Comparison of Physiological and Metabolic Changes between Phosphine Resistant and Susceptible Strains of *Rhyzopertha dominica* (Fabricius) and *Tribolium castaneum* (Herbst)

Ihab Abdul-Kreem Alnajim

This thesis is presented for the degree of

Doctor of Philosophy

at

College of Science, Health, Engineering and Education

Murdoch University

Perth, Western Australia

Australia

Declaration

I hereby declare that this thesis is my own account of my research and contains as its main content work which has not been previously submitted for a degree at any tertiary educational institution.

Ihab Abdul-Kreem Alnajim 30 July 2019

Acknowledgements

I gratefully acknowledge the Iraqi Government, represented by the Ministry of Higher Education and Scientific Research, for providing the wonderful opportunity to complete my doctoral study in Australia. I greatly appreciate its financial support allowing me to focus solely on my research without any obstacles.

I also extend my sincere gratitude to my supervisors, Professor YongLin Ren, Dr Manjree Agarwal and Dr Tao Liu; you have been fabulous supporter for me. I would like to thank you for supporting my research and assisting me to think as a scientist. Your advices and guidance regard with my PhD study have been appreciated. I would also like to thank Professor Wujun Ma as an advisory committee chair for assisting me with my PhD study and providing valuable feedback

Many thanks also going to stuff members, James Newman, Xin Du, Amy Xiao, Sue Pan, Myuki Taniguqi and George Li from the post-harvest biosecurity and food safety laboratory for their helpful laboratory technical support.

I am also hugely grateful to Rob Emery from Department of Primary Industries and Regional Development for providing me with the insect samples.

I am also very grateful to my friends Pauline Barratt, Desmond Barratt and Catherine Barratt for their support and encouragement.

I would also like to thank my colleague and my friend Basman Aljalely, you were my best supporter during my stay in Australia.

I would like to thank my colleagues Nadia Hanon, Thamer Alshowili, Farhan Albehadili, Mohammed Ibrahim, Hassan Hadi, Ahmed Auda and Khalid Mohammed. Your commitment, intelligence, persistence and hopefulness were more appreciated than you could ever imagine.

I greatly appreciate the support that I have received from my dear wife and my best friend, Doaa Altuama I thank you for being so sympathetic with me through the hardest days of my life, without your encouragement and support I can't complete my PhD study. I can also not forget my wonderful children Yousif Alnajim Lial Alnajim and Rose Alnajim; I love you more than anything.

Abstract

The lesser grain borer, *Rhyzopertha dominica* (Fabricius) and the red flour beetle, *Tribolium castaneum* (Herbst) are serious insect pests of stored grain and a broad range of other stored products. Phosphine is widely used as a fumigant to protect the stored commodity from insect damage. However, phosphine resistance by stored grain insects has been occurred worldwide. The mechanism of phosphine resistance reported that it might include changes in respiratory and metabolic function. Lower respiration of the resistant insects may reduce the rate of phosphine entering insect bodies. Changes in the metabolism of resistant strain insects may prevent metabolic crises or supplies more energy.

In this thesis, the respiration rate, the emission of VOCs and lipid content of phosphine susceptible and resistant strains of *R. dominica* and *T. castaneum* were investigated. Two novel methods have been explored and validated, including the headspace solid phase microextraction (HS-SPME) and direct immersion solid phase microextraction (DI-SPME) technologies. The optimal conditions for both methods were investigated, and the optimized conditions were used for our study to determine the volatile organic compounds (VOCs) from insects and the insect cuticular hydrocarbons.

This thesis has confirmed that the respiration rate of the susceptible strain insects was significantly higher than the resistant strains. Phosphine can reduce the respiration rate of both susceptible and resistant strains when treated with average concentrations ranged from 2 to 20 ppm. However, the rate of respiration of the resistant strains was unexpectedly increased under a high level of phosphine (320 ppm) which indicated that resistant strain insect metabolism was elevated.

Moreover, two characteristics were used as indicators to determine the metabolic differences between phosphine susceptible and resistant strains of *R. dominica* and *T. castaneum* including VOCs and lipid contents. The GC-MS response of most of the VOCs detected in susceptible strains of both species used in this study were higher than that from the resistant strains. However, 2, 3-Butanediol, [R-(R*, R*)] was significantly in abundance in the resistant strain of *R. dominica*; while dodecanal was only detected from the susceptible strain of *T. castaneum*.

Lipid contents including cuticular hydrocarbons, glycerolipids and phospholipids were analysed with different methods, such as cuticular hydrocarbons were analysed using our optimized (DI-SPME) method; glycerolipids and phospholipids were determined by HPLC. The results showed that resistant strains of both insect species containing higher levels of lipids

than the susceptible insects. This higher content of lipids may have significant role in phosphine resistance.

This study concludes that respiration rate was found higher in the susceptible strain than the resistant; however, treating the resistant strain with a high concentration of phosphine caused an increase in the rate of the respiration. Some VOCs were found significantly different which indicated that metabolism is different according to the susceptibility of the strains. 2,3-Butanediol, [R- (R*, R*)] and dodecanal were suggested to be used as a biomarker to differentiate susceptible and resistant strains of *R. dominica* and *T. castaneum* respectively. The resistant insects of both species used in this study had a higher amount of lipids than the susceptible insects, shows that the higher lipid contents in the resistant strains may prevent the fumigant from entering the insect bodies, provide energy to the insect and participate in avoiding phosphine effect.

LIST OF PUBLICATIONS

Alnajim, I., Agarwal, M., Liu, T., Ren, Y. 2019. A Novel Method for the Analysis of Volatile Organic Compounds (VOCs) from Red Flour Beetle *Tribolium castaneum* (H.) Using Headspace-SPME Technology. *Current Analytical Chemistry* **15**, 1-9. (Chapter3).

Alnajim, I., Du, X., Lee, B., Agarwal, M., Liu, T., Ren, Y. 2019. A novel non-derivatization method for analysis of lipids in *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Fabricius) using gas chromatography–mass spectrometry (GC–MS) (Insects 2019, 10(10), 363; https://doi.org/10.3390/insects10100363

Alnajim, I., Lee, B., Agarwal, M., Liu, T., Li, B., Du, X., Ren, Y. Preliminary study on the differences in hydrocarbons between phosphine susceptible and resistant strains of *Rhyzopertha dominica* (Fabricius) and *Tribolium castaneum* (Herbst) using direct immersion solid-phase microextraction coupled with GC-MS. (Under review Molecules)

LIST OF INTERNATIONAL CONFERENCE PRESENTATIONS

Oral presentation:

2nd International Conference on Advances in Biotechnology. Gavin Conferences, July 23-25, 2018, Kuala Lumpur, Malaysia.

-Development and validation a method based on headspace-SPME to analyse volatile organic compounds (VOCs) from red flour beetle *Tribolium castaneum* (H.).

Poster presentation:

10th international conference on controlled atmosphere and fumigation in stored product, New Delhi, 7-11 November 2016.

-New tool to diagnose phosphine resistant and susceptible strains of lesser grain borer *Rhyzopertha dominica* (F.).

Abbreviation

µg	Nanogram
µM	micromole
ADP	Adenosine diphosphate
AMDIS	Automatic Mass Spectral Deconvolution and Identification System
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
DG	Diglyceride
DGDG	Digalactosyldiacylgylcerol
DI-SPME	Direct immersion solid phase microextraction
DVB/CAR/PDMS	Divinylbenzene/Carboxen/Polydimethylsiloxane
ETC	Electron transport chain
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
HBWs	high boiling point waxes
HS-SPME	Headspace solid phase microextraction
LBWs	Low boiling point waxes
LC-ESI-MS	Liquid chromatography-electrospray ionisation-mass
LD_{50}	Lethal dose kills 50 % of experimental animal
LOD	Limit of detection
LPA	Lysophosphatidic acid
LPG	Lysyl-phosphatidylglycerol
LPI	Lysophosphatidylinositol
MF	Match factor was generated from the NIST database
mg	Milligram
MG	Monoglyceride
MGDG	Monogalactosyldiacylglycerol
MIM	mitochondrial inner membrane
mL	Millilitre

