. All the peaks were integrated and quantified with the aid of Enhanced ChemStation Software (version E.02.021431, Agilent Technologies, Inc.). In this study only the compounds eluting from *n*-C_{21} onwards were considered and this was decided based on the fact that earlier eluted compounds are most likely internal body compounds and not from the cuticle layer of the insects' body. The hydrocarbons were then identified using a library search (NIST2.2, 2014), the diagnostic fragmented ions[124] and the Kovats Index[125] along with matching the mass spectra of the detected compound to that available in the literature.

The mass of the fragmented ions, in particular of the molecular ion, [M+.], has a significant role in hydrocarbons' identification. In straight chain alkanes, the molecular ion is essential to determine the number of the carbons in the backbone. Whilst this was present in most of the linear hydrocarbons detected in the tested thrips species, largely, it was not detected in branched compounds. However, the molecular mass was identified (for both mono- and dimethyl CHC) using the $[M-15]^+$ ion, which corresponds to loss of the methyl group (15 = the mass of CH_3). In Fig. 2.2, are shown examples mass spectra corresponding to straight chain alkane, monomethyl alkane and a monoalkene. The top spectrum (Fig. 2.2a) belongs to a pentacosane and its molecular ion is 352 (in red circle). It can be seen that the mass of the ions are odd numbers, except for the molecular ion, which is an even number. Also, it can be observed that the mass difference between the ions is 14. This is due to each CH_2 group in the backbone is adding14 atomic mass units to the total molecular mass: the mass of C = 12, H_2 = 2 (due to two hydrogen atoms), hence their sum is 14. In methyl

branched compounds, as aforementioned, in most of the cases, the molecular ion is missing, but some of the ions (in the middle part of the spectra) have higher intensity as compared to nearby ions. The pairs of high intensity ions in the middle of the spectrum, as shown by the blue circles in Fig. 2.2c, indicate where the chain was cleaved and by dividing their mass value by 14 and then subtracting 1, the result will reveal the position of the methyl branch. Thus, in the below example, the ion *m/z* = 112 indicates that the methyl group is attached at the seventh carbon of the chain. The mass spectra of alkenes have a distinctive shape due to ion *m/z* = 69 whose intensity is lower, as compared to the side ions, causing a 'V' shape drop as shown in the Fig. 2.2b. Moreover, in alkenes the pattern of the high intensity ions – indicated by the green circles - (*m/z* = 41, *m/z* = 55, *m/z* = 69, *m/z* = 83, *m/z* = 97) corresponds to lower masses as compared to the same region in alkanes (*m/z* = 43, *m/z* = 57, *m/z* = 71, *m/z* = 85, *m/z* = 99). Thus, at a quick glance, based on the 'shape' of the fragmentation pattern in the mass spectra, one can classify a compound, as one of the above. However, the identification of the hydrocarbons is more complex, due to the large number of isomers present in insect samples[127], but also due to co-elution of two isomeric compounds.


a. Pentacosane (n-C25)
Abundance
m/z-->
57.0
41.0
71.0
85.1
99.0
113.1
127.0
141.1
155.0
169.0
183.1
196.0
225.1
239.0
253.2
267.0
295.5
309.0
323.4
[M+]
352.4


b. Pentacos-1-ene (C25:1)
Abundance
m/z-->
43.0
55.0
69.0
83.0
97.0
111.1
125.0
139.1
153.0
168.2
182.0
195.2
208.0
238.2
264.1
282.0
293.1
323.3
[M+]
350.4

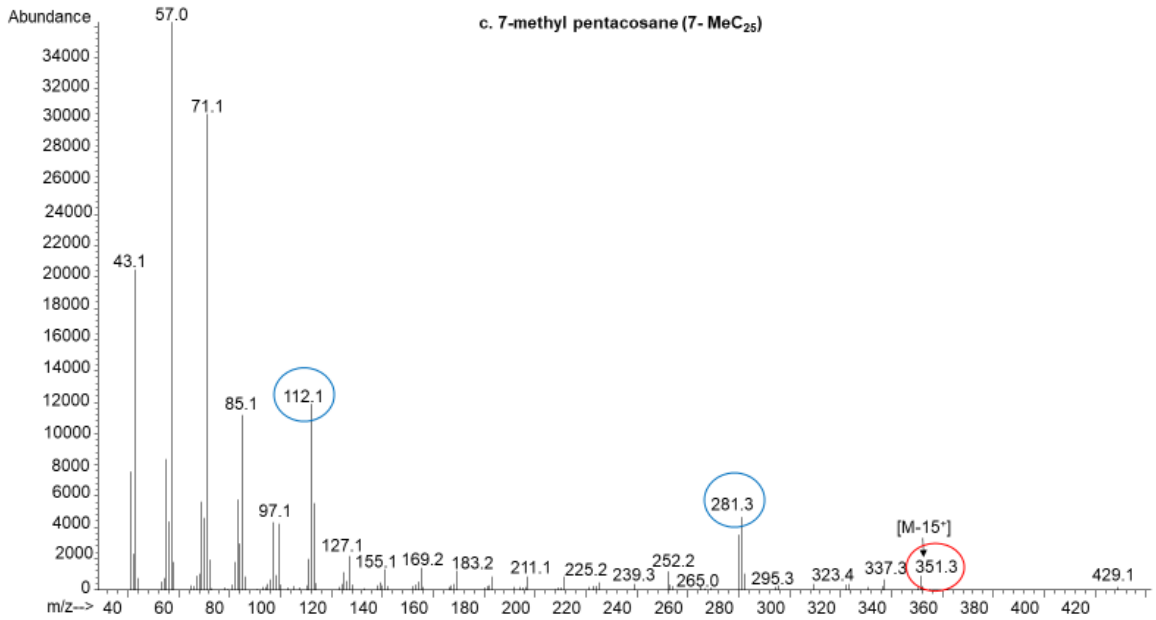


Fig. 2.2 Examples of mass spectra of typical *n*-alkane (a), *n*-alkene (b) and mono-methyl branched alkane (c) identified in the present study. Red circles represent the molecular ion; green circles represent the fragmentation ions associated with *n*-alkane and *n*-alkene, respectively; blue circles indicate the position of the methyl group. All the identifications are tentative

Alongside the fragmentation pattern, the Kovats Index equation[125], as seen in Fig. 2.3, was used to calculate the index retention value and based on this to identify the various CHCs in thrips. Specifically, the retention time of the compound of interest was compared with the retention time of an alkane series. In the equation, the Kovats Index (KI) is represented by *I*, *n* is the number of carbon atoms in the small *n*-alkane, *N* is the number of carbon atoms in the large *N*-alkane, Log t_r is the logarithm of the retention time value. Kovats Index is based on a quantitative index that is relating the retention of an unknown compound to the retention time of a known standard so that system-independent constants are generated and used to describe the elution pattern of the considered molecules[125] .

$$I = 100 \times \left[(n + (N - n) \frac{\text{Log } t_{r(\text{unknown})} - \text{Log } t_{r(n)}}{\text{Log } t_{r(N)} - \text{Log } t_{r(n)}} \right]$$

Fig. 2.3 Kovats Index Equation. *I* = Kovats index; *n* = number of carbon atoms in the small *n*-alkane; *N* = number of carbon atoms in the large *N*-alkane; Log t_r = logarithm of the retention time value (Carlson *et al*., 1998)

2.12 Statistical analysis

Statistical analysis was carried out using several statistical software like Minitab® 20.3 (Minitab Inc., USA), GraphPad Prism 6.01 (GraphPad Prism Inc., USA), Microsoft Excel, version 2013.

Microsoft Excel was used to determine and visualise the optimum number of thrips per glass capillary by generating a series of box plots. To ensure best quality of results, major peaks were selected across the samples containing different insect densities and a box plot was produced for each peak in particular. Major peaks refer to those specific peaks that constantly, in all samples, exhibited strong abundance (peak areas).

GraphPad Prism was used to analyse the efficiency of the solid injection technique. Therefore, chromatographic peak areas of *n*-alkanes and branched monomethyl and dimethyl alkanes extracted from *F. occidentalis* were used and a series of unpaired *t* - tests (with Welch's correction to test whether the means of the two data sets are equal) were run to compare the profile content obtained through whole-body analysis with the one obtained through liquid extraction. Welch correction was

Additionally, Shapiro-Wilk normality tests were run prior to *t* - test to determine whether the data were following a Gaussian distribution.

Finally, Minintab was used to run Principal Component Analysis (PCA) in order to compare the CHC profiles across the five species of thrips. This is a multivariate technique that allows visualisation through the generated plots of the summarised information contained within a large set of data and the presence or the absence of a pattern. The summarised information is also called the principal component and it describes the similarities and differences within a dataset. The first principal component describes the gross average features of the datasets while the second and subsequent principal component introduce further specific features of decreasing significance[126].

Examples of large datasets that are suitable for PCA analysis include chromatographic or spectral data. When such data are processed, a PCA program commonly yields a plot that indicates how much a variable correlates with a principal component (factor loading). The generated plot converts the correlation (or lack of it) among the samples into a 3- or 2-dimension graph (a 2D representations was used in here). With the aid of orthogonal linear transformation, the original data will be converted into a new set of variables or principal components. In other words, the PCA program will display an overview regarding the relationship between variables and groupings of samples that are chemically similar and represented by similar loadings.

In terms of data processing, largely in this research, for descriptive statistical analysis, the relative abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample. In all datasets apart from *Hydatothrips. adlofifriderici*, six principal components were generated (four for *H. adlofifriderici* due to the dataset was smaller). Multiple combinations of PCs

were plotted (starting with PC1 vs PC2) until the best separation was obtained between the extraction species. On the PC plot, for each species, it is expected to see two cluster of points that would represent the genders of that particular species. Tight cluster indicates no significant variation in terms of CHC composition between the two groups (females and male), whereas a more scattered plot indicates a difference in chemical composition between the males and females of the same species.

For the principal component analysis, the less common hydrocarbons (in here, they are referred to as those compounds that were found only in one or two of the processed replicates) were not included in the data analysis. Samples that were clearly not representative (referred to as those samples with only a few compounds detected in the profile) from the whole data set were not included in the data analysis. The reason some samples were excluded is because they would be plotted as outliers and could introduce scatter within the PCA data. .

Not representative samples were excluded from *F. borinquen*, (one male and one female samples), *F. schultzei* (one from each gender) and *T. tabaci* (one female and all three male samples). In *T. tabaci* male samples were also removed because the number of replicates was too low for further considerations. Overall, beside hydrocarbons, other molecules were detected (for example cholesterol and potential aldehydes, esters and alcohols) and they were also excluded from the data processing to fit the aim of this research.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Results for sample density optimisation

The CHC composition at three densities of thrips per micro-capillary was investigated to determine the optimum number (Table 3.2). In Fig 3.1 are presented the total ion chromatogram (TIC) from samples containing a single *F. occidentalis* adult female per micro-capillary (a), two individuals (b) and three specimens in a micro-capillary (c). Overall, in all samples, a mixture of linear alkanes (*n*-C_{25} – *n*-C_{29}) and branched alkanes (mono – and dimethyl) were detected, yet the density of three insects revealed trace amounts of monoene alkenes with 31 and 32 carbons in the chain (data not shown because the focus was on samples with two insects per glass tube, see below). For best quality of data, 12 major compounds detected across eight peaks (whose abundance was constantly high in all samples) were selected and used in producing the box plots in Fig. 3.2. These compounds were identified as: *n*-C_{25}, 9-MeC_{25}, 3-MeC_{25}, *n*-C_{27}, 11-Me, 13-Me, 15-MeC_{27}, 9-MeC_{27}, 3-MeC_{27} and 11-Me, 13-Me, 15-MeC_{29} (mixture of three co-eluting mono methyl branched hydrocarbons) and they were used in further statistical analysis. From a quick glance over Fig. 3.1, it can be seen that the peaks labelled on top, which are corresponding to the selected major hydrocarbons, have quite strong signals as compared to the background noise.

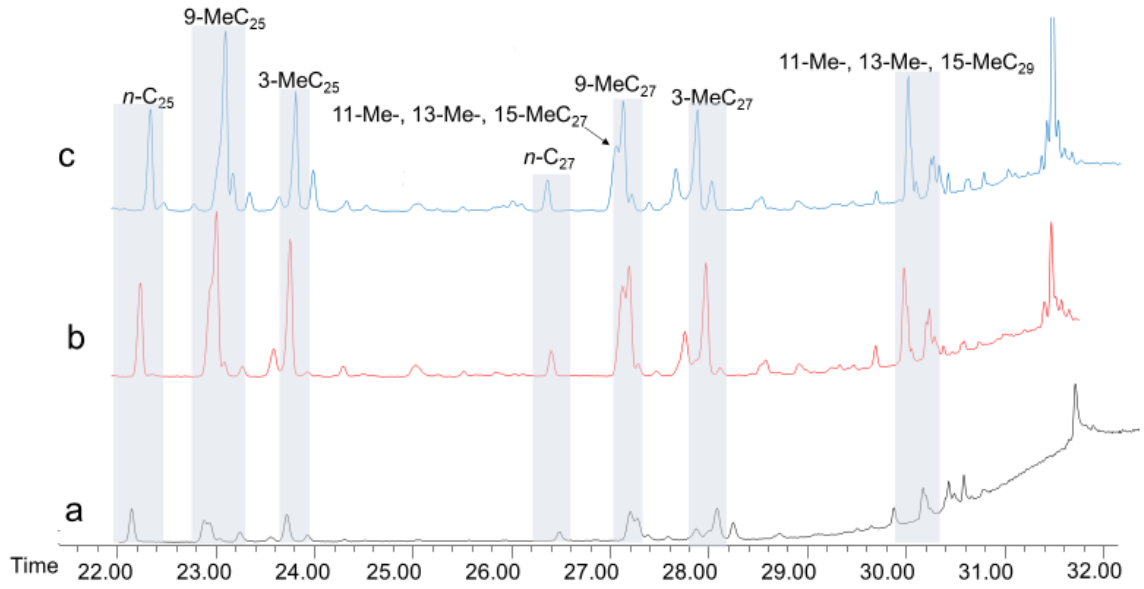


Fig. 3.1 Total ion chromatograms (TIC) from samples with various densities of western flower thrips (WFT) adults showing the selected major compounds used for box plot analysis. a – 1 adult female/micro-capillary (n=6); b – 2 adult female/micro-capillary (n=5) and c – 3 adult female/micro-capillary (n=6)

Table 3.2 provides an overview of the hydrocarbons that were present or absent from samples with various density levels. Also, it is important to be mentioned that whole-body analysis of a single thrips insect displayed valid information only in two out of six replicates. Basically, in four of the replicates containing a single adult female, the TIC indicated that no compounds were detected. Even so, the data obtained in these two replicates for density 1 showed that from a total of 37 hydrocarbons 35% were detected from of a single specimen. This difference between a single insect and two insects in terms of whole-body analysis could be due to their small body size which would lead to an inter-individual variation. As mentioned in section 3.1.2 a particular specimen, depending on its age, satiety status, etc., may have produced more or less CHCs as compared to a different specimen from the same cohort. Moreover, when the CHCs from a single individual are assessed, it is possible that the amount of CHCs

produced by that individual to be too low, so will be under the limit of the detection of the instrument. Whereas when the CHCs of two individuals are assessed, their cumulative composition would then be detectable by the instrument.

From Table 3.2 it can be seen that by increasing the density size, solid injection technique allowed the detection of a wider range of alkanes. Moreover, in Fig. 3.1 can be observed that the abundance of the selected compounds increased when the number of insects per micro-capillary was increased. Because the selected compounds were found in low proportions in samples with only one insect, these were not subjected in statistical analysis, but it was unclear whether their concentration was comparable in the rest of the samples. To investigate this, statistical analysis was conducted to verify whether the mean (±SE) of the major compounds' abundance measured at density of two insects (n=5) was significantly different from the one measured at density of three insects (n=6). To determine whether a parametric or non-parametric test would fit best for data analysis, the distribution of the dependent variable (CHC abundance) for density of two (D2) and three (D3) was tested a-priori using a Shapiro-Wilk test (*W*). The results showed that the Shapiro-Wilk test, in case of both tested densities, did not show evidence of non-normality. This information was extracted from the distributions where *p* values were higher than 0.05, as seen in Table 3.1.

Table 3.1 Results of data normality tests of the selected CHC compounds found in samples with 2 and 3 insects per glass capillary

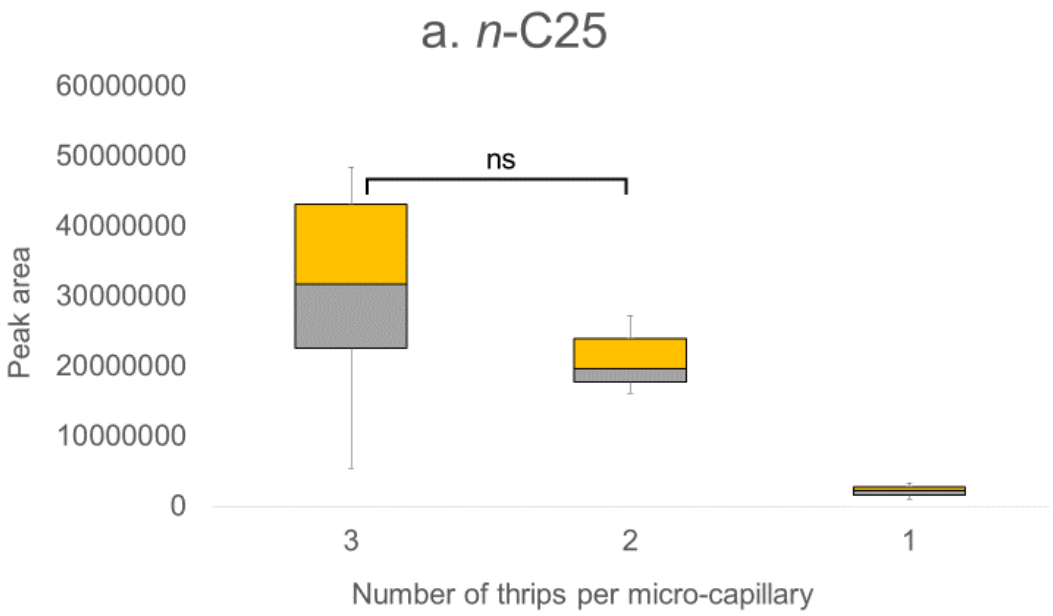
	D2[a]			D3[b]			Normal distribution
Compound[c]	*W*	*p*	Significance	*W*	*p*	Significance	(Yes/No)
n-C_{25}	0.943	0.685	ns	0.941	0.670	ns	Yes
9-MeC_{25}	0.917	0.511	ns	0.760	0.725	ns	Yes
3-MeC_{25}	0.962	0.824	ns	0.990	0.988	ns	Yes
n-C_{27}	0.712	0.071	ns	0.910	0.434	ns	Yes
11-Me, 13-Me, 15MeC_{27}	0.940	0.676	ns	0.926	0.553	ns	Yes
9-MeC_{27}	0.942	0.679	ns	0.813	0.076	ns	Yes
3-MeC_{27}	0.940	0.668	ns	0.813	0.076	ns	Yes
11-Me-, 13-Me-, 15-MeC_{29}	0.940	0.668	ns	0.813	0.076	ns	Yes

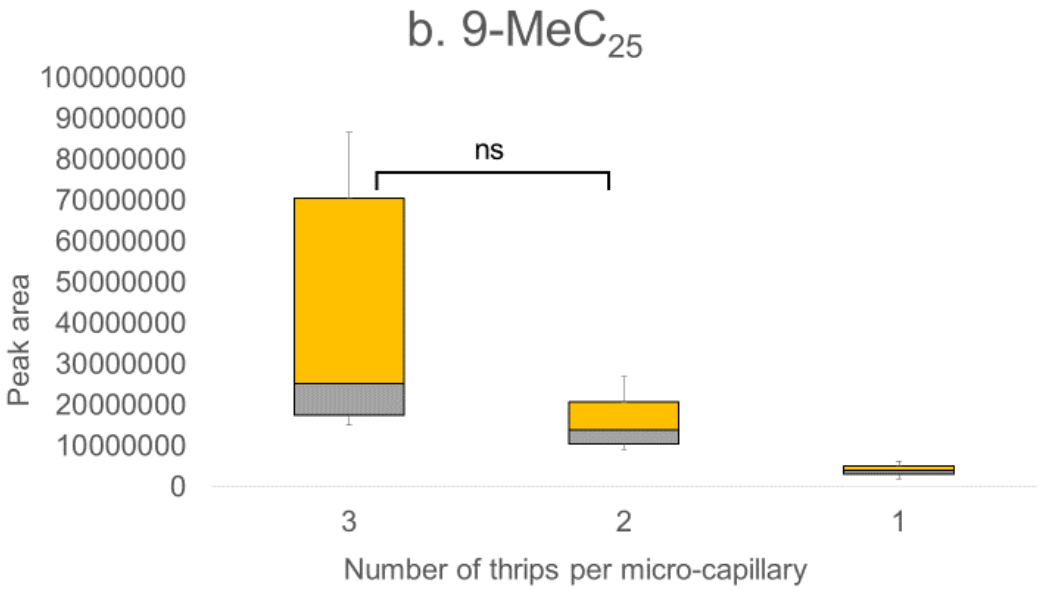
a - CHC abundance of the compounds for density 2 (D2) or two adult females per glass capillary (n=5)
b - CHC abundance of the compounds for density 3 (D3) or three adult females per glass capillary (n=6)
c - major CHC compounds whose relative abundance was constantly high in samples with 2 respectively 3 insects per glass capillary
W - Shapiro-Wilk test largely used to verify the normal distribution within data sets
ns = not significant as *p* value is higher than 0.05

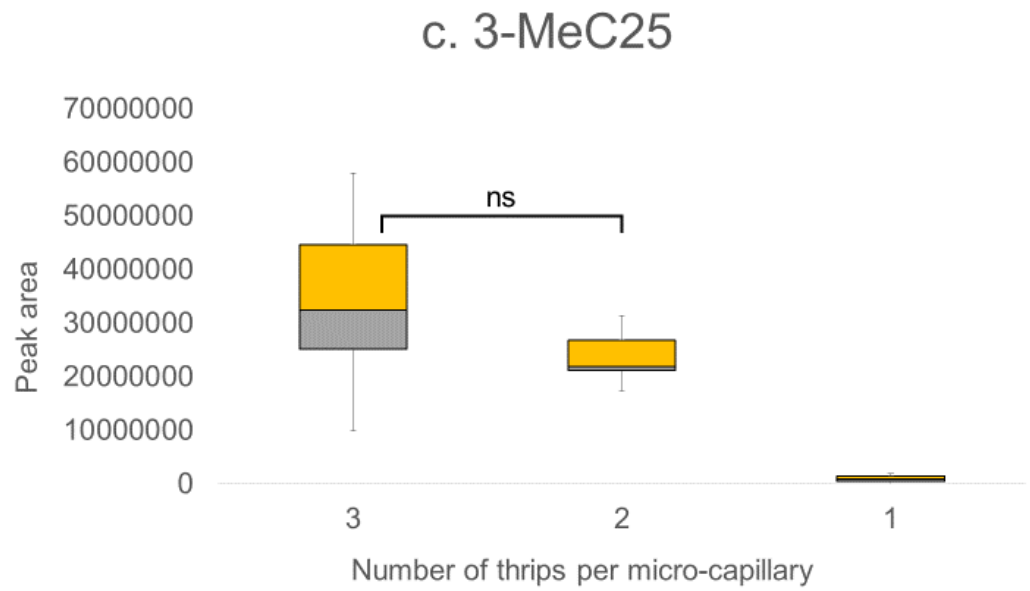
Table 3.2 Overview of the CHC pattern found in *F. occidentalis* at three different densities using the solid injection method. Symbols indicate that the compound was either present (+) or absent (-) in the samples with the specified density

	Number of thrips per micro-capillary		
Compound	**1 (n=6)**	**2(n=5)**	**3 (n=6)**
7-MeC$_{23}$	–	+	+
n-C$_{25}$	+	+	+
11-Me-, 13-Me-, 15-MeC$_{25}$	+	+	+
9-MeC$_{25}$	+	+	+
7-MeC$_{25}$	+	+	+
5-MeC$_{25}$	–	+	+
9,11-diMeC$_{29}$, 9,13-diMeC$_{25}$	+	+	+
3-MeC$_{25}$	+	+	+
5,9-diMeC$_{25}$, 5,11-diMeC$_{25}$	+	+	+
n-C$_{26}$	–	+	+
10-Me-, 12-Me-, 14-Me-,16-MeC$_{26}$	–	+	+
6-MeC$_{26}$	–	+	+
4-MeC$_{26}$	–	+	+
3-MeC$_{26}$	–	+	+
n-C$_{27}$	+	+	+
11-Me-, 13-Me-, 16-MeC$_{27}$	+	+	+
9-MeC$_{27}$	+	+	+
7-MeC$_{27}$	–	+	+
5-MeC$_{27}$	–	+	+
9,11-diMeC$_{27}$, 9,13-diMeC$_{27}$	+	+	+
3-MeC$_{27}$	+	+	+
5,9-diMeC$_{27}$, 5,11-diMeC$_{27}$	+	+	+
X,X-diMeC$_{27}$	–	+	+
10-Me-, 12-Me-, 14-Me-,16-MeC$_{28}$	–	+	+
4-MeC$_{28}$	–	+	+
n-C$_{29}$	–	+	+
11-Me-, 13-Me-, 16-MeC$_{29}$	+	+	+
9-MeC$_{29}$	–	+	+
7-MeC$_{29}$	–	+	+
11,15-diMeC$_{29}$	–	+	+
9,11-diMeC$_{29}$, 9,13-diMeC$_{29}$	+	+	+
7,11-diMeC$_{29}$	+	+	+
3-MeC$_{29}$	–	+	+
5,9-diMeC$_{29}$, 5,11-diMeC$_{29}$	–	+	+
11-Me-, 13-Me-, 16-MeC$_{31}$	–	+	+
11,15-diMeC$_{31}$	–	+	+
7,11-diMeC$_{31}$	–	+	+

Taking into account the normality test results, unpaired *t* – tests with Welch's correction were run and two-tailed *p* value was calculated. No significant difference was found between the two datasets (D2 vs D3) for each compound for which two-tailed *p* value was lower than 0.05. This was the case for all selected compounds (Fig. 3.2). Therefore, since the same quality of information was revealed for both densities, the optimum density size was set by using the lowest number of insects (two) in a glass capillary sample.




b. 9-MeC25
100000000
90000000
80000000
70000000
60000000
50000000
40000000
30000000
20000000
10000000
0
Peak area
ns
3
2
1
Number of thrips per micro-capillary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30000000
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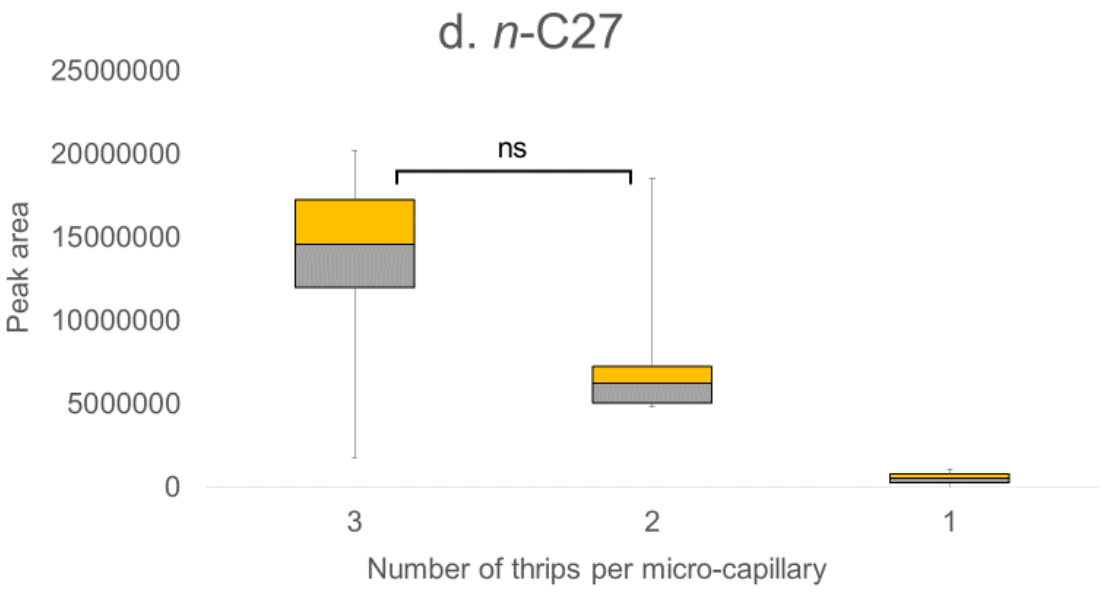
10000000
0
Peak area
ns
3
2
1
Number of thrips per micro-capillary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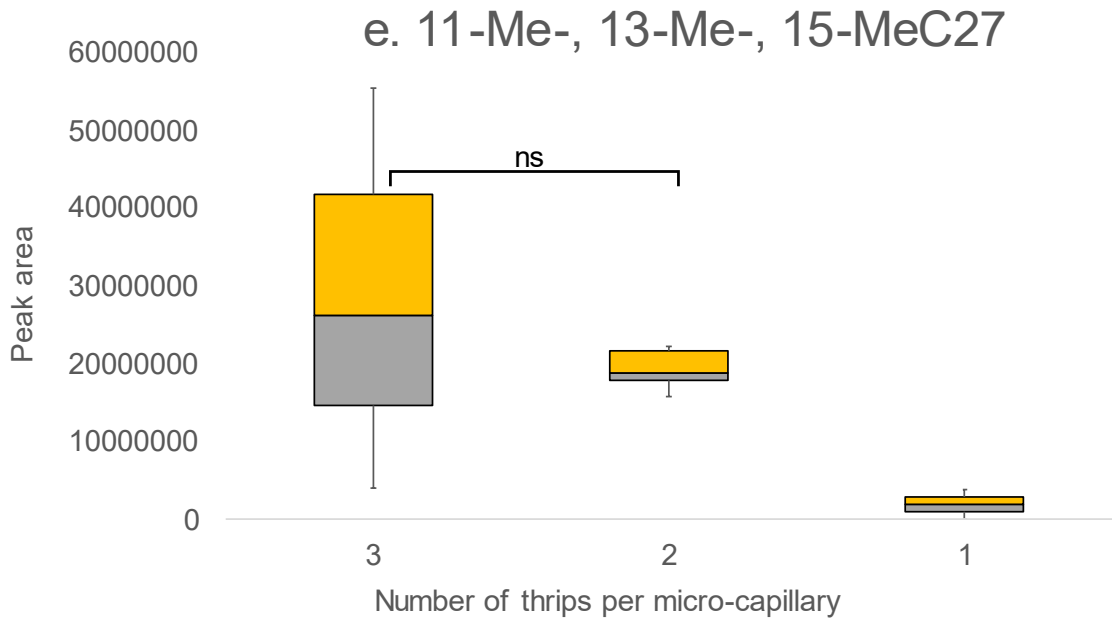
15000000
10000000
5000000
0
Peak area
ns
3
2
1
Number of thrips per micro-capillary