MSD	Mass spectrometry detector
NADH	Nicotinamide adenine dinucleotide
NIST	National Institute of Standards and Technology
NRI	Retention index obtained from the NIST database
O_2	Oxygen
PA	Polyacrylate
PC	Phosphtatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PH_3	Phosphine
PI	Phosphatidylinositol
PLS-DA	Partial Least Squares - Discriminant Analysis
ppm	Part per million
PS	Phosphtatidylserine
R^2	Regression coefficient
RD	*R. dominica*
RD-SR	Resistant *R. dominica*
RD-SS	Susceptible *R. dominica*
RI	Retention index
RT	Retention time
SPME	Solid phase microextraction
TC	*T. castaneum*
TC-SR	Resistant *T. castaneum*
TC-SS	susceptible *T. castaneum*
TG	Triglyceride
TIC	Total signal chromatograms
VIP score	Variable Importance in Projection of Partial Least Squares –
VOCs	Volatile organic compound

v/v	Volume to volume
w/w	Weigh to weight

Table of Contents

Chapter 1

Literature Review

1.1 Background

Settled agriculture began about 10,000 years ago, while food grain storage to preserve stocks of food for humans began about 4,500 years ago (Saxena et al., 1988). Storage is essential for preserving the quantity and the quality of commodities. The storage process is defined as "the art of keeping the quality of agricultural materials and preventing them from deterioration for a particular period, beyond their normal shelf life" (Kiaya, 2014). Food supply is a worldwide concern due to rapid population growth and the degradation of agricultural land (Boserup, 2017). The growing demand for high-quality grain is apparent worldwide. Storage method efficiency regulates grain quality and eventually market prices. The time gap between harvest and transport, operation and consumption is often time-consuming. Consequently, both short and long-term storage are required post-harvest. Insects are, however, a very critical issue in stored products. Storages offer an ideal environment for the development of insects in comparison to any other external environment (Nansen et al., 2008; Rees, 2004). Therefore, efficient strategies to protect storages from the infection are vital to the sustainability of the food.

1.2 Stored grain

Two billion tons of grain or more are produced yearly for feeding and supplying a large portion of protein. Two hundred million tons of total production are traded internationally (Tubiello et al., 2007). Grains, with their high starch content, serve as energy sources (Evers & Millar, 2002). Predictions indicate that the demand for grains would rise in future in response to the population increasing, reaching four billion tons annually (Tubiello et al., 2007). Therefore, storage is an important part of the process for obtaining a high quantity and quality stock of grains. However, the safe storage of grains requires controlling many factors that affect the stored product, including physical, chemical and biological aspects (Jayas & White, 2003). To achieve safe grain storage, farmers need to consider critical issues like hygienic practices, including avoiding the mixing of infested and healthy grains, and burning the yield remains as well as other essential procedures to avoid contamination by stored-grain insects (Upadhyay & Ahmad, 2011). There is a rising concern regarding the protection of stored grains against insects that cause hydrolysis and oxidation, which reduces the level of stored grain; many stored-grain insects have become global due to the human's activities of transferring and distributing grains (Padın et al., 2002). Therefore, the safe storage of grains is an important consideration in providing markets with high-quality grains.

1.3 Stored-grain insects

Stored-grain insects as pests are an ancient problem for stored cereals, grain products and legumes. Flour beetles that were found in an Egyptian tomb date back to 2500 B.C. and are the earliest record of insects associated with stored food products. Beetles and weevils were even found in the tomb of Tutankhamen (1390-1380 B.C.) (Munro, 1966). Insects are the most significant pests of stored grains (USDA, 1986). Most stored-grain insects are found worldwide, according to commodity and weather conditions, although a few species are not always found in some countries (e.g. *Trogoderma granarium* (Everts) in Australia). The economic significance of the insect species varies depending on the importance of the stored products they infect (Champ & Dyte, 1976). Stored-grain insects are largely consumers; some species damage whole cereals, some consume grain products, flour and dried fruit, and others are scavengers or mould feeders. These insects not only consume these materials but also reduce the quality of the products by contaminating them with insect fragments, faeces, webbing and metabolic products in addition to mould contamination (Hagstrum et al., 2012; Snelson, 1987). Some insect pests like cockroaches, silverfish, ants and termites spread in grain stores and cause off odour food, particularly in wet places (Upadhyay & Ahmad, 2011).

Approximately 300 species can infest stored products; however, only 18 are of primary economic importance (Boyer et al., 2012). Beetles (Coleoptera) and moths (Lepidoptera) are the main insect pests that damage stored products. Beetles have a more severe effect compared to moths because both larvae and adult beetles attack stored food while only the larvae of moths are harmful (Upadhyay & Ahmad, 2011), causing economic damage before and after harvest by an extensive range of 10% to 100% in tropical countries (Mugisha-Kamatenesi et al., 2008). The losses in developed countries range from 5% to 10% (Adam et al., 2006). *R. dominica* (F.), *Sitophilus granarius* (L.), *S. zeamais* (M.) *T. castaneum* (H.), *T. confusum* (D.), *Callosobruchus chinensis* (L.), *C. maculatus* (F.), *Oryzaephilus surinamensis* (L.), *Acanthoscelides obtectus* (S.), *Prostephanus truncatus* (H.), *Lasioderma serricorne* (F.) and *Ephestia elutella* (H.) are the main species of insects that cause a significant damage to stored products (Benhalima et al., 2004; Talukder & Howse, 1994; Talukder, 1995).

This study focuses on two main beetle species in Australia, *R. dominica* (F.) and *T. castaneum* (H.). These species can survive on the generally dry grain under dry Australian weather conditions. The first targeted species is the lesser grain borer *R. dominica* (Bostrichidae: Coleoptera), which is a destructive, worldwide pest (Cogburn et al., 1984; Edde, 2012;

Hagstrum & Flinn, 1994; Khorramshahi & Burkholder, 1981). This insect is a serious and primary pest that affects various commodities including stored rice, barley, wheat, maize and sorghum (Nguyen, 2006). The adults live for four months or more. During this time, the female may lay more than 500 eggs, which can severely reduce grain quality (Oppert & Morgan, 2013). The second targeted species is the red flour beetle, *T. castaneum* (Tenebrionidae: Coleoptera), a worldwide major secondary pest infecting a wide range of commodities (Arbogast, 1991; Donahaye EJ, 2007). The economic importance of this species derives from its contamination of the products with insect parts, ecdysis skin and individuals at each life stages (Hameed et al., 2012), which results in significant loss of grain (Campbell et al., 2004; Herron, 1990), and cause critical infestations of flour (Campbell et al., 2004; Campbell et al., 2010). The adult is reddish-brown, and the antennae end in three large segments (Bousquet, 1990). The head is visible from above, and the thorax has slightly curved sides. The insects can live for more than three years (Baldwin & Fasulo, 2003; Walter, 1990).

1.4 Management of stored insects

Long-term effective control of stored-grain insects is one of the main goals of entomologists all over the world (Talukder, 1995). Many control methods are currently being used, but researchers are trying to develop safer and more economical means.

There are various successful non-chemical methods used for protecting commodities from insects including refrigeration, heating, aeration and a controlled atmosphere. Methods also include biological controls such as the use of parasites and predators against insects. However, these methods are often expensive, not totally effective nor as quick or effective as chemical methods. Chemicals, such as fumigants, pesticides, growth regulators and pheromones, have all been used to control stored product pests (Talukder, 2009). Synthetic pesticides are the major tools in the grain pest management system (Harein & Davis, 1992; Perez-Mendoza, 1999). These chemicals have played a progressively more important role in crop protection, animal health and public health. Moreover, they have been developed to be more specific regarding the targeted pest, have less impact on the environment and usually used at low concentrations.

Fumigation is one of the chemical methods used and is the art of using gaseous substances, particularly for disinfection. In many situations, fumigation has become the only practicable method for pest control as it does not require the commodity to be moved, specialised

apparatuses, electricity or a workforce, and it is relatively easy to apply in comparison to other methods such as heating and irradiation (Banks, 1990).

1.4.1 Fumigants

Fumigants are chemicals that can be converted to gases, acting as bactericidal, fungicidal, insecticidal and nematocidal pesticides. The first use of fumigants occurred about 1000 B.C. when "sulphur fumes" were used to sterilise homes, however, the modern usage of fumigants dates back over 100 years as a consequence of the discovery of carbon disulphide and hydrocyanic acid in 1854 (Winks, 1975)

The toxic characteristic of the gaseous form of fumigants at ordinary temperatures allow them to penetrate the airspace around products, and into cracks and crevices in the storage structure, to kill insects at all life stages, both between and within grains. With this advantage, fumigants are widely used for insect, rodent and fungal control of many stored products, including food, flour and timber. Many chemical compounds can be included within the definition of fumigants as they are volatile at ordinary temperatures and sufficiently toxic. However, most gases have been eliminated because of adverse properties, the most important being chemical instability and damaging effects on products (Bond, 2007).