e. 11-Me-, 13-Me-, 15-MeC27
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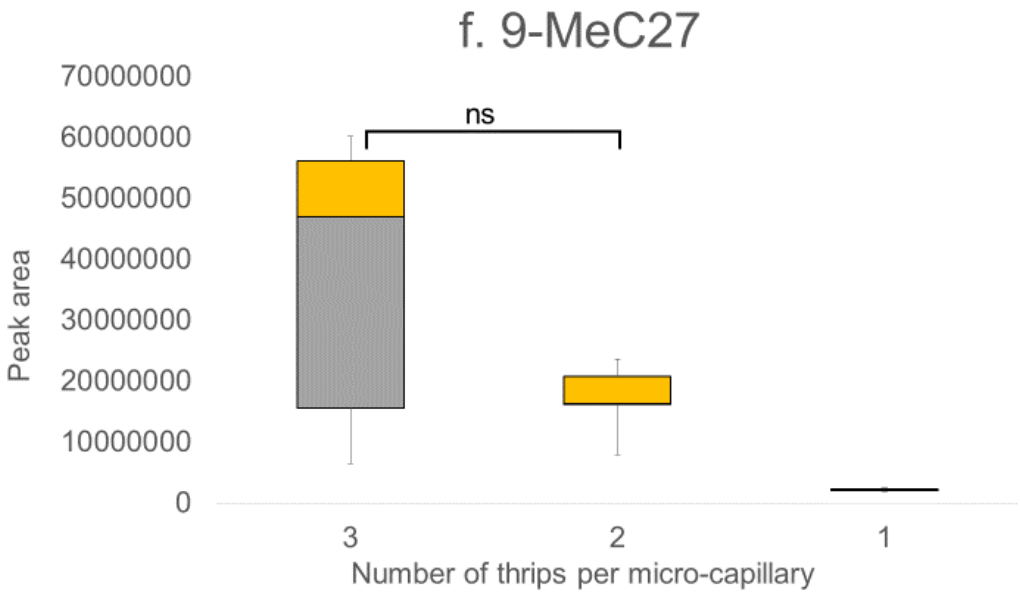
50000000
40000000
30000000
20000000
10000000
0
Peak area
ns
3
2
1
Number of thrips per micro-capillary


f. 9-MeC27
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30000000
20000000
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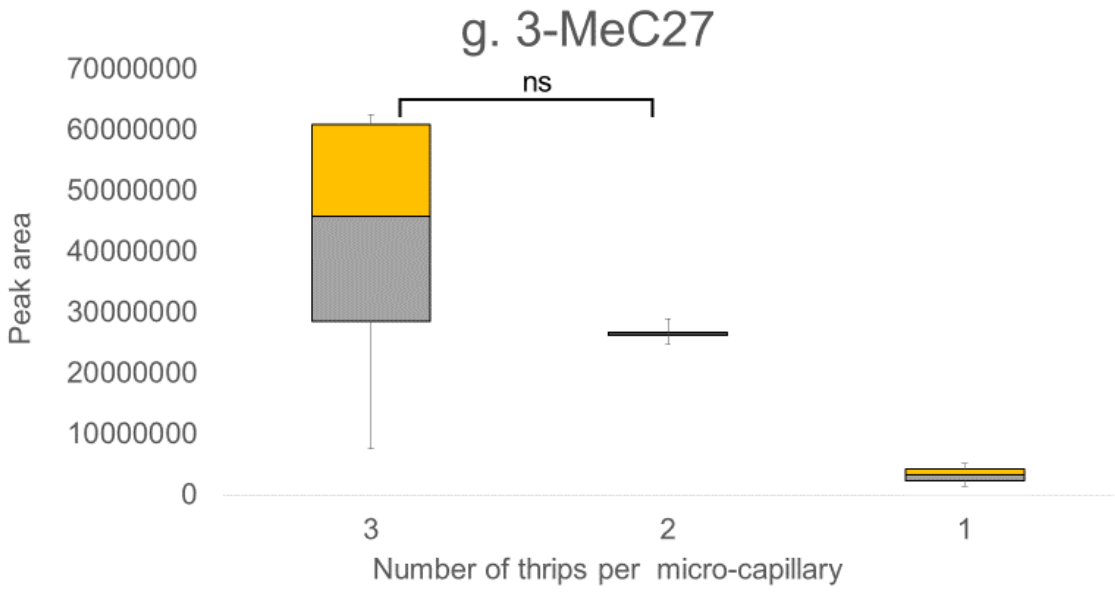
0
Peak area
ns
3
2
1
Number of thrips per micro-capillary


g. 3-MeC27
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0
Peak area
ns
3
2
1
Number of thrips per micro-capillary

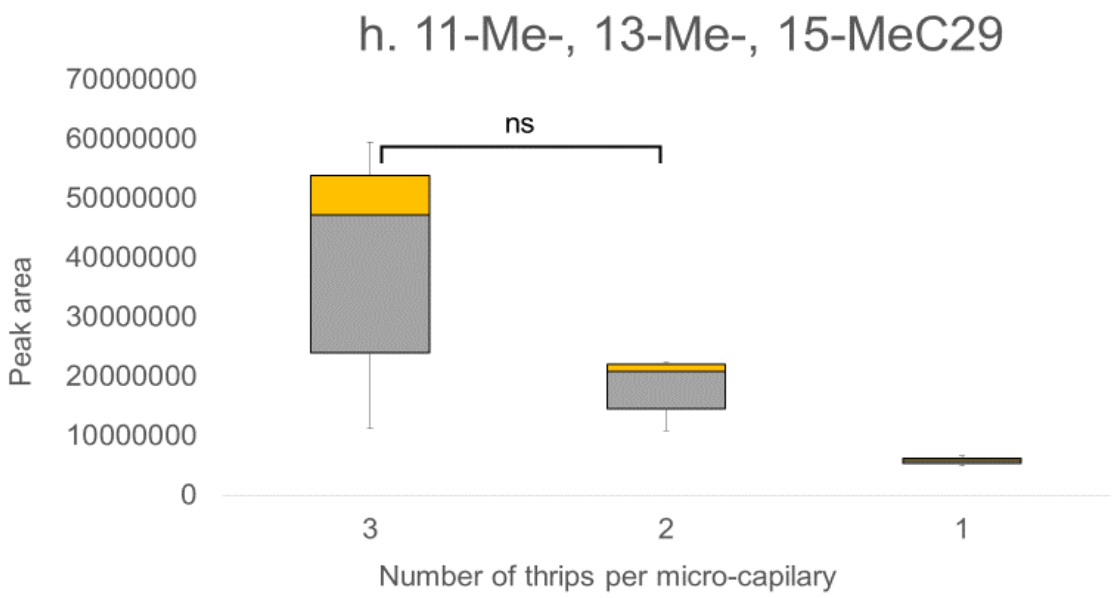


Fig. 3.2 Box plots (a-h) indicating the GC peak area of the dominant CHCs identified in *F. occidentalis* at three different density sizes (1, 2 and 3 adult females/micro capillary). The boxes show interquartile range (from 25th to 75th percentiles) and the whiskers bars indicate the minimum and maximum values. In the middle of the boxes the horizontal bars represent the median values. *ns* = not significant

3.1.2 Discussion on the results for sample density optimisation

So far, the most common analytical method for analysing hydrocarbons in thrips is based on liquid extraction[34,40,127]. However, a solvent-free technique was tested in this study based on direct analysis of the whole body with the aid of a custom-made solid injection device. In order to evaluate the efficiency of the method, a-priori, the optimum number of insects per sample was tested. Adult females of *F. occidentalis* were used as the model species to prepare samples with one, two or three individuals in a glass tube. In order to investigate the optimum number of thrips in a sample, the peak area of the eight most abundant peaks in samples with two and three females were statistically compared and the results indicated that two insects per microcapillary should be used. Unfortunately, it was not possible to run optimisation

trials for each of the species delivered from Kenya, because at the collection time, there were low populations of insects in the field.

The GC analysis of samples with a single insect per glass capillary indicated an inter-individual variation regarding the produced hydrocarbons. Out of six replicates, data was recorded only in two of them. This could be related to intrinsic factors like, age[128], gender or fecundity status[40]. Notably, in this study, laboratory reared thrips were used and the insects' age and satiety and fecundity status were unknown. Still, majority of the hydrocarbons found in a single individual of thrips are consistent with those reported by Chen *et al.*[121], who also used solid injection analysis (based on a commercially available device) to test samples containing only one *F. occidentalis* adult. Chen and co-workers[121] reported that the insects were starved 24 h before the GC analysis. In the current research, the samples were collected straight from the culture and immediately freeze-killed before insertion into the micro-capillary. Therefore, it can be speculated that the satiety status may have a minimal impact on the CHC composition. Chen and collaborators[121] have also reported the use of cultured insects, but it was not specifically mentioned. Hence it can be assumed that the fecundity status and the age of the insects use for their samples, was also not known. Thus, more investigations are required to verify the effect of these two factors in the production of CHCs in thrips insects. Additionally, the variation could be linked to the device. A commercially-produced device, like the thermo separation probe, is probably more efficient in transferring compounds as compared to a custom-made one.

Additionally, regarding the samples with a single insect per glass capillary, in their work Chen and collaborators[121] tested two different populations of western flower thrips, from Beijing and Holland, and dimethyl alkanes were not reported to be present in either of the tested populations. These compounds were, however, found in the

population from Keele University (UK), indicating qualitative differences among populations. Thus, as compared to Chen *et al.*'s[121] work this suggests, to an extent, an intra-specific variation[129].There were also several differences in terms of the relative abundance of the hydrocarbons among the profiles which are likely related to the geographical location of the samples and/or the host plant used for laboratory rearing. In an age-grading study conducted on *Aedes aegypti*[130], a mosquito species, it was suggested that geographical location could explain the CHC variation between samples collected at two different sites. The effect of the host plant on the biosynthesis of CHC has already been proved in mustard leaf beetles (*Phaedon cochleariae*)[131]. The variation of the CHC pattern caused by host plant, led to mating behavioural changes in leaf mustard beetle and the laboratory bioassays showed that males preferred to mate with females reared on the same plant species. Whether this is the case in thrips species, it would be of interest to be researched. The outcome could help to understand better their multilevel interactions (with their host-plants and between the individuals of opposite gender).

In the prior studies that investigated the solid injection analysis in thrips, there are no mention of optimisation tests related to the sample size, hence no records for samples with two insects in a sample, the focus was on a single individual. There is a practical advantage of dealing with sample sizes as low as one specimen, especially when in early stages of monitoring a pest, as the number of insects is reduced. However, because CHCs in thrips, as a research subject, has been very little explored, collecting various type of data would make a significant contribution to the literature and would help to discover whether there are any patterns in their behaviour associated with their chemical profile. It is worth mentioning that by increasing the density of the insects per glass vial, the diversity of the detected hydrocarbon classes

has increased as well. Particularly, trace amounts of alkenes with single double bond in the chain were seen in the samples with three adults per micro-vial (data not shown because the focus was on samples with density of two adults due to the established optimum density size). Monoenes have not been reported before in *F. occidentalis*. However, these alkenes were not seen in the hexane-extraction samples for which a significant number of 100 females were used (section 2.9). Given their low concentration, they could have been diluted even further through the steps of extraction and the remained amounts were, maybe, under the limit of the detection.

As further work, it would be interesting to test, with the solid injection, whether the range of CHC content increases by increasing further the number of insects or it remains stable. More importantly, considering that western flower thrips is a major pest of crops[8] it would be of practical interest to test whether the alkene compounds act as semiochemicals, as this could lead to new methods for its biological control.

3.2 Results for solid injection efficiency test

The mirrored total ion chromatograms of CHCs from *F. occidentalis*, detected through whole-body analysis and hexane extraction in both cases using the solid-injection method, are shown in Fig. 3.3. The chemical compounds included in these two profiles along with their correspondent Kovats Index (KI) value and relative abundance can be seen in Table 3.3. The GC-MS analysis of the hexane extracted samples revealed a comparable hydrocarbon profile to the one presented in section 3.1 (Table 3.2) analogue for the samples with two insects per microcapillary. Thus, a mixture of *n*-alkanes (*n*-C_{25} to *n*-C_{31}) and branched monomethyl alkanes (-MeC_{23}, -Me$C_{25\text{-}29}$, -MeC_{31}) were identified in these samples as well.

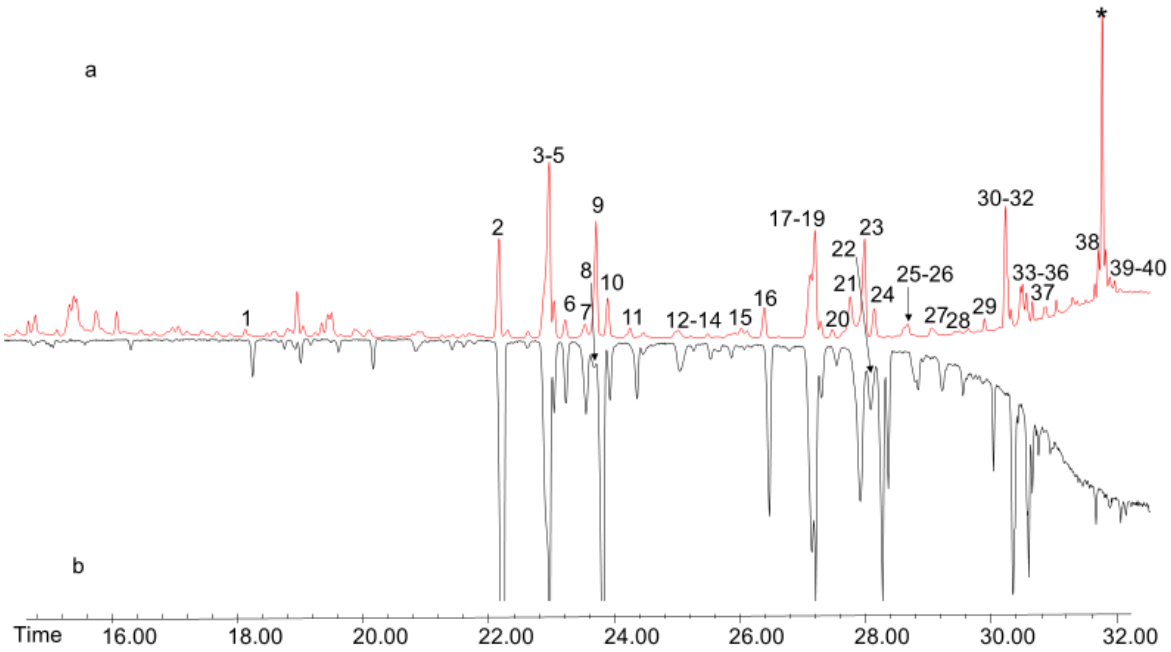


Fig. 3.3 Solid injection-based gas chromatograms mirroring the CHC profile for *F. occidentalis* obtained through whole-body analysis (a) and hexane extraction (b). Peak identification listed in Table 3.3 under the same peak number; * cholesterol

As per Fig 3.3, it can be seen in both chromatograms (a and b) that the same dominant hydrocarbons are well represented. These dominant hydrocarbons are found at peaks 2 - 4, 9, 16-18, 23, 30, 31 and they correspond to: *n*-C_{25}; 11Me-,13Me-,15-MeC_{25}; 9-MeC_{25}; 3-MeC_{25}; 11Me-,13Me-,15-MeC_{27}; 9-MeC_{27}; 3-MeC_{27}; 11Me-,13Me-,15-MeC_{29} and 9-MeC_{29}, respectively. Three compounds, corresponding to peaks 8, 22 and 25 in Table 3.3 (7,11-diMeC_{25}, 7,11-diMeC_{27} and *n*-C_{28}, respectively) were not found through the whole-body analysis. In terms of the compound at peak 8, although present in the hexane extract samples, it was found only in trace amounts (Fig. 3.3b). The hydrocarbon at peak 22 seems to be produced in higher concentration as compared to the one produced at peak 8. Still, it was absent from the whole-body analysis. Regarding octacosane, *n*-C_{28}, (at peak 25) it is represented through a small shoulder due to co-elution with peak 26 (a -diMeC_{27} with unknown positions of the

methyl groups). Most likely, the octacosane could be also present in the solid samples, but the low intensity of the ions and the lack of molecular ion in its mass spectrum did not allow tentative identification. This could be due to this compound being, overall, produced in low concentration, perhaps under the limit of detection, thus its measurement from only two individuals was not revealing enough.

The CHC corresponding to peaks 28 and 40 (Fig. 3.3a) were found in trace amounts in the whole-body samples, but absent in hexane extract samples. Considering the small amounts found in the solid samples it could be that, in the hexane extract samples, the compounds at peak 28 and 40 were lost during the liquid extraction protocol (section 2.9).

The efficiency of the solid injection method was assessed through comparison of the peak areas of each individual compound, detected through both hexane extraction and whole-body analysis. For instance, *n*-C_{27} was found in both profiles and the abundances (peak areas) measured with each type of analysis were statistically compared. Thus, 31 comparisons were conducted altogether. Beforehand, to ensure the appropriate statistical method was chosen, especially considering the small size of the samples, it was important to determine the distribution of the data. For this purpose, a Shapiro-Wilk test (*W*) was performed and the results indicated no evidence of non-normality. Considering this outcome, a series of unpaired *t* – tests were conducted and for each variable (the compound) the mean value and the standard error of the mean (±SEM) was calculated. Overall, the unpaired *t* – tests result revealed *p* values lower than 0.05 which suggested no significant difference (Table 3.3) between the peak areas of individual compounds contained in the two profiles. There is, however, an exception for 11,15-diMeC_{29} (peak number 33) whose abundance was significantly higher ($t = 4.186$, d.f. = 2.141, $p = 0.046$) in the samples

prepared for whole-body analysis. On further examination, this *p* value is slightly under the conventional threshold of 0.05 which makes this particular result debatable and it should be disregarded. Across the series of *t* - tests, unpaired two-tailed *p* value and Welch's correction was considered.

Beside CHC, internally produced molecules like cholesterol (the peak labelled with an asterisk in Fig. 3.3a) were found in the chemical composition of the cuticle *F. occidentalis*, but were not taken into account in any of the data analysis, as the focus of this project is exclusively on hydrocarbon compounds.

Table 3.3 Comparison of the CHC profiles detected in adult females of *F. occidentalis* through hexane extraction and whole-body analysis using the solid injection technique. The relative abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample

			Relative abundance (%)[b]		
Peak number	**Compound[a]**	**KI**	**Hexane extraction analysis**	**Whole-body analysis**	**Statistical significance**
1	7-MeC$_{23}$	2341	0.45±0.13	0.33±0.19	ns
2	*n*-C$_{25}$	2500	13.54±5.64	6.79±0.57	ns
3	11-Me-, 13-Me-, 15-MeC$_{25}$	2534	4.30±1.61	4.78±1.51	ns
4	9-MeC$_{25}$	2536	7.37±2.93	5.30±1.10	ns
5	7-MeC$_{25}$	2540	0.99±0.39	1.26±0.31	ns
6	5-MeC$_{25}$	2548	1.12 0.43	1.44±0.30	ns
7	9,11-diMeC$_{29}$, 9,13-diMeC$_{25}$	2562	1.76±0.70	1.43±0.46	ns
8	7,11-diMeC$_{25}$	2568	0.61 ±0.24	ND	N/A
9	3-MeC$_{25}$	2574	7.42±3.09	7.79±0.95	ns
10	5,9-diMeC$_{25}$, 5,11-diMeC$_{25}$	2579	1.13±0.46	2.05±0.63	ns
11	*n*-C$_{26}$	2600	1.20±0.45	0.82±0.10	ns
12	10-Me-, 12-Me-, 14-Me-,16-MeC$_{26}$	2633	1.09±0.49	0.81±0.20	ns
13	6-MeC$_{26}$	2644	0.15±0.08	0.15±0.01	ns
14	4-MeC$_{26}$	2657	0.37±0.14	0.29±0.02	ns
15	3-MeC$_{26}$	2672	0.31±0.16	0.37±0.06	ns
16	*n*-C$_{27}$	2700	3.17±1.30	2.64±0.69	ns
17	11-Me-, 13-Me-, 15-MeC$_{27}$	2735	5.29±2.17	6.27±0.35	ns
18	9-MeC$_{27}$	2739	5.86±2.49	5.52±0.88	ns
19	7-MeC$_{27}$	2743	0.84±0.32	1.65±0.33	ns
20	5-MeC$_{27}$	2751	0.30±0.16	0.71±0.14	ns
21	9,11-diMeC$_{27}$, 9,13-diMeC$_{27}$	2765	3.96±1.64	3.69±0.60	ns
22	7,11-diMeC$_{27}$	2771	1.32±0.55	ND	N/A
23	3-MeC$_{27}$	2777	4.18±1.78	8.67±0.14	ns
24	5,9-diMeC$_{27}$, 5,11-diMeC$_{27}$	2781	1.91±0.80	2.88±1.36	ns
25	*n*-C$_{28}$	2800	1.06±0.66	ND	N/A
26	X,X-diMeC$_{27}$	2804	0.94±0.40	1.45±0.33	ns
27	10-Me-, 12-Me-, 14-Me-,16-MeC$_{28}$	2835	0.75±0.39	0.98±0.11	ns
28	4-MeC$_{28}$	2875	ND	0.33±0.09	N/A
29	*n*-C$_{29}$[d]	2900	1.98±0.16	0.27±0.27	N/A
30	11-Me-, 13-Me-, 15-MeC$_{29}$	2934	5.27±2.01	5.85±0.65	ns
31	9-MeC$_{29}$	2937	2.11±0.68	2.15±0.26	ns
32	7-MeC$_{29}$	2943	0.49±0.25	1.32±0.22	ns
33	11,15-diMeC$_{29}$[c]	2959	2.38±0.45	3.00±0.47	*
34	9,11-diMeC$_{29}$, 9,13-diMeC$_{29}$	2962	2.79±0.83	2.91±0.34	ns
35	7,11-diMeC$_{29}$	2969	1.43±0.71	2.49±0.44	ns
36	3-MeC$_{29}$[d]	2976	0.21±0.21	1.31±0.20	N/A
37	5,9-diMeC$_{29}$, 5,11-diMeC$_{29}$	2979	1.47±0.74	1.45±0.30	ns

38	11-Me-, 13-Me-, 15-MeC$_{31}$[d]	3131	2.17±2.17	3.72±0.26	N/A
39	11,15-diMeC$_{31}$[d]	3159	1.68±1.68	3.38±0.55	N/A
40	7,11-diMeC$_{31}$[d]	3171	ND	3.72±0.48	N/A

Peak number corresponding to those in Fig. 3.3; *ND* = not detected; *N/A* = not applicable due to not tested; *ns* = not significant; X = the unknown position of the methyl group

[a] Abbreviations: *n*-C$_{23}$ = *n*-tricosane, etc., 7-MeC$_{23}$ =7-methyletricosane, etc., 9,11-diMeC$_{25}$, 13-diMeC$_{25}$ = 9,13-dimethylpentacosane, etc. Tentative identification based on Kovats Index values and a match with NIST2.2 Library Database

[b] Mean percentage of CHCs ± SE for hexane extraction analysis (n = 3 glass capillaries containing 1 µl of liquid extract for which 100 mixed age adult females were used) and whole-body analysis (n = 5 glass capillaries each containing two adult females per glass tube)

[c] significant at $p = 0.0467$

[d] *t*-test was not run due to the compound was found only in one of the replicates

3.2.1 Discussion on the results for solid injection efficiency test

The results presented in this work, in regards to sample optimization, revealed that a low density of insects would be sufficient to extract CHCs from tiny insects like thrips. The optimum number of thrips per sample, was established as two adults per micro-capillary (section 3.1). To test the efficiency of the solid injection method, a proof-of-concept experiment was performed. In a nut-shell, the CHC dataset obtained through whole-body analysis was compared with the one obtained through hexane extraction and the results indicated that, overall, quantitatively, there was no statistical difference between the two hydrocarbon patterns. There were, though, some compounds that were present in the whole-body extracts but absent in the hexane extracts and vice-versa. Considering that for both experiments thrips specimens came from the same culture, is unlikely that this variation to be linked to the origin of the insects. However, as previously mentioned, intrinsic factors like age and fecundity status, which were unknown, may explain some of the differences between the two hydrocarbon patterns.

Over time, different researchers sought ways to detect directly (solventless) the volatile constituents from small insects by using either body parts or the whole body. For instance, in 1972, Morgan and Wadhams[122], proposed the customised solid-

sampler used in the present work. They tested the sensitivity of the device by injecting a single Dufour gland collected from an ant species. The description of the device and how it is used, has been explained in Chapter 2 (section 2.5). Later on, in 1985, Brill and Bertstch[132] tested the efficacy of a so called Pyroprobe Injector. Their techniques involved the use of the device under thermal desorption conditions and its efficacy was also tested on ant samples, but they used the whole body of the insects. More recently in 2016 and 2020, a commercially available Thermal Separation Probe (TSP) which also involves the thermal desorption for sample analysis, was used to assess the CHCs in body parts from fruit flies and from the whole body of six species of thrips, respectively[121,133].

The use of a solid injection method provides a series of benefits: i) it does not involve solvent, thus there is no first strong peak related to the solvent to overshadow any early eluting compounds; ii) the sample size can be very small; iii) it is comparable to liquid injection technique in regards to the quality of data (as proved in this study, as well); iv) can detect internally produced compounds; v) the device is cheap. Moreover, the sensitivity of the solid-sampler device has been tested for the detection of the aggregation pheromone produced by the adult males of *F. occidentalis* (Covaci, Kirk and Drijfhout, unpublished data). The aggregation pheromone was initially identified through liquid extraction and for each replicate, tens of adult males were required[33,134]. This high number of males per sample, is quite difficult to achieve, simply because males are less abundant in the population. However, with the solid injection, the detection of the aggregation pheromone, was possible from as few as 10 individual thrips for each sample (Covaci, Kirk and Drijfhout, unpublished data unpublished data). Therefore, this proves that the solid injection technique can be also employed in the discovery of pheromone compounds in thrips insects. A better understanding of

thrips behaviour could also enhance the use of pheromones in pest management (in monitoring, push pull or mass trapping). However, several downsides were associated with this method: i) the samples must be prepared in a small glass capillary, which can be a slightly time-consuming process; ii) the instrument must be adjusted to accommodate the solid injection device; iii) septa (from the inlet oven and device) must be regularly checked to avoid low pressure in the inlet oven; iv) the method relies solely on manual injection and this can be quite time consuming if large number of samples must be analysed. Nevertheless, the advantage of using the solid injection method to detect the hydrocarbon profile and its capability to detect potential pheromone compounds, outweigh the limitations. Even more, due to its proficiency in analysing body parts, it could be used to detect the production site of the aggregation pheromone in western flower thrips which as of yet, is not known for sure, only assumed. Additionally, valuable information would be revealed in terms of the CHC distribution on thrips' body parts.

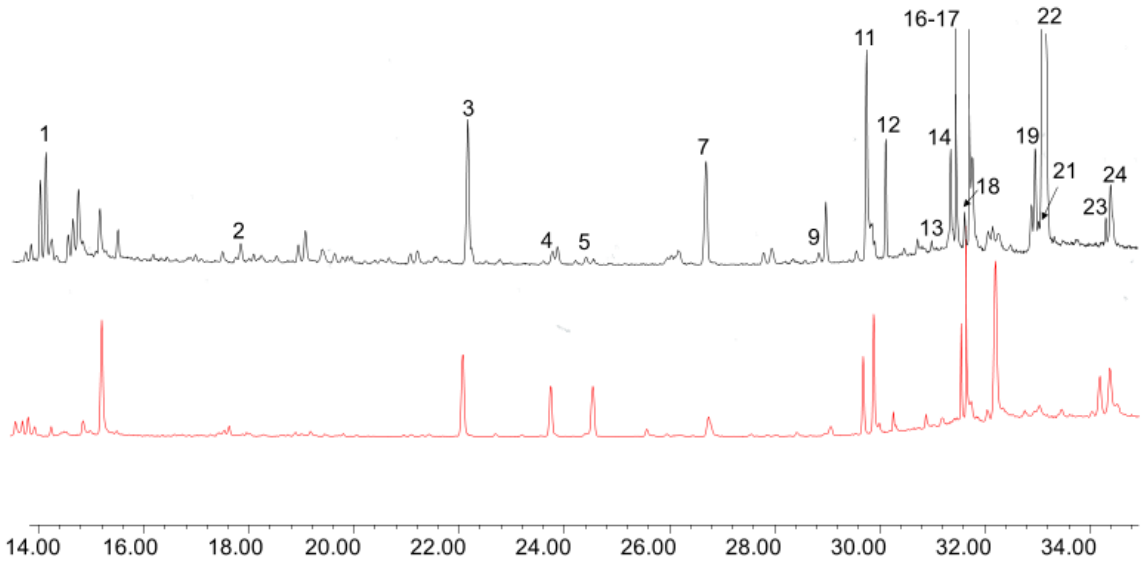
3.3 Results for solid injection analysis of CHCs in five different species of thrips

The investigation of the CHCs in five species of thrips (including *F. occidentalis* by considering the whole-body extract), revealed various linear alkanes with one or two methyl groups attached to their chain length (resulting in mono- and dimethyl branched alkanes), as well as unsaturated alkenes and alkynes. The gas chromatograms for the four species of thrips delivered from Kenya are given in Fig. 3.4 and for each species the TIC for females and males (except *T. tabaci*) is presented. Also, in Tables 3.4 – 3.7 are presented the species/gender specific hydrocarbon

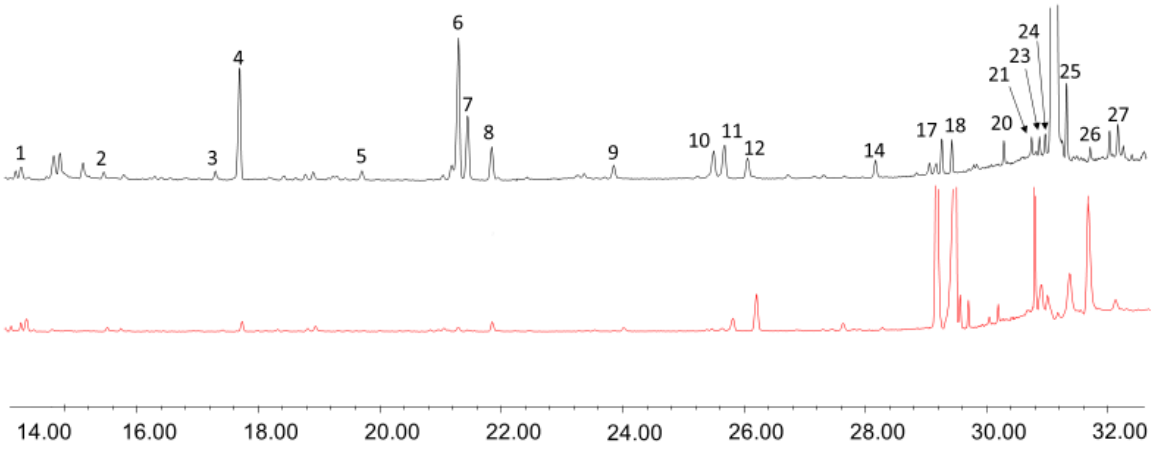
patterns and the relative content values of the individual compounds detected in each profile.