Currently, fumigants are the most effective pesticides for managing stored-grain insects and avoiding the losses caused by insects (Donahaye, 2000). However, environmental and economic considerations have limited the number of chemicals that are available for application in the grain industry (Collins, 2006). Consequently, in recent years, there has been a decline in the number of fumigants used to control stored grain pests, and only two fumigants, methyl bromide and phosphine, are available for use (Leesch, 1992). International agreements regarding methyl bromide residue and its effect on the ozone layer may lead to a phasing out of methyl bromide (Casanova, 2002). Therefore, phosphine is the only fumigant that can be used widely because of its low cost, rapid spread and absence of residues (Chaudhry, 2000; Chaudhry, 1997; Nayak & Collins, 2008; Nayak et al., 2013).

1.4.2 Phosphine

Although phosphine was discovered in 1700, the application of this fumigant as a pesticide started in the 1930s (Bond, 1984; Fluck, 1973). From the 1980s, domestic and international markets alike increasingly started requesting the use of chemicals with low residue on grains. Hence, phosphine became the optimal alternative, which significantly increased its use and has

made it become the premier chemical to control pests in the store-product industry (Collins, 2006). The fumigant has many characteristics that qualified it to be widely used to protect stored grain all around the world, including the absence of residue (Bruce et al., 1962; Scudamore & Goodship, 1986), and its having no negative effect on seeds (Ahmad, 1976; Krishnasamy & Seshu, 1990; SITTISUANG, 1985). However, the long-term, extensive use and insufficient application of phosphine had led to the development of resistance toward phosphine (Daglish et al., 2002; Daglish et al., 2015; Opit et al., 2012). This resistance to phosphine is a critical issue that threatens the grain industry worldwide (Bell & Wilson, 1995; Price & Mills, 1988). Therefore, studying the resistance phenomenon will significantly influence the sustainability of using this fumigant.

1.5 Toxicity of phosphine

Our studies focused on the changes in metabolic aspects that were expected to be related to phosphine resistance. In this section, we briefly address the mode of action of phosphine that may explain the expected participation of respiration and metabolism in phosphine resistance. Despite the fact that the mode of action of phosphine is still not understood enough (Zuryn et al., 2008), the toxic effects generally includes reduction in metabolic activities and oxidative stress (Nath et al., 2011). The time that is required for the appearance of toxicity symptoms in insects depends on the concentration of phosphine. Severe symptoms in cockroaches start to appear after 6-10 min of exposure to 12 mg/L of phosphine. These symptoms included tremor of labials and legs, knockdown, loss of the control of muscles and inhibition of respiration (Bond et al., 1969). It was reported that when human workers were exposed to phosphine, it caused headache, cough, dizziness, numbness, lethargy and tightness in the chest (Misra et al., 1988). Phosphine causes hyperactivity in both humans and animals, followed by lethargy that is called "anaesthesia in humans and animals" (Nath et al., 2011). The neural symptoms include vomiting, nausea and tremor (Wilson et al., 1980). Phosphine was reported to reduce the activity of the acetylcholinesterase enzyme of *Ephestia cautella* in and humans, resulting in the induction of body activity (Al-Hakkak et al., 1989; Al-Azzawi et al., 1990; Potter et al., 1993; Sher et al., 2004). Because acetylcholine is a neural transmitter, affecting acetylcholine esterase leads to an increase in movement (Nath et al., 2011).

Studies have confirmed that phosphine is nontoxic in the absence of oxygen (Kashi, 1981). Phosphine was found to only be absorbed into the insect bodies when oxygen was present (Bond et al., 1969). Studies have proven the inhibitory effect of phosphine on aerobic

respiration (Dua & Gill, 2004; Jian et al., 2000; Zuryn et al., 2008). Phosphine causes oxidative stress by increasing the reactive oxygen species, resulting in metabolic inhibition (Chaudhry & Price, 1992; Hsu et al., 2000; Hsu et al., 1998; Valmas et al., 2008). Mitochondria are the sites of aerobic respiration, producing the majority of cellular ATP, using redox energy to conduct the phosphorylation of adenosine diphosphate (ADP) by the FOF1-ATPase (Valmas et al., 2008). Mitochondria membrane is an important element in energy production; the process relies on energy that is produced by the electron transportation, which is conducted through the electron transport chain (ETC) (Nath et al., 2011). Phosphine causes a reduction in the cytochrome c oxidase (complex IV), which is responsible for transfer of the electron to its final site, as well as a reduction in oxygen consumption, resulting in inhibition of respiration and energy production (Chefurka et al., 1976; Chin et al., 1992; Dua & Gill, 2004; Price, 1980b; Price & Dance, 1983; Singh et al., 2006). A study on the effect of phosphine on the dynamic characteristics of cytochrome C oxidase and energy metabolism of rat liver revealed that phosphine caused a severe reduction of cytochrome oxidase activities and affected nicotinamide adenine dinucleotide (NADH) and succinic dehydrogenase activities, leading to reduced synthesis of ATP (Dua et al., 2010). Moreover, phosphine causes extensive organ damage (Chefurka et al., 1976; Nakakita et al., 1971) and raises the oxidative deterioration in the brain, lung and liver of rats (Hsu et al., 2000). The protein synthesis and enzymatic activities of the mitochondria of the lung and cardiac cells were also found to be inhibited by phosphine (Bumbrah et al., 2012). The inhibitory effect of phosphine on cytochrome c oxidase was only reported when the mitochondria was treated in vitro (Chefurka et al., 1976; Nakakita et al., 1971; Price, 1980a; Price, 1980b; Zuryn et al., 2008). In contrast, phosphine did not or only slightly affected cytochrome c oxidase activities when the organisms were treated in vivo (Price, 1980a; Price & Dance, 1983; Zuryn et al., 2008). Moreover, phosphine disturbed mitochondrial morphology and reduced the oxidative respiration of *Caenorhabditis elegans* after five hours of exposure in addition to its effect on the mitochondrial membrane potential (Zuryn et al., 2008).

1.6 Insect resistance to phosphine

The first report of resistance toward insecticides was in 1914 when scale insects showed resistance toward sulphur-lime, an organic pesticide (Melander, 1914). By 1983, hundreds of cases of resistance were recorded in arthropods (Georghiou, 1986). Insects are considered resistant to a particular pesticide if their response to this pesticide is much less than the regular

response (Georghiou & Mellon, 1983). In the grain industry, this resistance to fumigants is a severe threat all around the world (Collins, 2006; Price & Mills, 1988).

In our study, we focused on the insect resistance toward hydrogen phosphide. The phenomenon threats the use of this fumigant, which is considered the most important pesticide used in grain industry. Resistance to phosphine by stored-grain insects began to be documented in the 1970s (Champ & Dyte, 1976). A survey conducted by the Food and Agriculture Organisation (FAO) with regard to the susceptibility of the stored product insects in different countries revealed for the first time that 10% of the insect populations had resistant insects (Champ & Dyte, 1976). Subsequently a study in Bangladesh revealed strong resistance in five stored grain species including *R. dominica* (F.), *Oryzaephilus surinamensis* (L.), *Cryptolestes ferrugineus* (S.) and *T. castaneum* (H.) (Mills, 1983). In 1997, the levels of resistance started to increase, including the high resistance of the lesser grain borer, and since then, strong resistance to phosphine has been revealed in most species of major grain insects (Collins, 2009). This increasing resistance to phosphine is threatening the effectiveness of this fumigant and the entire grain industry (Collins, 2009). In resistant insects, the resistance level was found to be stable over the years, even when the insects were not treated with phosphine during the years of rearing (Chaudhry, 2000). At present, phosphine is one of the most effective control methods. However, resistance to it means that there is a need to use much higher concentrations to control insect populations in the future, and in some cases, it may not be possible to complete the control (Lilford et al., 2009).