As a first general observation, in the case of the samples for which both females and males were recorded (*F. borinquen, F. schultzei* and *H. adlofifriderici*), it was noticed that a higher number of CHC were detected in females, as compared to males. Also, by considering the 73 hydrocarbon structures found in all adults, the odd-numbered-chain hydrocarbons are more abundant, they represent 77% of the total number of compounds. A second general observation, refers to the fact that a different group of hydrocarbons was found to be dominant in each species profile. For instance, alkenes predominate in *F. schultzei* (52%) and *F. borinquen* (33%). Both monomethyl and dimethyl alkanes were mainly found in *T. tabaci* and *F. occidentalis*, where they account for 80% and 89%, respectively. Finally, linear alkanes, were prevalent in *H. adolfifriderici* where they represent 83% of the total number of compounds. When looking at the overall chemical composition of the tested insects, it can be seen that the chain length of alkanes does not significantly differ among the thrips species. However, in this study, CHCs with less than 21 carbons in the backbone, were not integrated, hence not recorded, but they could potentially be present in the samples. Below, in subsections 3.3.1- 3.3.5, specific characteristics related to the hydrocarbon pattern will be presented separately for each species of thrips.

a. *Frankliniella borinquen* ♀ and ♂



b. *Frankliniella schultzei* ♀ and ♂



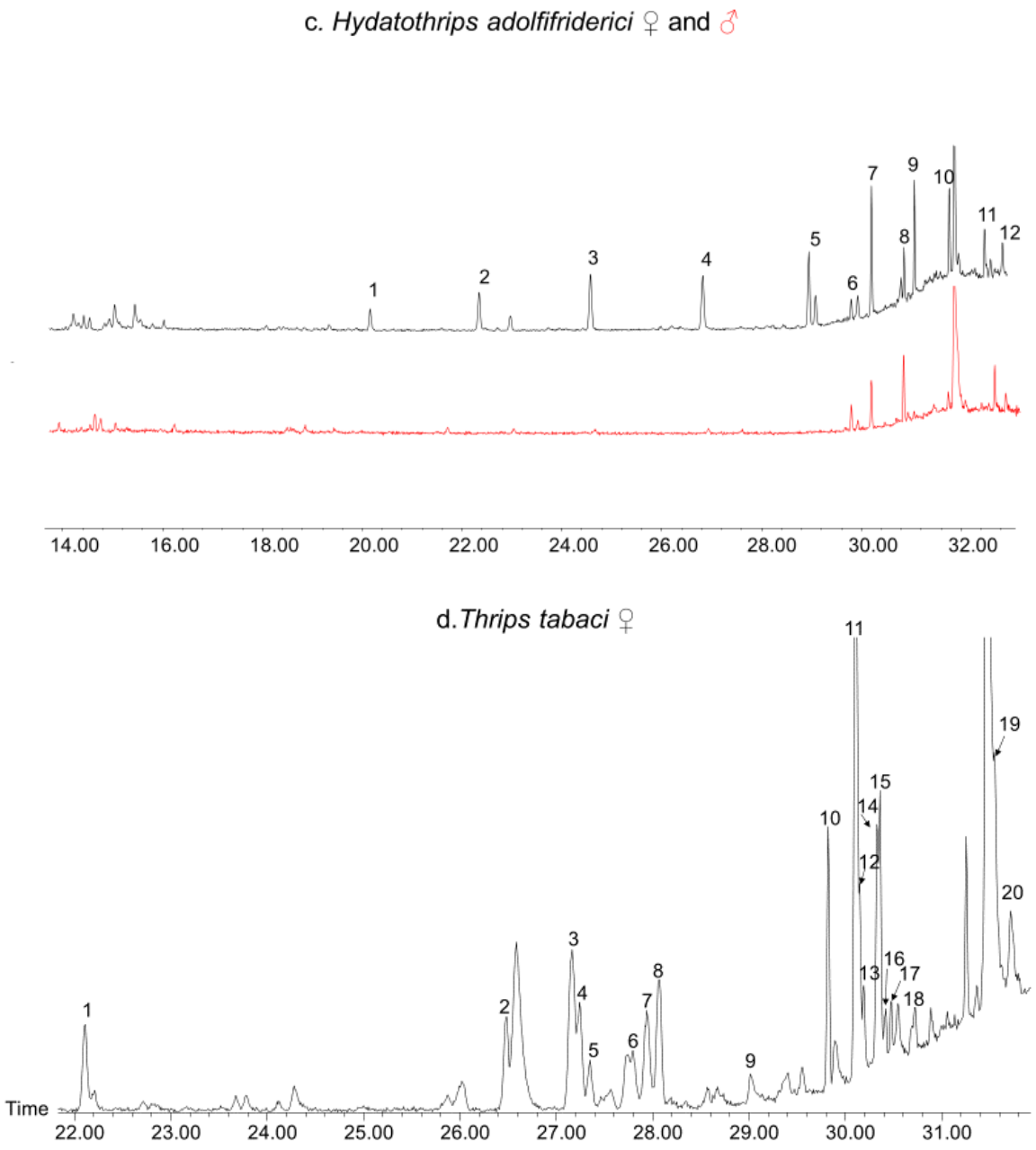


Fig. 3.4 Total ion chromatograms of adults from four (a-d) species of thrips. In figures a, b and c the top TIC (in black) is correspondent to adult female whereas the bottom TIC (in red) to adult male. Peak numbers are matching the CHCs found in Table 3.4, 3.5, 3.6 and 3.7 and are corresponding to *F. borinquen*, *F. schultzei*, *H. adolfifriderici* and *T. tabaci* respectively

3.3.1 CHCs in *Frankliniella borinquen*

A total of 24 hydrocarbons were identified from the cuticle layer of *F. borinquen*, as presented in Table 3.4. Notably, compounds with up to 35 carbons in the chain were detected, which was only found in this species of thrips. As compared to the rest of the species in which the CHC profile is mainly based on compounds that belong to a single group or maximum two groups of hydrocarbons, *F. borinquen*'s profile is more diverse, as it contains hydrocarbons from three different classes: mono or tri- methyl branched alkanes (50%), alkenes with one or two double bonds (33%) and branched alkynes (17%). Another important note is that among the tested insects, *F. borinquen* is the only species of thrips in which alkynes and alkadienes were detected. In terms of most abundant compounds, as shown in Table 3.4, in the adult females, the highest concentration was calculated for $C_{33:1}$ (21%) whereas in males the most abundant was for $C_{31:1}$, accounting for circa 18% from the total peak area of the sample.

Table 3.4 CHCs compounds detected in *F. borinquen* along with the relative content percentage (RA%) of each compound and Kovats Index (KI) for their identification. The relative abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample

			RA%[b]	
Peak number	**Compound[a]**	**KI**	**Females (n=9)**	**Males (n=8)**
1	*n*-C_{21}	2100	3.30±0.45	2.29±0.39
2	*n*-C_{23}	2300	1.00±0.19	1.13±0.14
3	*n*-C_{25}	2500	7.97±0.81	13.51±1.10
4	3-MeC_{25}	2572	0.43±0.19	6.15±0.68
5	*n*-C_{26}	2600	0.91±0.27	ND
6	X,8,X-triMeC_{26}	2607	ND	6.01±0.65
7	*n*-C_{27}	2700	5.19±0.48	4.26±0.25
8	3-MeC_{27}	2776	ND	0.53±0.25
9	*n*-C_{28}	2800	0.82±0.12	ND
10	$C_{29:2}$	2856	ND	5.48±0.47
11	$C_{29:1}$	2872	6.60±1.17	9.19±0.67
12	*n*-C_{29}	2900	3.83±0.21	2.77±0.19
13	*n*-C_{30}	3000	2.08±0.34	2.91±0.72
14	11- Me$C_{31\text{- alkyne}}$	3051	6.80±0.60	ND
15	$C_{31:2}$	3052	ND	7.26±1.20
16	$C_{31:1}$	3069	11.05±0.69	17.98±3.16
17	$C_{31:1}$	3074	9.86±0.35	ND
18	*n*-C_{31}	3100	4.05±0.63	4.32±1.39
19	11 - Me$C_{33\text{- alkyne}}$	3251	2.95±0.75	ND
20	$C_{33:2}$	3251	ND	5.02±1.17
21	13 - Me$C_{33\text{- alkyne}}$	3258	2.78±0.87	ND
22	$C_{33:1}$	3268	21.06±1.77	11.20±2.78
23	11- Me$C_{35\text{- alkyne}}$	Unknown[c]	1.31±0.56	ND
24	$C_{35:1}$	Unknown[c]	8.01±0.98	ND

Peak numbers correspond to those in Figure 3.4 (a)

[a] The abbreviation of most of the compounds is explained in the footnotes of Table 3.3, except: $C_{29:1}$ = nonaocosene or C_{29} alkene or C_{29} mono -ene, etc; $C_{29:2}$ = C_{29} alkadiene, etc., $C_{31\text{-alkyne}}$ = hentricontyne, etc., 11-Me$C_{31\text{-alkyne}}$ = 11-methylhentriacontyne, etc. Tentative identification based on Kovats Index values and a match with NIST2.2 Library Database

[b] Mean percentage of cuticular lipids ±SE from samples with two adults per capillary tube

[c] Retention Index value not calculated as to the retention time for the larger alkane (N value in the Kovats Index equation) was unknown

In Table 3.4, two hydrocarbons (at peak 14 and 15) with similar values for their retention index were identified as distinct compounds. Specifically, the compound found in the cuticular layer of the female sample (KI value of 3051) was identified as 11-C_{31} alkyne (peak 14) whereas the cuticular lipid found in the males (KI value of 3052) was identified as an alkadiene (peak 15) with 31 carbons ($C_{31:2}$). Their mass spectra are shown in Fig. 3.5.

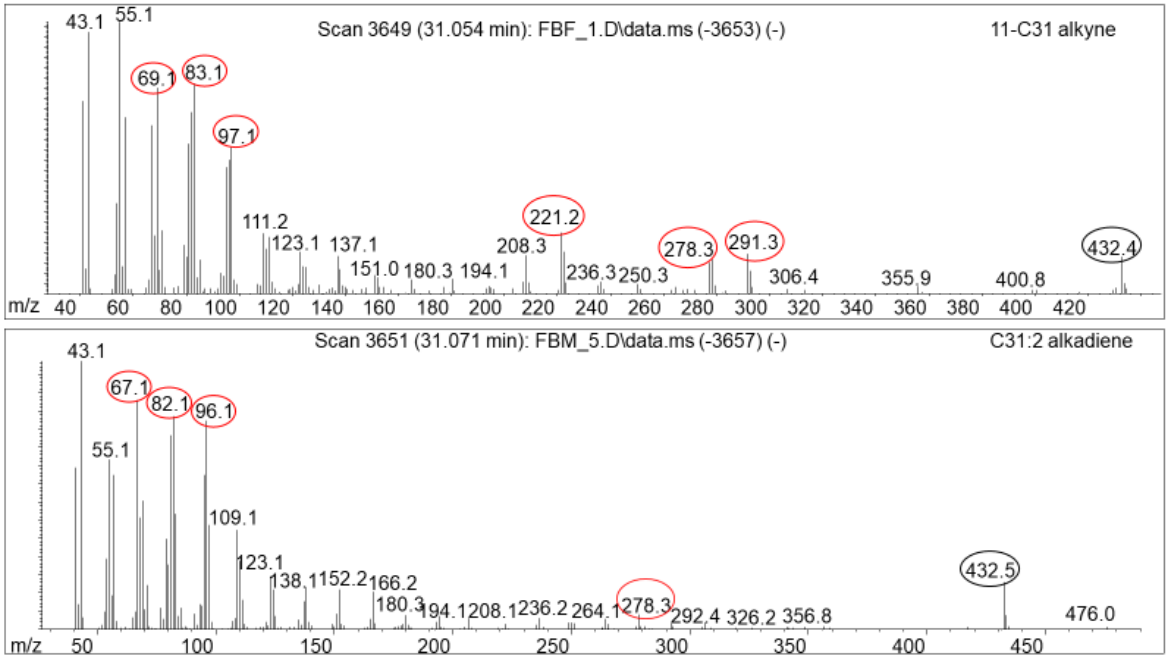


Fig. 3.5 Mass spectra from the GC of *F. borinquen*: from females (top) and males (bottom). Red circles indicate the differences between the spectra whereas black circles show that the both of them have similar molecular ion

According to Fig. 3.5, the molecular ion is indicated as 432 in both spectra. However, in the top spectrum (11-C_{31} alkyne), the major ions are consisting of fragments *m/z* = 69, *m/z* = 83 and *m/z* = 97 as compared to the bottom spectrum ($C_{31:2}$) where these are represented by the fragments *m/z* = 67, *m/z* = 82 and *m/z* = 96). Other differences, between the two mass spectra, are seen in relation to ions at *m/z* = 221, *m/z* = 278 and *m/z* = 291. Firstly, ion 278, although present on both spectra, in $C_{31:2}$ its intensity is as low as of the ions next to it. Secondly, in the top mass spectrum ions

221 and 291, along with the aforementioned ion (278) have a greater intensity as compared to the surrounding ions. This suggests that the chain has cleaved and these ions could indicate the position of the methyl group. The eleventh position of the methyl group in this alkyne was identified using the NIST Library data base where another branched alkyne revealed a high intensity in ion 221. In regards to alkadiens, the double bond position can be located by using an established method based on dimethyl disulfide (DMDS) adduction[135,136]. This method has not been pursued in this research, but the alkadiens were identified by matching the spectrum of the hydrocarbon detected in *F. borinquen* with that found in the literature[135–137] (Fig. 3.6 and 3.7).

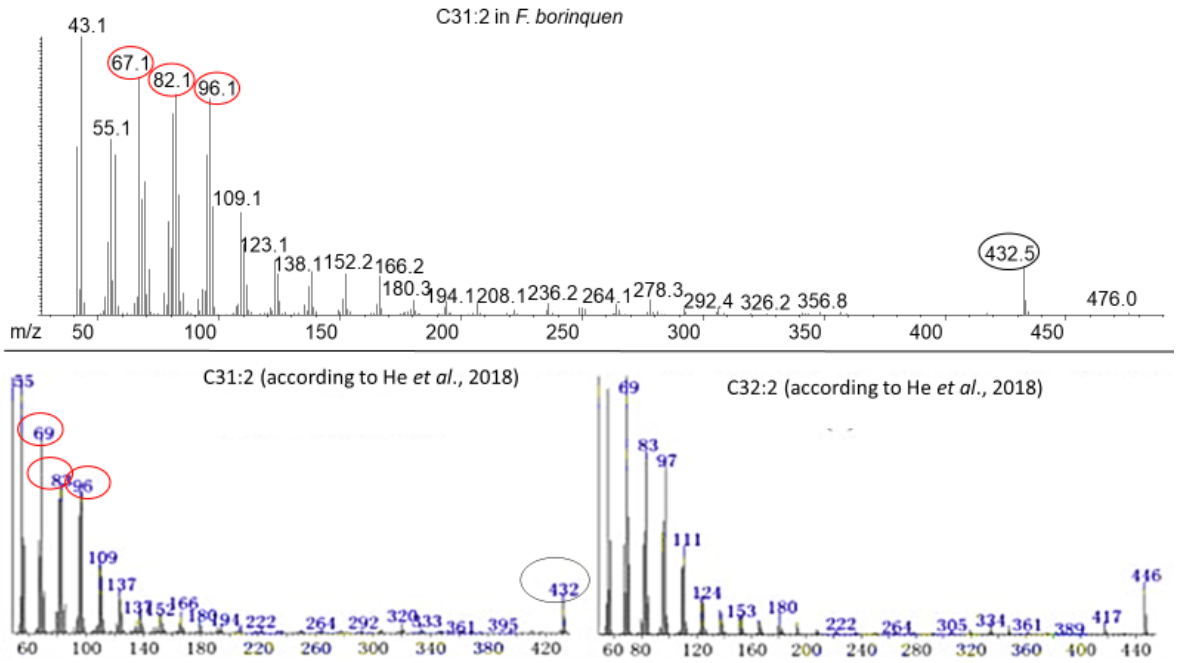


Fig. 3.6 Comparison of a mass spectrum of a hydrocarbon from two *F. borinquen* males (top) with the one identified in the literature (by He *et al*., in 2018) as C_{31} alkadiene (bottom-left). Red circles indicate the differences between the spectra whereas black circles show that the both of them have similar molecular ion

As seen in Fig. 3.6 the mass spectra of the two heptadecadienes are quite similar, the middle fragment ions, marked in red (*m/z* = 221, *m/z* = 278 and *m/z* = 291)

have very low intensities, which excludes them to be branched hydrocarbons. Still, these two mass spectra are not a perfect match (the differences are recorded in red circles on the image). The different mass values in the three major ions could be most likely explained by double bond position. In here the double bond position is unknown, it could be to the side of the molecule, but it could also have a central location. As reported by Wolf and co-workers[138] during the ionization process a hydrogen rearrangement takes place, thereby the double bond migrates along the chain. Based on this, further research in the literature revealed that the double bond migration along the chain could indeed cause slight differences in the mass spectra of an alkadiene as demonstrated by Ikeda and colleagues[135]. An example from the work of Ikeda and colleagues[135] is shown Fig. 3.7.

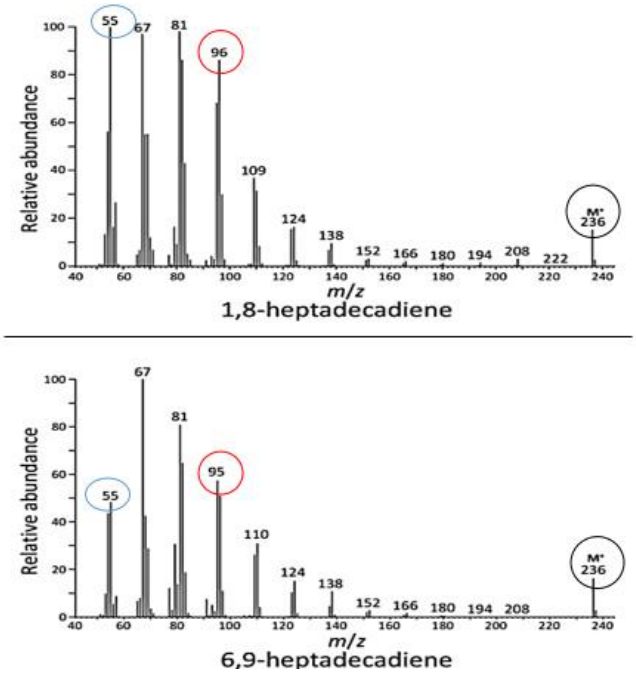


Fig. 3.7 Mass spectra of 1,8- heptadecadiene versus 6,9-heptadecadiene identified in lichen species (according to Ikeda *et al*., 2021) that shows the double bond migration along the chain can cause slight differences in the mass spectra of an alkadiene (blue circle – intensity changes, in red circle – mass changes, black circle – similar molecular ion)

The two heptadecadienes in Fig. 3.7, although strongly similar, it can be observed that they differ in the mass spectrum where the double bond shifted towards the centre of the chain (in 6,9-hepadecadiene), the at ion *m/z* = 95 is one of the major ions (instead of *m/z* = 96), also the intensity of ion *m/z* = 55 is significantly lower as compared to 1,8-hepadecadiene. Although DMDS was not performed to discern peak 15 as an alkadiene, by considering the almost identical mass spectra found in the literature, there were strong reasons to identify it as an alkadiene with 31 carbons in the backbone. A similar situation to the one described above was observed in this species (*F. borinquen*), in the subsequent odd chain of carbons, in C_{33}, at peak 19 (11-C_{33} alkyne) and 20 ($C_{33:2}$). This suggests these cuticular non-polar lipids could subtly separate the females from males and they could potentially act as pheromones. Still, further investigations are required to confirm this hypothesis. Two other compounds, found at peak 3 and 4, may have a contribution to the gender separation, as well. Both *n*-C_{25} (peak 3) and 3-MeC_{25} (peak 4) are common in females and males. However, in males, *n*-C_{25} was found in almost double amounts, whereas the proportion of 3-MeC_{25} was 14 times higher as compared to females.

3.3.2 CHCs in *Frankliniella schultzei*

There were 27 compounds detected, in both females and males of *F. schultzei*, as shown in Table 3.5. The chemical composition in this species is less diverse, it contains only two classes of hydrocarbons, straight-chain alkanes and alkenes. However, what makes *F. schultzei* distinctive, is the fact that monoene alkenes are quite predominant in this species, in particular, are more prevalent in males as compared to females. As aforementioned, alkenes represent over 50% of the total compounds, whereas the other group of hydrocarbons (*n*-alkane) was found

in a proportion of 44%. As per Table 3.5, monoenes were mainly found in odd-numbered chains ($C_{23:1}$ -$C_{33:1}$) and it can be seen that for $C_{29:1}$ and $C_{31:1}$ up to three different isomers were detected. Also shown in Table 3.5, are the relative abundance values for each compound and it can be noticed that the most abundant compound in females is $C_{25:1}$ (15.5%), whereas in males is $C_{29:1}$ which accounts for circa 25% from the total peak area of the sample.

Among alkanes and alkenes, an unknown compound was detected at peak 25. This was commonly found in both genders, but its relative abundance in males is almost twice as much. Another interesting thing about this unknown molecule is that its mass spectrum is similar to the monoene $C_{29:1}$. However, C_{29} monoene was also detected both genders and the elution pattern and Kovats Index suggested that it should elute before the straight-chain alkane C_{29}. Therefore, the retention time and Kovats Index value of this unknown compound indicated that there is in fact a different compound. The matching mass spectra and non-matching retention time (marked in red) for $C_{29:1}$ and the unknown can be seen in Fig. 3.8.

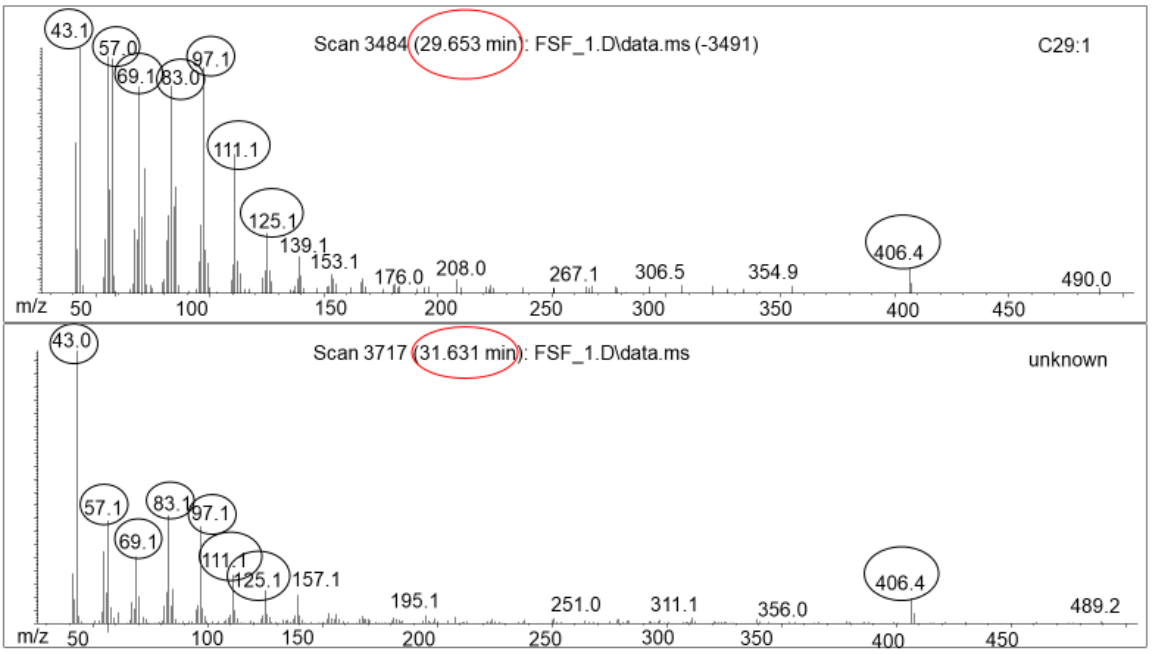


Fig. 3.8 Matching mass spectra from the GC of the females of *F. schultzei* for two hydrocarbons (top - $C_{29:1}$; bottom - unknown) with different retention times. Black circles indicate that both spectra have similar fragmentation pattern including similar molecular ion, whereas the red circles show their different retention time.

Table 3.5 CHCs compounds detected in *F. schultzei* along with the relative content percentage (RA%) of each compound and Kovats Index (KI) for their identification. The relative abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample

			RA (%)[b]	
	Compound[a]	**KI**	**Females (n=8)**	**Males (n=10)**
1	*n*-C_{21}	2100	2.65±0.38	2.69±0.89
2	*n*-C_{22}	2200	0.62±0.07	ND
3	$C_{23:1}$	2280	0.72±0.18	ND
4	*n*-C_{23}	2300	11.09±0.70	1.02±0.16
5	*n*-C_{24}	2400	1.21±0.08	ND
6	$C_{25:1}$	2475	15.54±0.91	ND
7	$C_{25:1}$	2482	5.13±0.76	ND
8	*n*-C_{25}	2500	4.47±0.21	1.08±0.16
9	*n*-C_{26}	2600	2.34±0.25	ND
10	$C_{27:1}$	2676	5.24±0.93	ND
11	$C_{27:1}$	2683	3.93±0.39	1.39±0.30
12	*n*-C_{27}	2700	3.23±0.24	3.87±0.24
13	$C_{28:1}$	2769	ND	0.36±0.13
14	*n*-C_{28}	2800	2.37±0.25	ND
15	$C_{29:1}$	2873	ND	24.71±1.07
16	$C_{29:1}$	2881	ND	14.26±0.77
17	$C_{29:1}$	2887	2.93±0.28	2.64±0.32
18	*n*-C_{29}	2900	3.10±0.25	2.00±0.13
19	$C_{30:1}$	2970	ND	0.99±0.28
20	*n*-C_{30}	3000	3.80±0.45	ND
21	$C_{31:1}$	3067	2.10±0.78	17.61±1.61
22	$C_{31:1}$	3078	ND	3.82±0.68
23	$C_{31:1}$	3087	4.14±0.66	2.68±0.79
24	*n*-C_{31}	3100	5.60±0.38	ND
25	Unknown[c]	3141	8.15±1.36	13.36±1.73
26	*n*-C_{32}	3200	2.77±0.85	ND
27	$C_{33:1}$	3273	8.88±0.64	7.53±1.48

Peak numbers correspond to those in Figure 3.4 (b)
[a] The abbreviation of the compounds is explained in the footnotes of Table 3.3 and 3.4. Tentative identification based on Kovats Index values and a match with NIST2.2 Library Database
[b] Mean percentage of cuticular lipids ±SE from samples with two adults per capillary tube
[c] Retention Index value not calculated as to the retention time for the larger alkane (N value in the Kovats Index equation) was unknown

At a glance, by comparing the profile of females and males it can be seen that three of the common hydrocarbons, at peak 4, 8 and 11 (*n*-C_{23}, *n*-C_{25} and $C_{27:1}$, respectively) were more abundant in females than in males. The relative abundance

of the straight alkane with 23 carbons tricosane was roughly 10 times higher, whereas the abundance of the other two compounds was roughly 4 times higher in females as compared to males.

3.3.3 CHCs in *Hydatothrips adolfifridericí*

The CHC profile detected in *H. adolfifridericí* is different as compared to other thrips species. More specifically, it has the lowest number of compounds, only 12 were detected (Table 3.6). Therefore, the profile composition of this species is classified as the least diverse profile, due to the identified compounds belonging to a single class of hydrocarbons (alkanes). Among the alkanes, in Table 3.6 two compounds were identified as methyl-branched, one with 28 carbons in the backbone (peak 6) and the other with 29 carbons (peak 8). The rest of the ten compounds were identified as linear alkanes and they form a series ranging from *n*-C_{24} to *n*-C_{33} with high abundance of the compounds. It is noticeable that the profiles of males are represented by only three compounds of which 3-MeC_{29} was the most abundant (48%). On the other hand, in females the most abundant compound was *n*-$C_{31,}$ which represents approximately 15% of the total peak area of the sample. The three compounds detected in males, were also found in females, yet in female samples their concentration was considerably lower: (approximately) 3 times lower for *n*-C_{29}, 7 times lower for 3-MeC_{29} and 10 times lower for 2-MeC_{28}. It is important to mention that the body size of *H. adolfifridericí* was the smallest among all tested species, hence its surface-volume ratio is even larger, thus in order to fight desiccation they may have adjusted their CHC to the weather conditions[141].