Nath et al. (2011) described the mechanism of the effect of phosphine in his review under three categories, including neural, metabolic and respiratory stress. In the respiratory category, phosphine is described as a respiratory inhibitor (Chaudhry, 1997; Chefurka et al., 1976; Dyte et al., 1983; Price, 1980a; Price, 1980b; Valmas et al., 2008; Zuryn et al., 2008). The inhibitory function of phosphine was verified in vitro on mitochondria in different parts of several animal species, such as the rat liver (Dua et al., 2010; Nakakita et al., 1971), insects and mites (Chefurka et al., 1976; Jian et al., 2000; Price & Dance, 1983) and nematode (Zuryn et al., 2008). The exclusion of phosphine from the respiratory system by taking in a lesser amount of phosphine was reported to be the mechanism of insect resistance toward phosphine (Chaudhry & Price, 1992; Pimentel et al., 2007). The lower phosphine uptake was found to be linked with a low rate of respiration in resistant individuals in comparison to sensitive insects (Pimentel et al., 2007; Pimentel et al., 2008).

1.7 Phosphine-resistant insects and metabolism

Based on the modes of action of phosphine, two possible factors may contribute in phosphine resistance: respiratory and metabolic (Nath et al., 2011; Price, 1984; Price & Dance, 1983; Zuryn et al., 2008). The metabolism of resistant strains may prevent metabolic crises or supply the energy demand in resistant individuals (Nath et al., 2011). This may be a result of the genetic differences that were unquestionably recognised in many resistant insect species (Nguyen et al., 2015; Schlipalius et al., 2002; Subramanian S, 2016). These inherited changes led us to assume the presence of metabolic changes in susceptible and resistant strains of *R. dominica* and *T. castaneum*. Three aspects are discussed below as either possible factors contributing to phosphine resistance or indicators of metabolic changes resulting from phosphine resistance.

1.7.1 Respiration

Since phosphine is acting as a respiratory inhibitor, studying respiration was crucial for understanding the mechanism of phosphine resistance (Bond & Monro, 1967; Bond et al., 1969; Chaudhry, 2000; Chaudhry, 1997; Chefurka et al., 1976; Hobbs & Bond, 1989; Price, 1980a; Price, 1980b; Valmas et al., 2008; Zuryn et al., 2008). These studies confirmed the relationship between respiration rate and the resistance level of the organism (Pimentel et al., 2008; Price, 1980b; Zuryn et al., 2008). Phosphine causes respiratory stress by disturbing the mitochondrial electron transport chain (Chaudhry, 1997; Chefurka et al., 1976) in addition to its effect on mitochondrial morphology and mitochondrial membrane potential (Valmas et al., 2008; Zuryn et al., 2008). The plausible mechanism of phosphine resistance includes the elimination of this fumigant from the respiratory system by resistant insects taking in less gas when compared to susceptible insects (Chaudhry & Price, 1992; Pimentel et al., 2007; Price, 1984; Price, 1981). This was observed when resistant strains of *R. dominica* showed lower uptake of phosphine in comparison with susceptible strains despite treatment with the same concentration (Price, 1984; Price, 1981). Similarly, Pratt (2003) confirmed that resistant *T. castaneum* absorbed more phosphine than susceptible insects of the same species. The higher rate of phosphine uptake by susceptible insects was observed to continue even when the susceptible insects were dead, compared to resistant alive insects (Pratt, 2003; Price, 1981). Accordingly, studies confirmed that resistance to phosphine is linked to the decreased respiration rate of resistant populations (Pimentel et al., 2007; Pimentel et al., 2008). . However, a different response to this fumigant was reported from different treatment types. For

instance, phosphine reduced the respiration rate of susceptible insects in vivo but did not affect the respiration of resistant individuals, whereas, in the same studies, the inhibition of isolated mitochondria treated in vitro with phosphine was similar in both resistant and susceptible types (Price, 1980a; Zuryn et al., 2008). This parallel response suggested that the effect of phosphine on mitochondria in vitro is not related to phosphine resistance (Price, 1980b). Hence, the relationship between respiration and phosphine resistance is a significant to provide a clearer image of the mechanism of phosphine resistance (Chaudhry et al., 2004). This is supported by the fact that the mortality of resistant insects is much lower than the mortality of susceptible insects despite treatment with the same concentration of phosphine (Chaudhry & Price, 1990), which suggests that there are more biochemical processes involved in avoiding the toxicity of phosphine than respiration alone (Price, 1980a; Price, 1980b).

Respiration is a physiological response to the environment in which the insects are exposed (Emekci et al., 2002). Therefore, the atmosphere affected the respiration rate of *T. castaneum* and was proportional to the oxygen levels (Emekci et al., 2002; Emekci et al., 2001). Results showed that respiration was higher under low levels of oxygen than at normal atmospheric air (Emekci et al., 2002). Phosphine is reported to be nontoxic unless oxygen is present (Bond & Monro, 1967). Hence, studying the effect of oxygen on insect respiration is essential in understanding phosphine resistance.

1.7.2 Volatile organic compounds

Volatile organic compounds (VOCs) are carbon compounds that evaporate at room temperature (Bennett & Inamdar, 2015). In organisms, they are released as products of metabolic activities or tissue damage. The profile of VOCs may provide very valuable evidence about a variety of relationships and biological status, such as the relationship between insects and their hostage plants (Du, 2001; Jhumur et al., 2007; Kesselmeier & Staudt, 1999; Verheggen et al., 2008; Wei, 2001). Also, the inhibitory effect of VOCs on other organisms such as bacteria and fungi (Arroyo et al., 2007; Liu et al., 2004; Sekine et al., 2007), the attractive effect on the other sex (Landolt & Phillips, 1997), and as aggregation or defensive secretions against other species (Arnaud et al., 2002; Villaverde et al., 2007). So because VOCs are affected by biological status, they have been used as indicators for studying metabolic changes (Goh et al., 2016). Hence, VOCs were reported to be robust tools for diagnosing specific diseases that infect humans through the investigation of the differences in the VOCs emitted by patients and healthy people (Belda-Iniesta et al., 2007; Miekisch et al., 2004; Song et al., 2010). Examples of diseases diagnosed using VOCs include respiratory issues, inflammatory diseases,

cholesterol biosynthesis and cancer diseases in the human body (Miekisch et al., 2004). For instance, a study about the effect of smoking on human respiration showed that non-smokers produced more saturated hydrocarbons through their respiration, whereas hydrocarbons were predominant in smokers' breath along with other compounds including furan, acetonitrile and benzene (Buszewski et al., 2009). A study of children with asthma and healthy children showed that eight VOCs in exhaled breath could be used as a discriminating tool to differentiate the children with asthma from healthy children (Dallinga et al., 2010).

In the agricultural industry, studies on the potential use of VOCs as biomarkers to investigate the infestation of some plant pathogens such as *Phytophthora spp.* was also proved in many studies (Crespo et al., 2008; De Lacy Costello et al., 2001; Laothawornkitkul et al., 2010; Qiu et al., 2014b). Qui et al. (2014) used VOCs as a tool for identification of *Phytophthora cinnamomi* in different growing environments, using specific VOCs like 2-methoxy-4-vinyl phenol like to determine the infestation.

Stored-grain insects release a variety of abundant VOCs such as quinones released by *T. castaneum* into the airspace surrounding stored products, which cause undesirable contamination (Villaverde et al., 2007). VOCs released by stored-grain insects are also a reliable potential method of diagnosis and identification of insect species such as *R. dominica* (Fabricius) and *T. castaneum* (Herbst) (Laopongsit et al., 2014; Niu et al., 2012; Niu et al., 2015). VOCs were used by Niu et al. (2016) to identify the infestation of wheat flour by the red flour beetle *T. castaneum*, and the use of 2-ethyl-2,5-cyclohexadiene-1,4-dione as a biomarker was suggested.

Studies on phosphine resistance proved that resistant strains are genetically varied from susceptible strains (Kocak et al., 2015; Nguyen et al., 2015; Subramanian S, 2016). We assume this genetic difference to be a reason for the metabolic differences that affect the emission of the VOCs. Therefore, VOCs are a potential diagnostic tool for differentiating between susceptible and resistant strains.