Table 3.6 CHCs compounds detected in *H. adolfifriderici* along with the relative content percentage (RA%) of each compound and Kovats Index (KI) for their identification. The relative abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample

			RA%[b]	
Peak number	**Compound[a]**	**KI**	**Females (n=8)**	**Males (n=10)**
1	*n*-C_{24}	2400	2.04±0.60	ND
2	*n*-C_{25}	2500	5.12±0.73	ND
3	*n*-C_{26}	2600	8.03±0.86	ND
4	*n*-C_{27}	2700	8.20±0.87	ND
5	*n*-C_{28}	2800	8.77±0.95	ND
6	2-MeC_{28}	2868	1.73±0.33	20.04±1.27
7	*n*-C_{29}	2900	11.16±0.78	31.50±1.51
8	3-MeC_{29}	2976	7.09±0.81	48.46±1.46
9	*n*-C_{30}	3000	12.16±0.85	ND
10	*n*-C_{31}	3100	15.22±1.69	ND
11	*n*-C_{32}	3200	14.39±2.10	ND
12	*n*-C_{33}	3300	6.09±2.29	ND

Peak numbers correspond to those in Figure 3.4 (c)
[a] The abbreviation of the compounds is explained in the footnotes of Table 3.3. Tentative identification based on Kovats Index values and a match with NIST2.2 Library Database
[b] Mean percentage of cuticular lipids ±SE from samples with two adults per capillary tube

3.3.4 CHCs in *Thrips tabaci*

No male samples were recorded for *T. tabaci*, due to insufficient field abundance to allow the preparation of ten replicates, hence the data presented in Table 3.7 only refers to female samples. As seen in Table 3.7, twenty peaks (which account for 30 CHCs) are included in the chemical profile of adult *T. tabaci*, and they are all classified as alkanes. Exactly half of these cuticular lipids, are dominantly monomethyl branched alkanes (most of them with an odd number of carbons), whereas the other 50% are represented by dimethyl compounds (30%) and *n*-alkanes (20%). In this species, the proportion of 11-Me-, 13-Me-, 15-MeC_{29} was the highest and accounts for almost 16% of the total peak area of the sample. However, the second most abundant hydrocarbon is the corresponding compound with 31 carbons

in the chain (11-Me-, 13-Me-, 15-MeC_{31}), whose relative abundance was calculated as almost 13%.

Table 3.7 CHCs compounds detected in *T. tabaci* along with the relative content percentage (RA%) of each compound and Kovats Index (KI) for their identification. The relative abundance of each compound was quantified as percentage of the total peak area from the total ion chromatogram for each sample

			RA%[b]
Peak number	**Compound[a]**	**KI**	**Females (n=11)**
1	*n*-C_{25}	2500	2.27±0.32
2	*n*-C_{27}	2700	2.43±0.23
3	11-Me-, 13-Me-, 15-MeC_{27}	2734	7.12±0.66
4	9-MeC_{27}	2737	3.76±0.38
5	7-MeC_{27}	2743	1.74±0.22
6	9,11-diMeC_{27}	2764	1.58±0.28
7	7,11-diMeC_{27}	2771	3.63±0.55
8	3-MeC_{27}	2777	3.46±0.53
9	10-Me-, 12-Me-, 14-Me-,16-MeC_{28}	2836	0.89±0.16
10	*n*-C_{29}	2900	3.63±0.33
11	11-Me-, 13-Me-, 15-MeC_{29}	2932	15.63±1.56
12	9-MeC_{29}	2935	4.34±0.46
13	7-MeC_{29}	2941	2.41±0.25
14	11,15-diMeC_{29}	2957	5.73±0.70
15	9,11-diMeC_{29}, 9,13-diMeC_{29}	2960	6.33±1.06
16	7,11-diMeC_{29}	2966	1.45±0.25
17	3-MeC_{29}	2973	1.90±0.20
18	*n*-C_{30}	3000	2.93±0.41
19	11-Me-, 13-Me-, 15-MeC_{31}	3123	12.59±1.93
20	11,15-diMeC_{31}	3150	8.75±0.98

Peak numbers correspond to those in Figure 3.4 (d)
[a] The abbreviation of the compounds is explained in the footnotes of Table 3.3. Tentative identification based on Kovats Index values and a match with NIST2.2 Library Database
[b] Mean percentage of cuticular lipids ±SE from samples with two adults per capillary tube

3.3.5 CHCs in *Frankliniella occidentalis*

The chemical profile detected through whole-body analysis for samples with two insects per microcapillary was considered for *F. occidentalis* and is shown in Table 3.3. Also, the TIC chromatogram is presented in Fig. 3.3a. As in *T. tabaci*, only female

samples were recorded for this species. As this is a cultured insect, male availability was not an issue. However, initially it was only considered as model species and, unfortunately, the decision to include it as a fifth species of thrips, was taken too late to allow male data production in time.

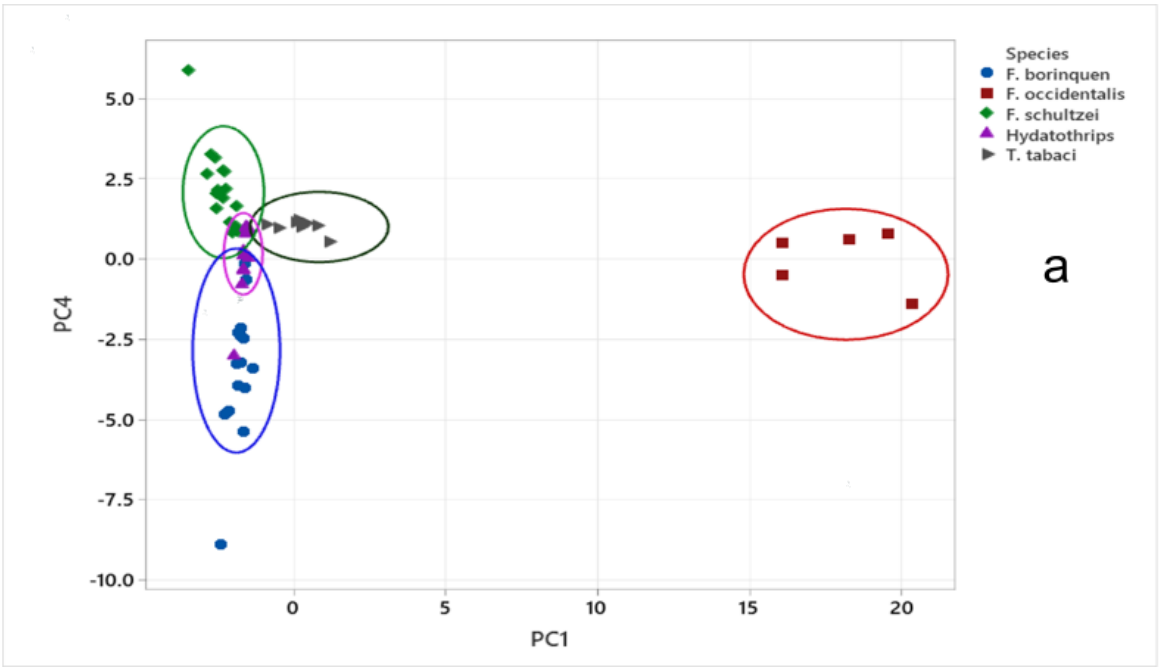
Among the tested species, western flower thrips has the most extensive chemical profile, a total of 37 CHCs were identified. Similar to *T. tabaci*, all the chemical structures in the *F. occidentalis'* profile are alkanes. Yet, the difference is that there were twice as many in this species than in *T. tabaci*. However, the proportion of the three types of alkanes is slightly different to the proportion found in *T. tabaci*: monomethyl - approx. 59%, dimethyl - circa 30% and straight chain alkanes – around 10%. Also, in this species, both monomethyl and dimethyl structures have dominantly an odd number of carbons in their chain. As shown in Table 3.3, the chemical structure with the highest relative abundance was identified as 3-MeC$_{27}$. Interestingly, a similar pattern as in *T. tabaci* is observed in western flower thrips, in terms of the second most abundant compounds, which also, in this case, is a corresponding compound of the first most abundant compound (3-MeC$_{25}$).

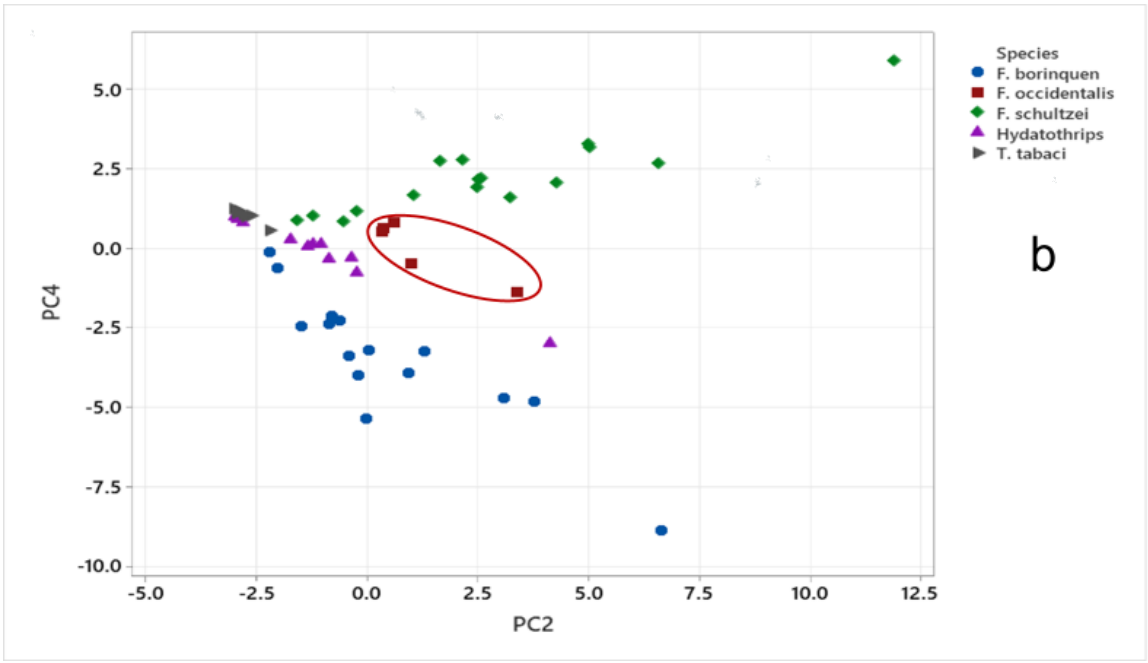
3.3.6 Multivariate analysis

The chemical composition dataset was analysed using Principal Component Analysis. PCA plots were produced to visualize the differences in the CHC profile among the tested thrips species (Fig. 3.9). Additionally, PCA plots were generated to visualize whether there was a difference in the hydrocarbon composition between females and males (Fig. 3.10 - 3.12). This was possible only for *F. boriquen, F. schultzei* and *H. adolfifriderici*, as males were not recorded in *F. occidentalis* and *T.tabaci*.

Before conducting the statistical analysis, the initial dataset was adjusted. The less representative samples and least common hydrocarbons were removed from statistical analysis (as explained in Chapter 2, section 2.11). The principal component analysis was initially performed including the data from all the species and then a second PCA was performed excluding *F. occidentalis* data. Generally, the PCA was carried out using six principal components, except for the smaller dataset from *H. adolfifriderici*, for which four PCs were used.

When the dataset including *F. occidentalis* was analysed (Fig. 3.9a), the six PCs described 82% of the variation. The first four PCs comprised 41%, 13%, 9.2% and 8.4%, respectively. PC1 and PC4 were used to plot the relevant scores for this data set. As seen in Fig. 3.9a, the plot shows five clusters, each representing a certain species of thrips. It also shows that *F. occidentalis* is well separated from the rest of the species, while the insects delivered from Kenya, are located toward the centre of the plot.


Species
F. borinquen
F. occidentalis
F. schultzei
Hydatothrips
T. tabaci
PC4
PC1
5.0
2.5
0.0
-2.5
-5.0
-7.5
-10.0
0
5
10
15
20
a


Species
F. borinquen
F. occidentalis
F. schultzei
Hydatothrips
T. tabaci
PC4
PC2
5.0
2.5
0.0
-2.5
-5.0
-7.5
-10.0
-5.0
-2.5
0.0
2.5
5.0
7.5
10.0
12.5
b

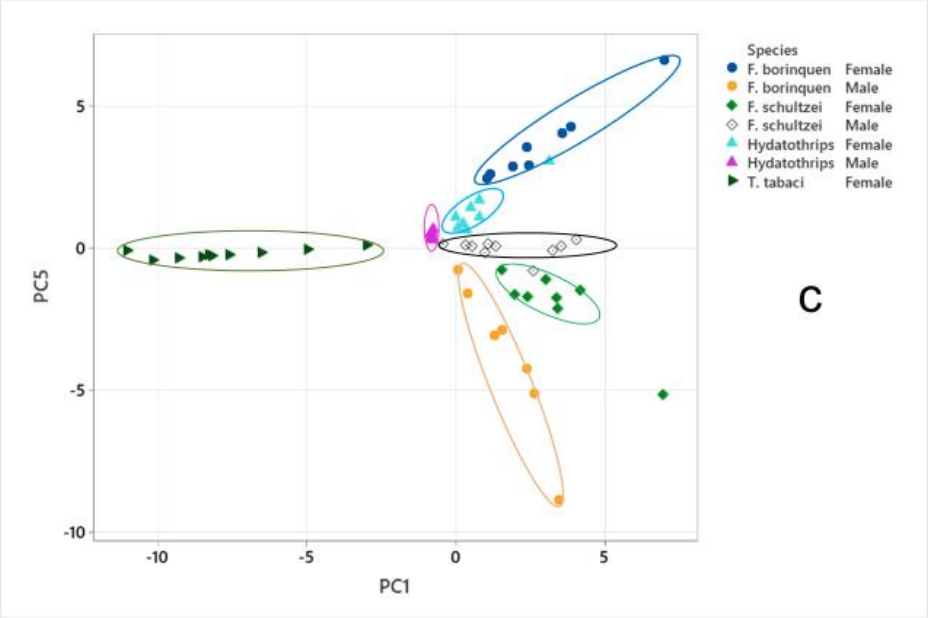


Fig. 3.9 PCA plot showing the multivariate variation among five different species of thrips, including (a and b) and excluding *F. occidentalis* (c)

When PC2 was plotted against PC4, *F. occidentalis* is not shown as a separate cluster anymore (Fig. 3.9b), yet the rest of the data appear more scattered. In Fig. 3.9c, western flower thrips data were removed, and the new dataset shows the separation of the thrips species from Kenya. For this new set, the six PCs explained 86% of the variation. By plotting PC1 (which described 29% of the variables) against PC5 (which described 9.8% of the variation) it can be seen that the thrips species from Kenya are better separated (Fig 3.9c). Additionally, in Fig. 3.9c, a clear separation of the genders, can be noticed, as well.

The PCA plots of PC1 versus PC2 from figures 3.10 - 3.12 indicate that for the species in which both genders were recorded, there is a difference regarding the chemical profile in males and females.

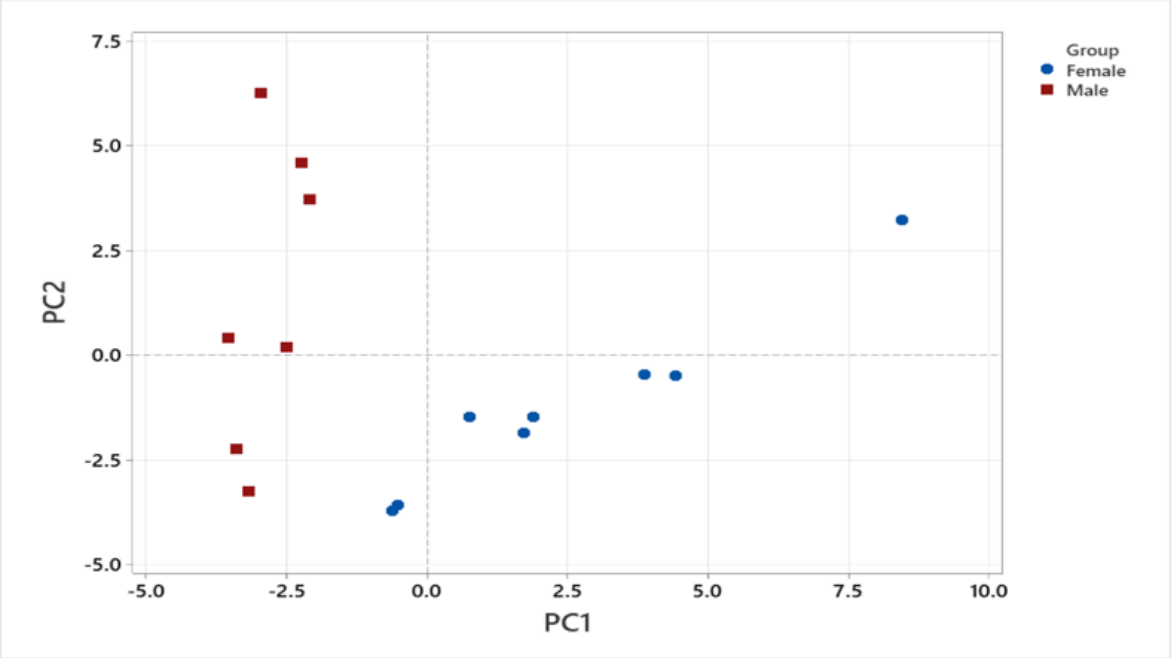


Fig. 3.10 PCA plot showing PC1 vs PC2 for *F. borinquen* using data extracted from males and females

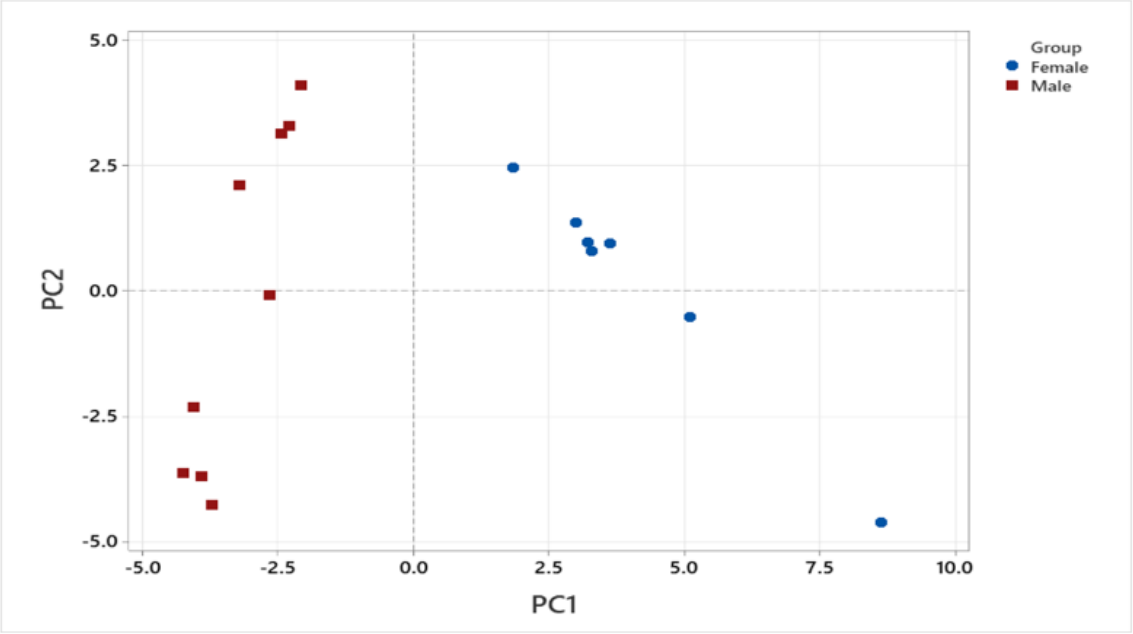


Fig. 3.11 PCA plot showing PC1 vs PC2 for *F. schultzei* using data extracted from males and females

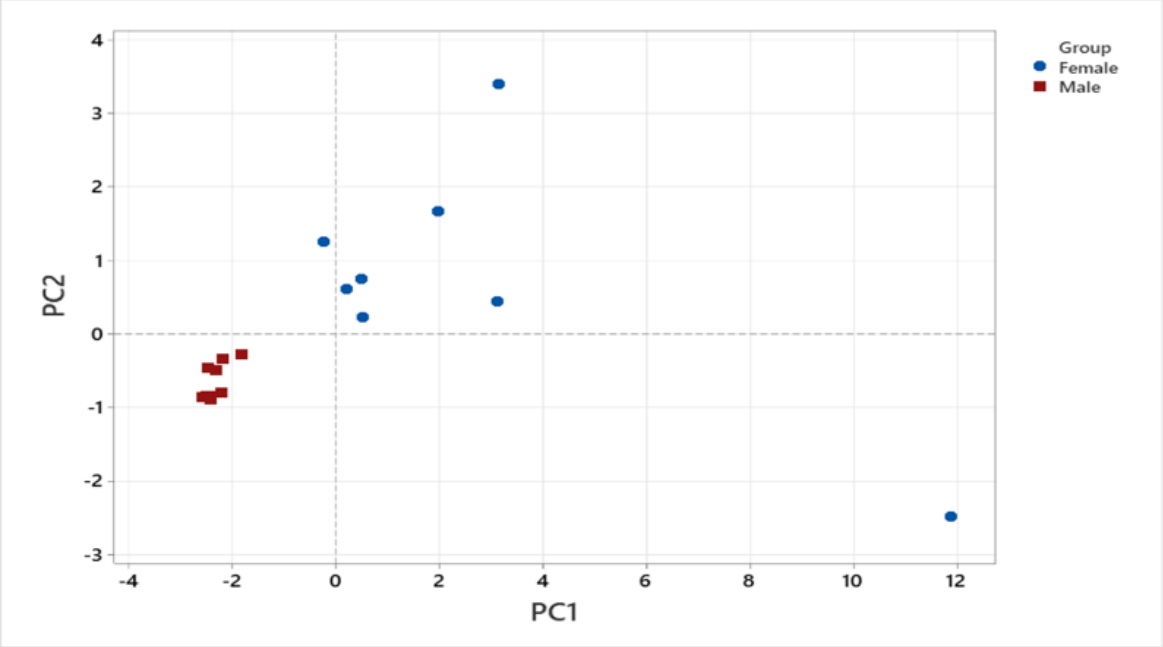


Fig. 3.12 PCA plot showing PC1 vs PC2 for *H. adolfifriderici* using data extracted from males and females

3.3.7 Discussion on the results for CHCs in five species of thrips

A mixture of various groups of hydrocarbons (*n*-alkanes, *n*-alkenes and methyl branched compounds) are found in the epicuticle of the insects[108] and their two main functions refer to their role in communication[139,140] and protection against desiccation[141,142]. CHC have been investigated in a number of studies for their role in chemotaxonomy[114,116,121,129]. However, for chemotaxonomy purposes, but not only, selecting a suitable extraction method is very important to obtain a complete profile. For instance, in this research, it was shown that in western flower thrips, alkenes were detected through direct extraction from three whole insects, but not through solvent extraction from 100 insects.

According to Blomquist and Guinzel[139] hydrocarbon compounds account for less than 0.1% of the total body mass of an insect. However, their composition can be quite informative in separation of individuals from the same taxonomic group or opposite sex. In the present study, the composition and content of CHCs in five

species of thrips were described and the results showed that each species of thrips is characterized by the presence of a different class of hydrocarbons. The results also revealed a distinct biochemical description among the species included in the same genus (*Frankliniella*). For instance, *F. borinquen* was characterized by the presence of alkynes and alkadiens, whereas *F. schultzei* by the dominance of monoene and lack of methyl branched alkanes and finally, *F. occidentalis* was described by the prevalence of different types of alkanes. Interestingly, the chemical profile of *H. adolfifridericі* consists mainly of *n*-alkanes. Additionally, in *T. tabaci* the CHC pattern was based on alkanes as in *F. occidentalis*, yet these species had distinct patterns. In terms of the species in which both genders were recorded, males and females were distinguished based on qualitative and quantitative variation of their CHC content. Moreover, the multivariate analysis confirmed that the species and genders studied in the present work, appear as separate entities. Thus, the identification of the five species of thrips based on their CHC profile, provides evidence that hydrocarbons can be used as biochemical markers for species and gender separation.

It has been reported that linear alkanes have been mainly associated with the role in water-loss prevention whereas the methyl branched and alkenes with the role in communication[108]. Due to their increased surface-to-volume ratio, at high temperatures, insects need to prevent dehydration and a way of doing so is to adapt their CHC profile by producing more hydrocarbons with straight chains[108,109,140]. By considering this, the *n*-alkane-based CHC profile in *H. adolfifridericі* adult females could be explained as an adaptation to the high temperature in Kenya. Although, in males, as aforementioned, the CHC contained two methyl branched alkanes and a single linear alkane. However, a recent study on aphids[142], concluded that methyl branched hydrocarbon compounds display a higher plasticity to fight water-loss.

Therefore, it could be that the two methyl branched alkanes, found in *H. adolfifriderici* males to be more involved in preventing dehydration as compared to the linear alkane. This adaptation in the males of *H. adolfifriderici* would be justified by their minute size. Considering that in general in Thysanoptera, males are smaller than females[123] and that *H. adolfifriderici* species was observed to be smaller than the rest of the species studied in here, it can be speculated that *H. adolfifriderici* males, as compared to females, have developed divergent traits in fighting desiccation. Still, more laboratory experiments, and eventually some genetic tests would help to test further this theory.

In 2018, Akynyemi[40], reported for the first time the CHC in a European thelytokous *T. tabaci*, however, even though he detected a similar profile based on mono and dimethyl alkanes, the chemical content differs to the one detected in this study. Firstly, in the strain studied here, there were detected compounds with up to 31 carbons in the chain but a maximum of 29 carbons were reported by Akyneyemi. Secondly, 5-MeC25, n-C26, 5-MeC27 were absent in this project's strain, whereas 7,11-diMeC27; 10-Me-, 12-Me-, 14-Me-, 16-MeC26; 11,15-diMeC29; n-C30; 11-Me-, 13-Me-, 15-MeC31 and 11,15-diMeC31 were not detected in Akynyemi's. Also, in the profile described by Akynyemi, six 'unknown' and two ketone compounds are listed. Furthermore, in his experiment, Akinyemi used virgin females whereas the fecundity status of the strain used in this current study was unknown. Additionally, the mode of reproduction (thelytokous or arrhenotokous) is not known on the strain studied in here, hence all these unknowns along with potential extrinsic factors (temperature, humidity, diet, etc.) could, most likely, explain the CHC variation between the two strain of *T. tabaci*.

As additional findings, in this research the CHC profile of three species of thrips are reported for the first time (*F. borinquen*, *F. schultzei* and *H. adolfifriderici*). Moreover, monene alkenes are also reported for the first time in *F. occidentalis*. As of yet, there is a significantly low number of publications on the composition of CHC in thrips[34,40,120,127]. Given that all the studied species are pest insects, along with the few records in thrips chemistry, the results obtained in this study represent an essential contribution to the literature as background information to research new biological methods for thrips control.

3.4 Conclusion

In this study, five species of thrips belonging to the Terebrantia sub-order were used to analyse their CHC profile: *F. occidentalis*, *F. borinquen*, *F. schultzei*, *H. adolfifriderici* and *T. tabaci*. The aim of this project was to test a new CHC extraction technique based on the solid injection of various species of thrips, but at the same time to describe the hydrocarbon pattern in the tested species.

This work is presented in three main sections: i) literature review (Chapter 1); ii) materials and methods (Chapter 2) and iii) results and discussion section (Chapter 3). Overall, valuable information was obtained from this research experimental work. Firstly, CHCs may be used as a tool in thrips separation and this could further on contribute to their chemotaxonomical classification. Secondly, by considering the fact that quite a number of the thrips species are major pests and that some of the detected hydrocarbons are known to act as pheromones, the interest in investigating thrips' chemistry could lead to discovering sustainable measures for their biological control.

All the investigations on CHCs were performed using the GC-MS technique, which is commonly used in the analysis of such compounds. The CHCs were

separated based on their boiling point using the GC and then were identified and quantified with the aid of the MS. Traditionally, GC-MS analysis may require the extraction of the CHCs within a solvent like hexane, but in this research a new solventless method was tested, using a custom-made solid-sample injector. The results have suggested that the solid injection extraction method has the same potential of detecting CHCs in thrips as the hexane extraction method. In addition, the main advantage of the solid injection method is that it requires a small number of insects. Thus, two insects have been shown to be the optimum number of thrips per glass capillary. Therefore, for research purposes, all of these make the solid injection method a suitable choice when dealing with low populations of thrips.

The analysis of the CHCs across the five tested species has indicated the presence of linear alkanes with one or two methyl groups attached to their chain (resulting in mono- methyl and dimethyl branched alkanes), as well of unsaturated alkenes and alkynes. Additionally, a different group of hydrocarbons was found to be dominant in each detected profile of the five tested species. For instance, alkenes predominate in *F. schultzei* (52%) and *F. borinquen* (33%). Both monomethyl and dimethyl alkanes were mainly found in *T. tabaci* and *F. occidentalis*, where they account for 80% and 89%, respectively. Finally, linear alkanes, were prevalent in *H. adolfifriderici* where they represent 83% of the total number of compounds.

For statistical processing, in this work the PCA was used. Therefore, when the CHC data sets were inputted into a PCA model it indicated that the species were separated, by visually displaying five clusters, each representing a certain species of thrips. Similarly, for the species in which both genders were recorded, PCA has indicated that there is a difference regarding the chemical profile in males and females, by displaying two separate clusters (each corresponding to a certain gender).

The CHC profile of *F. occidentalis* and *T. tabaci* has been investigated before, although using the solvent extraction method. However, this study reports for the first time the CHC composition of three thysanopteran species: *F. borinquen, F. schultzei* and *H. adlofifriderici*. In fact, the contribution of this research to the literature is highlighted by the fact that when the project was initiated in 2019, CHC records on thrips, were available only in two species. Moreover, alkenes are reported for the first time in *F. occidentalis*.

According to all of the above, the results of this study support the hypothesis mentioned in the final section of the general introduction (Chapter 1). Finally, it can be confirmed that with the aid of solid injection method specific CHC patterns can be detected and associated with a particular species of thrips, highlighting their role in thrips chemotaxonomy.

3.4.1 Future work

Based on the fact that some CHC in thrips may act as pheromones[34], they can be used in the field to monitor and potentially to control them. The results in section 3.1 showed that in western flower thrips, at increased density (at 3 insects per micro-capillary) a separate group of hydrocarbons was detected (alkenes). Therefore, it would be of interest to test alkenes' potential role in communication. Also, to verify whether the CHC composition increases further with increased density or stays steady.

Taking into account the proven efficiency of the solid injection method, both in the present work (section 3.2) and prior studies[122,143–145] it would be of both theoretical and practical interest to explore the production site of the aggregation pheromone in *F. occidentalis* adult males.

There are over 6000 species of thrips in Thysanoptera order and over 100 are known as pests[43]. The CHC profile has only been detected in 9 species so far (included the species in this study in section 3.3) hence there is still a lot to be explored. For a better understanding of thrips behaviour and their multilevel-interaction, CHC patterns must be investigated in individuals of different age, fecundity status, reared on different host plants, but also various developmental stage should be considered. Moreover, once the number of CHC patterns in various species/gender/developmental stage increases, an online data base should be created to allow the identification based on their chemical description.

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