The first step to identify the biological VOCs is applying a noble sampling technique that can deal with the complicated chemical structures and polarities (Zhang & Li, 2010). Solid-phase microextraction (SPME) fibres were developed and proposed for the first time by (Arthur & Pawliszyn, 1990; Pawliszyn, 1995). Applying this technique by only inserting SPME into the sample matrix made this method easy to use (Camarasu, 2000). By reducing the extraction steps and increasing efficiency, SPME was considered the most valuable invention in the field

of sample preparation (Balasubramanian & Panigrahi, 2011; Zhang & Li, 2010). Three modes to perform SPME were described, including direct extraction, membrane protection extraction and headspace extraction (Balasubramanian & Panigrahi, 2011). Headspace extraction can be implemented by exposing the fibre coating to the headspace of the extraction chamber that contains the biological sample (Laopongsit et al., 2014; Laothawornkitkul et al., 2010; Niu et al., 2012; Niu et al., 2015; Qiu et al., 2014b). In biology, the headspace solid-phase microextraction (HS-SPME) of VOCs was commonly applied for its suitability for sampling various types of biological samples, such as animals, plants and microbial samples (Zhang & Li, 2010). Using HS-SPME coupled with gas chromatography–direct deposition infrared spectrometry (GC–DD-IR) led to a rigorous absence of water and could be applied to trap unknown VOCs directly from within living organisms (Auger et al., 1998). The HS-SPME has been used to extract and sample VOCs from a variety of biological samples (Belliardo et al., 2006; Cai et al., 2007; Crespo et al., 2008; Ezquerro et al., 2003; Nakamura & Daishima, 2005; Perera et al., 2002; Rochat et al., 2000; Rodrigues et al., 2011). A study reviewed the significance of using HS-SPME in the analysis of the volatile fraction of aromatic and medicinal plants, showed that 108 articles published during 2000–2005 proved the importance of this method (Belliardo et al., 2006).

The HS-SPME techniques coupled with gas chromatography (GC) to analyse insect-derived VOCs was previously suggested as a method to detect infestations of stored grain (Bocchini et al., 1999; Farag et al., 2006; Nilsson et al., 1996; Pecoraino et al., 2008). However, this method requires further optimisation to enhance the efficacy of VOCs extraction (Laopongsit et al., 2014; Niu et al., 2012). The ability of SPME to absorb chemical compounds is affected by many factors, such as extraction time, temperature, fibre coating and compound types (Balasubramanian & Panigrahi, 2011). A range of commercial coatings of SPME has been used to extract VOCs from the headspace (Balasubramanian & Panigrahi, 2011). For example, 50/30 μm DVB/CARB/PDMS fibre coating can extract a large number of VOCs because it is covered by a mid-polarity coating (Balasubramanian & Panigrahi, 2011; Kataoka et al., 2000). In addition to the effect of the fibre type, the extraction time is also considered a critical factor in collecting more VOCs (Kataoka et al., 2000; Niu et al., 2012; Qui et al., 2012). Appropriate extraction time is required to maximise the extraction ability of the fibre coating (Kataoka et al., 2000; Niu et al., 2012; Qui et al., 2012). A short extraction time may not be appropriate to release most of the VOCs, specifically the low volatile compounds, whereas a more extended extraction period may result in degradation of some of VOCs, such as the high volatile

compounds (Laopongsit et al., 2014). Using a suitable extraction temperature is a significant factor for abundant extraction. High extraction temperatures cause an increase in the extraction rate, but it also reduces the distribution constant (Kataoka et al., 2000). All the above parameters should be taken into consideration to produce reliable and sufficient VOC data. Studying the VOCs from phosphine-resistant and -susceptible insect strains may provide significant information in understanding the resistance mechanism.

1.7.3 Insect lipids

Lipids are compounds that are naturally excreted in animals and plants (Cerkowniak et al., 2013). The significance of lipids is not only in their role as a main source of energy but also as an essential part of the cell membrane (Downer & Matthews, 1976). Free fatty acids are fundamental parts of biological lipids and are usually bound with other compounds to build more composite lipids such as triglycerides, which are considered stores of energy (Desbois & Smith, 2010). Fatty acids, such as linoleic and linolenic acids, are essential requirements for insect bioprocesses, such as metamorphosis formation.

The cuticle is a large part of the dry insect weight, protecting other tissues. Because of this lipids are a significant part of the cuticle, which proves that cuticular lipids are an essential part of insect lipid content (Locke, 1974). Oenocytes are the epidermal cells that synthesise cuticular lipids such as hydrocarbons (Diehl, 1973; Diehl, 1975; Romer, 1980), while the lipophorin transports lipids to their location in the cuticle layer (Chino & Downer, 1982; Haruhito & Haruo, 1982; Katase & Chino, 1984). Species and the developmental stage are affecting the cuticular lipid composition and quantity (Gołębiowski et al., 2011) in addition to the genetic structure and ecological conditions (Toolson, 1982; Toolson & Kuper-Simbrón, 1989). The composition of lipids is useful biochemical characteristics used for taxonomical purposes for the identification and differentiation of species (Cohen & Moussian, 2016). The cuticular lipids of the insect consist mostly of wax esters and hydrocarbons, in addition to fatty acid esters, triacylglycerols, aldehydes, alcohols, ketones and free fatty acids (Blomquist & Jackson, 1979; Cohen & Moussian, 2016; Gibbs, 1998; Gołębiowski et al., 2011). The cuticular lipids of adult *Acanthoscelides obtectus* contain a variety of chemicals such as hydrocarbons, triacylglycerols, fatty acid esters, free fatty acids, sterols, aldehydes, ketones and alcohols (Blomquist & Jackson, 1979; Cohen & Moussian, 2016; Gibbs, 1998; Gołębiowski et al., 2011; Gołębiowski et al., 2008b). Analysis of the cuticular lipids of *Calliphora vicina, Dendrolimus pini,* and *Galleria mellonella* show that larval cuticle contains three groups, including

hydrocarbons, free fatty acids and triacylglycerols (Gołębiowski et al., 2008a). Whereas, cuticular lipids of *Pogonomyrmex barbatus* consist only of wax esters and hydrocarbons (Nelson et al., 2001).

Hydrocarbons are the major lipid category the cuticle of insects, including straight-chain saturated and unsaturated hydrocarbons (Gołębiowski et al., 2011). Which make up in some species more than 90% of the cuticular lipids and are usually a mixture of components including n-alkanes, branched methyl-alkanes and ethyl-alkanes (Blomquist & Jackson, 1979; Cerkowniak et al., 2013; Cohen & Moussian, 2016; Hadley, 1984). Analysis cuticular lipids from adults of *Zygogramma exclamationis* showed that large amounts hydrocarbons in the cuticle were identified in males and females (64.4±1.1 and 100.9±10.1 μg/g insects) ranged from C23 (tricosene) to C56 (trimethyltripentacontane) (Nelson & Charlet, 2003). The functions of insect cuticular hydrocarbons evolved not only to preserve water in the body but also to play an essential role in communication between sex and the other sex (Cohen & Moussian, 2016). Besides, their role as communicational chemical tools use in communication among the population individuals (Blomquist et al., 1980; Blomquist & Jackson, 1979; Nelson, 1978). They also serve as a barrier between the living insect bodies and their environment (Andersen, 1979; Kolattukudy, 1976), and to prevent the penetration of microorganisms (El-Sayed et al., 1991; Gołębiowski et al., 2008a; Koidsumi, 1957; Wang & Leger, 2005) and avoid the absorption of pesticides and other chemicals from the environment (Nelson & Sukkestad, 1970). In addition, some wax components act as sex and aggregation pheromones (Carlson et al., 1971; Cohen & Moussian, 2016; Ginzel et al., 2003; Ginzel et al., 2006). The cuticular lipids of *Tribolium spp* were studied for their expected defensive role against predators and pathogens (Arnaud et al., 2002; Howard & Mueller, 1987; Yezerski et al., 2004).

Various methods were applied for the extraction of insect cuticular lipids, but the essential element was the use of an organic solvent. The analysis methods varied (Gołębiowski et al., 2012), such as using a solo solvent like hexane to extract cuticular lipids with gas chromatography-mass spectrometry (GC-MS) (Nelson et al., 1999; Ye et al., 2007), chloroform with high-performance thin-layer chromatography (HPTLC) (Buckner et al., 1999), and dichloromethane with gas chromatography-flame ionisation detection (GC-FID) (Saïd et al., 2005). Combinations of solvents have been used, such as hexane/chloroform with GC-FID and GC-MS (Buckner et al., 2009; Nelson et al., 2000), chloroform/methanol with high-performance liquid chromatography-mass spectrometry (HPLC) (Kofroňová et al., 2009) and

petroleum ether/ dichloromethane with GC-FID and GC–MS (Gołębiowski et al., 2010; Gołębiowski et al., 2008a).

SPME was commonly applied as a method to extract insect cuticular lipids (Bland et al., 2001; De Pasquale et al., 2007; Ginzel et al., 2003; Ginzel et al., 2006; Monnin et al., 1998; Roux et al., 2002; Sledge et al., 2000). The extrication was either conducted by rubbing the SPME fibre with the cuticle of the insects (Ginzel et al., 2006; Monnin et al., 1998; Roux et al., 2002), alternatively, by using the headspace microextraction technique (Bland et al., 2001; De Pasquale et al., 2007; Sledge et al., 2000). Ginzel et al. (2006) compared extraction using the SPME with whole body hexane extraction; the SPME extraction was implemented by gently rubbing the dorsal surface, resulting in a similar response to the whole body extraction. Bland et al. (2001) used the headspace microextraction method to extract the cuticular hydrocarbons of *Coptotermes formosanus* termites; heating was used to convert the cuticular hydrocarbons to volatile compounds that could be collected by SPME.

Lipids composition occurs naturally, performing an essential role in the metabolism of insects and plants (Ad et al., 1985; Cerkowniak et al., 2013). Insects commonly contain a high content of lipids, making up 50-75% of the dry weight of some insect (Bursell, 1970; Pino Moreno & Ganguly, 2016; Rumpold & Schlüter, 2013). The obvious advantages of lipids are their use as energy sources for reproduction, transformation and flight, and their vital role in the persistence of insects on this planet (Gilbert & Chino, 1974). The lipid oxidation process also produces a large amount of metabolic water, which plays a significant factor in insect survival specifically during non-feeding stages (Downer & Matthews, 1976). Lipids are stored in the form of triglycerides (TG) inside cells called fat bodies, which supply the insects with energy requirements (Arrese & Soulages, 2010). Fat bodies are considered the main cells for both synthesis and storage of lipids to provide haemolymph with lipids required during bioactivities such as flight (Ad et al., 1985). TGs and glycogen (sugar of multi-branched glucose) are the main sources of energy inside insect bodies (Arrese & Soulages, 2010). To maintain long-term energy production, lipids are considered the most resourceful and appropriate way to generate the required energy. TG can be stored in an anhydrous form while glycogen is stored in the form of massive wet masses, allowing the use of lipids as an essential substance for metabolism, which allows for the accumulation of a large reservoir of energy that can be used during long periods of energy demand (Downer & Matthews, 1976). Studies demonstrated that sugars are also considered as a source of lipids through a specific pathway process that proves the priority of lipids (Storey & Bailey, 1978; Walker & Bailey, 1970). TGs are the main lipids

in the insect bodies, representing in some cases more than 90% of total body lipids, while monoglycerides, diglycerides (DG), free fatty acids and sterol usually make up only a small percentage of total body lipids (Ad et al., 1985). Fatty acids that synthesise in the insect body are stored in the fat body as a form of TG (Ad et al., 1985). Fatty acids are vital sources of energy and required to build up the cell membranes. Therefore, they consider as important elements for organism growth, differentiation, reproduction and homeostasis (Carballeira, 2008). DG, however, are found to be the main lipids released when the lipids absorption proceeded (Ad et al., 1985). TGs hydrolysis inside the fat body is the main source for DG production (Hoffman & Downer, 1979; Spencer & Candy, 1976). Studies on a variety of insect species indicated that the major lipids in the insect hemolymph are DGs, in addition to TGs and free fatty acids (Chang, 1974; Cohen, 1972; Stevenson, 1972; Thomas, 1974). The conversion pathway of TG to DG was suggested to be through the cleavage of TG tomonoglycerides (MG) followed by the conversion to DG, releasing one fatty acid to the haemolymph (Arrese & Wells, 1997; Hoffman & Downer, 1979; Tietz & Weintraub, 1978; Tietz & Weintraub, 1980). The importance of DG was also described in studies about locust flight when the amount of DG was levelled up in the haemolymph to threefold of its normal concentration to supply the energy requirements (Beenakkers, 1973; Jutsum & Goldsworthy, 1976).

Phospholipids are a large group of lipids that contain a polar and non-polar end. They consist of two layers; a hydrophobic layer that contains two fatty acids and a hydrophilic layer having a phosphate group connected by glycerol or alcohol (Bohdanowicz & Grinstein, 2013; Li et al., 2015; Singh et al., 2017). The importance of phospholipids derives from their main function as a major part of cellular membranes, acting as a barrier to separate the cells from the surrounding environment and allowing each cell to perform its specific function (Bohdanowicz & Grinstein, 2013). This importance of phospholipids is more evident through the role of phosphatidic acid, which affects the membrane shape despite their small quantity in the total lipids of the cell (McMahon & Gallop, 2005; Vance & Steenbergen, 2005). Moreover, phospholipids contribute a large amount to the mitochondrial membrane lipids environment; virtually all types of lipids that are present in the cell membrane also exist in the mitochondrial membrane, affecting the mitochondrial respiratory chain by affecting the physical properties of the mitochondrial membrane (Horvath & Daum, 2013; Ren et al., 2014). Respiration was found to be affected by the reduction of the amount of phospholipids due to the effect on mitochondria, in addition to the effect on the mitochondrial respiratory chain. This reduction

was also observed to be synchronous with a significant reduction of 50% of adenosine triphosphate (ATP) (Baker et al., 2016). On the other hand, evidence provided by previous studies confirmed that phospholipids are one the main precursors for the synthesis of DG and TG in some insect species (Tietz, 1969; Weintraub & Tietz, 1973).

The composition of lipids is influenced by many factors including genetic factors, ecological conditions and nutritional status. Therefore, lipids are varied among insect species (Ad et al., 1985; Toolson, 1982; Toolson & Kuper-Simbrón, 1989).

1.8 Research gap and aim of the study

The continuation of the effectiveness of phosphine is an important target in the grain industry around the world. Resistance to phosphine is a rising threat that affects the sustainability of this unique fumigant. Therefore, new approaches are required to avoid the spread of phosphine resistance. To acquire such strategies, studies on the mechanism of phosphine resistance should be implemented. We assume the genetic differences to be an indicator of metabolic changes between the resistant and susceptible strains. These metabolic changes may be considered as the key to understanding this phenomenon. Hence, studying the metabolism of phosphine-resistant strains is an urgent requirement for interpreting the mechanism of phosphine resistance.

This thesis provides new information about the participation of metabolic processes in the mechanism of phosphine resistance. The project was conducted on two important stored-grain insects *R. dominica* (Fabricius) and *T. castaneum* (Herbst) to answer the following questions:

I. Are there any differences in the emission of VOCs as an indicator of different metabolic processes between the resistant and susceptible strains?
II. Are VOCs able to be used as biomarkers to differentiate the resistant from the susceptible strains?
III. Are resistant and susceptible strains different in terms of their respiration? What is the response of resistant and susceptible strains to sublethal and lethal doses of phosphine in terms of respiration? Is that related to the emission of the VOCs?
IV. Are the lipids of the resistant and susceptible insects different in abundance? If yes:
    a. What is the expected participation of cuticular hydrocarbon lipids as a barrier?
    b. What is the expected participation of glycerolipids as sources of the energy?
    c. What is the expected participation of phospholipids as important parts of the cell walls?

Statement of Contribution

Title of Paper	Comparison of respiration rate between phosphine resistant and susceptible strains of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae
Publication Status	○ Published ○ Accepted for Publication ○ Submitted for Publication ◉ Unpublished and Unsubmitted work written in a manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Ihab alnajim
Contribution to the Paper	concept, laboratory works, data analysis and writing manuscript
Overall percentage (%)	65%
Signature	[redacted] Date: 25/ 7/ 2019

Co-Author Contributions
By signing the Statement of Contribution, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all the co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	Tao Liu
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	YongLin Ren
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	15%
Signature	[redacted] Date: 26/ 7/ 2019

Please cut and paste additional co-author panels here as required.

Chapter 2

Comparison of Respiration Rate between Phosphine Resistant and Susceptible Strains of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

Abstract

Resistance to phosphine is a worldwide issue that threatens the continued use of this fumigant. There is a general belief that resistance occurs through the low uptake of phosphine by a decrease in the respiration rate of the resistant populations. However, there is no evidence to prove that the low respiration rate of resistant strains is associated with resistance to phosphine.

The study aims to determine the bio-response of susceptible and resistant strains of *Tribolium castaneum* (Herbst) to different oxygen and phosphine levels using the respiration rate as an indicator. The gas analyser Oxybaby was used to analyse the oxygen and carbon dioxide concentrations. To support the result of respiration, we implemented an additional experiment by measuring the VOCs from the resistant strain using the HS-SPME technique.

The respiration rate of the susceptible strain was significantly higher than that of the resistant strain. The results showed no significant effect of oxygen level on the respiration rate in both susceptible and resistant strains. Phosphine reduced the respiration rate of both susceptible and resistant strains when it was applied in average concentrations. However, the rate of respiration of the resistant beetles increased significantly under a high level of phosphine. Higher VOC emissions were obtained from resistant insects treated with a high concentration of phosphine, indicating that there was an elevation in the metabolism of treated insects compared to their untreated peers.

In summary, despite the common understanding that phosphine acts as a respiratory inhibitor, we found for the first time, a different response from a resistant strain of *T. castaneum* that resulted from the increased rate of respiration after exposure to a high level of phosphine. The respiration increase was associated with the higher emission of VOCs, which proves the acceleration of metabolic processes to resist the phosphine action for survival.

2.1 Introduction

Phosphine has been used as a fumigant to control stored product pests for decades (Chaudhry, 1997). It is the most widely used fumigant because of many advantages such as low cost, rapid penetration to commodities, rapid diffusion in the atmosphere and low residues (Chaudhry, 2000). Nevertheless, ineffective application resulting from the rapid loss of phosphine concentration was considered to be the most likely reason for the spread of resistance worldwide against this fumigant (Benhalima et al., 2004; Champ & Dyte, 1976; Mills, 1983; Tyler et al., 1983). Phosphine mechanism of toxicity needs, and inhibits, aerobic respiration

(Chaudhry, 1997; Valmas et al., 2008), resulting in respiratory stress in insects by turning off the mitochondrial electron transport chain (Chaudhry, 1997; Chefurka et al., 1976). It is acting as a respiratory inhibitor on the organisms that are treated with phosphine (Chefurka et al., 1976; Hobbs & Bond, 1989; Price, 1980a). Therefore, the elimination of this fumigant from the respiratory system by the resistant strains was reported to be a mechanism to avoiding the harmful effect of phosphine (Price, 1984). This may be achieved by taking in of less gas in resistant insects compared with susceptible insects (Chaudhry & Price, 1992; Pimentel et al., 2007; Pimentel et al., 2008). Accordingly, a significant relationship between respiration rate and phosphine resistance was reported in different insect species (Pimentel et al., 2007; Pimentel et al., 2008). A study on resistant populations to phosphine of *T. castaneum*, *R. dominica* and *O. surinamensis* revealed that populations with lower carbon dioxide production showed a higher resistance ratio, showing that the lower respiration rate is reducing the fumigant uptake in the resistant insects (Pimentel et al., 2008). However, the mechanism of eliminating for resisting phosphine is insufficient by itself to provide a solid explanation for phosphine resistance (Chaudhry et al., 2004). This comes from the fact that the mortality of resistant insects is usually lower than the mortality of susceptible populations when they are treated with the same concentration of phosphine (Chaudhry & Price, 1990). The dihydrolipoamide dehydrogenase (DLD) gene contributes to biochemical processes that are important for phosphine resistance (Schlipalius et al., 2012), proving that there are biochemical processes involved in reducing the toxicity of phosphine (Price, 1980a; Price, 1980b). However, there is still a lack of information about the relationship between respiration, metabolic processes and phosphine resistance, so the whole image of the mechanism is still unclear (Chaudhry et al., 2004).

Respiration is an indicator of the insect's physiological response to the environment to which it is exposed (Emekci et al., 2002). Studying respiration of *Tribolium* spp. indicated that carbon dioxide consumption related to body weight, sex, and food (Park, 1932). Oxygen levels play a role in the respiration rate of the insects (Emekci et al., 2001); studies have confirmed that respiration of adult *T. castaneum* was higher in a low level of oxygen than in normal atmospheric air (Emekci et al., 2002; Emekci et al., 2001). The objective of this study is to determine the physiological response phosphine resistant and susceptible strains of adults of *T. castaneum* to different levels of oxygen and phosphine using the respiration rate and VOCs as indicators.

2.2 Material and Method

2.2.1 The insect cultures

Resistant and susceptible adult insects, *Tribolium castaneum* were obtained from the Department of Primary Industries and Regional Development (DPIRD), Australia. The narrow aged insects (2-3 days) were cultured by incubating 3000 adult insects with 1000 g of food, in 2-L jars sealed with meshed lids. The Parents' insects were removed after three days, and the remaining cultural medium were incubated at 28±1°C and 70±2% RH. Newly emerged adults were narrowly aged and transferred to the jar containing new food. The insects used in the experiments were one month old. The flour was made from freshly harvested wheat (Australian Standard Wheat). Before using, the wheat was sterilised by keeping at -20°C freezer for seven days followed by storing at 4°C until use. The grain was milled using a Wonder Mill (Model WM2000, Korea), and the flour was kept at 4°C.

2.2.2 Chemicals and instruments

Commercial tablets of aluminium phosphide (Quickphos, United Phosphorus Limited (UPL), NSW, Australia) were used to generate phosphine. Pure oxygen and nitrogen (99.99% purity) were purchased from BOC CO., Australia. Two hundred fifty mL clear glass collection bottles equipped with screw caps (24 mm Mininert Valves; Cat No. 24904; RESTEK, USA) were used for implementing the respiration experiments. The headspace gas analyser Oxybaby Witt-Gasetechnik D-58454 (WITT Gasetechnik GmbH & Co, Germany) was used for oxygen and carbon dioxide measurement.

The 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre with 2 cm coating was supplied by Sigma-Aldrich, Bellefonte, USA. Prior to use, the fibre was activated according to manufacturer's recommendations. The Agilent Technology gas chromatograph 7820A coupled with a Flame Ionization Detector (FID) fitted with a HP-5MS non-polar column (30 m × 0.25 mm × 0.25 µm, RESTEK, Cat No 13423) was used to detect the VOCs. The temperature of the GC inlet (splitless mode) was 250°C. Helium was used as a carrier gas at a constant flow of 1.1mL/min. The oven temperature program was 40°C for 5 min, increased at a rate of 5°C/min to 250°C, and held for 5 min with a total run time of 52 min. The Flame Ionization Detector temperature was 280°C.

2.2.3 Generation of phosphine gas and determination of resistance factor

Phosphine was generated by dissolving commercial tablets (Quickphos, United Phosphorus Limited (UPL), NSW, Australia) of aluminium phosphide in 10% sulphuric acid solution, and the final purity of phosphine was 86% (FAO, 1975). To determine the resistance factor, bioassays were implemented by using different concentrations of phosphine (control (air), 3.65, 7.30, 14.60, 21.90, 29.20, 73.00, 219.00, 438.00, 730.00, 1460.00, 2190.00 and 2920.00 ppm) with three replicates of each concentration. Fifty adult insects in 1000 mL flasks for each replicate were fumigated (without food) with phosphine for 20 h for the susceptible and resistant strains of *T. castaneum*, followed by a one-week recovery period with food at 25±1°C and 65±5% RH.

2.2.4 Respiration rate

2.2.4.1 Effect of different levels of oxygen and phosphine

The respiration rate of the adult of phosphine-resistant and -susceptible of *T. castaneum* was compared under combinations of five oxygen levels (5, 10, 20.9, 30 and 40%) with five phosphine concentrations (0, 2, 5, 10, 20 ppm) with three replicates for each combination. One hundred adult insects without food were transferred into a 250 mL clear glass bottle equipped with a screw cap. The respiration of the insects of each oxygen and phosphine combinations was measured after different sealing periods (8, 16 and 24 h) at 30±1°C and 70±5% RH. The oxygen levels were achieved by injecting a specific volume of pure nitrogen and oxygen into the flasks after removing an equivalent volume of air using a gas syringe (Valco instrument Co., USA). Before incubating the samples, the concentration of oxygen was confirmed using the gas analyser Oxybaby Witt-Gasetechnik D-58454 (WITT Gasetechnik GmbH & Co, Germany). Oxybaby was also used for oxygen and carbon dioxide measurements.

2.2.4.2 Respiration rate of phosphine resistant strain of *T. castaneum* under higher phosphine concentrations

The respiration rate of the adults of phosphine-resistant of *T. castaneum* was measured under five oxygen levels (5, 10, 20.9, 30 and 40%) combined with higher phosphine concentrations (40, 80, 160 and 320 ppm) with three replicates for each combination. The respiration of the insects of each phosphine level was measured after different sealing periods (8, 16 and 24 h) at 30±1°C and 70±5% RH.

To determine the long-term response of the resistant beetles to a high level of phosphine, the respiration rate of the surviving resistant insects from the 20 h fumigation of 292.00 ppm phosphine was compared with the respiration of insects of same generation and strain without phosphine treatment (0 phosphine or untreated control). The respiration was measured after 1, 3, 5, 7, 10, 15, 21 and 30 days of fumigation with three replicates. The experiment was implemented under a 24 h sealing period.

2.2.4.3 Volatile organic compounds (VOCs) of resistant insects survived from 292 ppm

Volatile organic compounds (VOCs) of resistant insects survived from 292.00 ppm were analysed after ten days of the treatment. Headspace solid-phase microextraction (HS-SPME) coupled with GC-FID was used to detect the VOCs from the adult *T. castaneum*. The SPME fibre was inserted into the 100 mL Erlenmeyer flask that contained 100 adult insects for 4 hours and then pulled and injected directly into the port of the GC.

2.2.5 Data analysis

Respiration data was analysed by IBM SPSS Statistics 24. The means of the replicates were compared using T and Tukey tests. Masshunter Qualitative Analysis B.07.00 (Agilent Technology, USA) was used to generate and compare the GC chromatogram.

2.3 Results

The probit model concentration-mortality analyses showed the LC_{50} of the susceptible strains of *T. castaneum* was 0.009 mg/L while the LC_{50} for the resistant strains was 0.26 mg/L for, respectively. Consequently, the resistance ratio (RR) has been calculated according to the LC_{50} of the susceptible insects (RR = 28.8 fold).

The results in Figure 2.1 showed no significant effect on the insects' respiration (carbon dioxide production) at different oxygen concentrations (5.0, 10, 20.9, 30 and 40%) in both susceptible and resistant *T. castaneum*. However, the production of carbon dioxide was overall higher in the susceptible population than the resistant strain. At the 8 hours exposure period, statistical analysis showed significant differences regarding the carbon dioxide production between the susceptible and the resistant strains. At 10% oxygen level (0.044 and 0.037 mg/insects ($P \leq 0.041$) for susceptible and resistant populations, respectively), and at 40% oxygen level (0.042 and 0.033 mg/insects ($P \leq 0.007$) for susceptible and resistant populations, respectively). The difference was further evident at 16 and 24 h of exposure periods in most of the oxygen levels. The carbon dioxide production after 16 h of exposure at 5%, 10%, 20.9% and 30%

oxygen levels for the susceptible and resistant populations were 0.080 and 0.067 mg/insect ($P \leq 0.005$), 0.084 and 0.067 mg/insect ($P \leq 0.006$), 0.086 and 0.068 mg/insect ($P \leq 0.001$), and 0.084 and 0.069 mg/insect ($P \leq 0.0014$), respectively. While, the production of carbon dioxide after 24 h at 5%, 10%, 20.9% and 30% oxygen levels for the susceptible and resistant populations were 0.125 and 0.101 ($P \leq 0.023$), 0.118 and 0.103 ($P \leq 0.033$), 0.121 and 0.101 ($P \leq 0.046$), and 0.125 and 0.106 mg/insect ($P \leq 0.027$), respectively.

The hourly change of carbon dioxide production (Table 2.1) showed an increase (from 2.547 to 2.865 µL/insect/h) for the susceptible strain at a low oxygen level (5%) when the insects were exposed to this oxygen level for an extended period. While at 10%, 21% and 30% oxygen levels, there was a decreasing trend in respiration after being exposed for a more extended period. On the other hand, the hourly respiration change pattern of the resistant strain was increasing except at the 40% oxygen level.

Table 2.1. Hourly changes of carbon dioxide production µL/insect/h under different oxygen levels

Oxygen level	Susceptible		Resistant	
	from 8-16h	from16-24	from 8-16h	from 16-24h
5	2.547	2.865	2.038	2.165
10	2.547	2.165	1.910	2.292
20.9	2.993	2.229	1.847	2.101
30	2.865	2.611	1.910	2.356
40	2.292	2.356	2.356	2.165

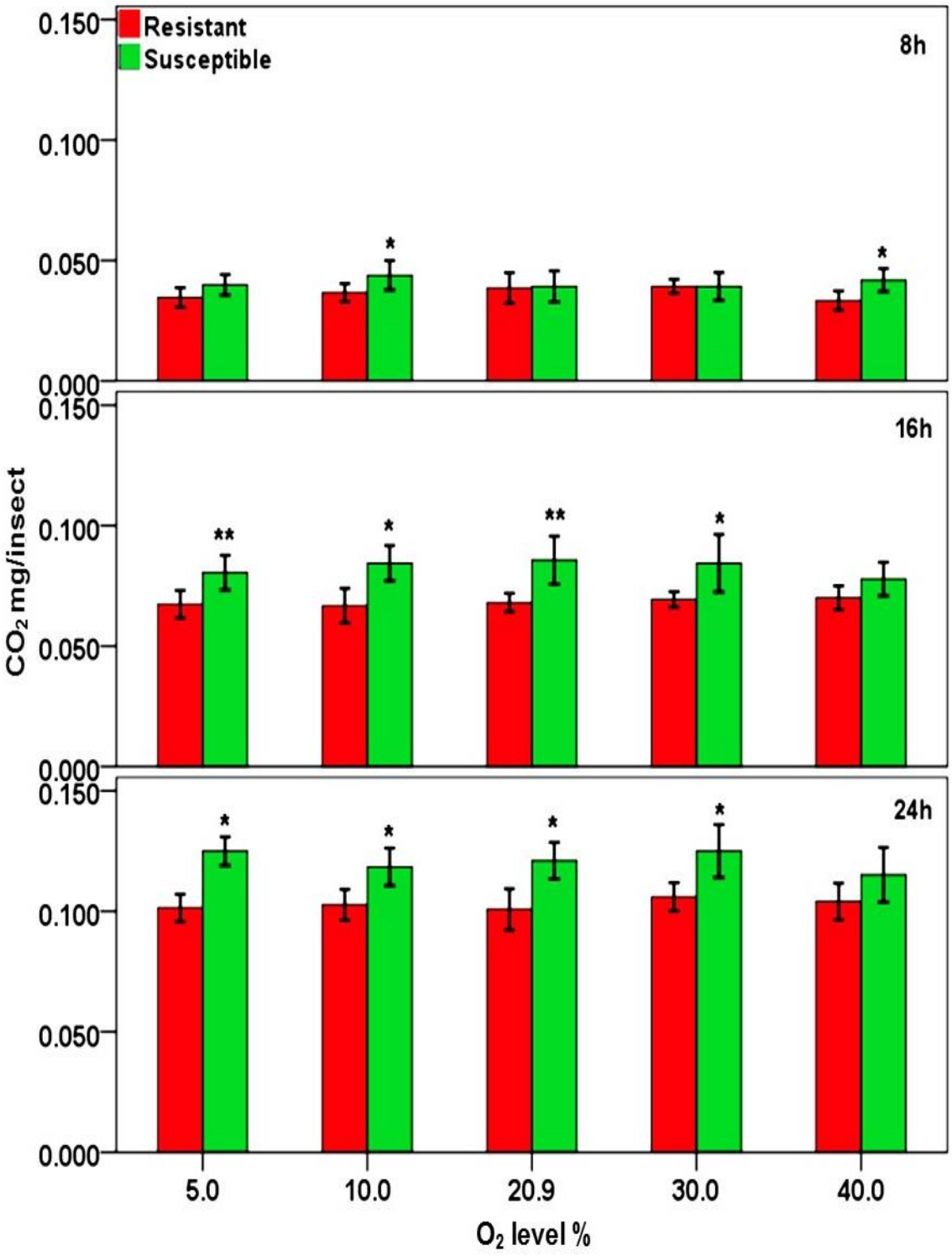

Figure 2.1. Respiration rate of *T. castaneum* under different oxygen level. Values represent the mean of CO_2 production; error bars are confidence intervals of 95% (n=15). *$p \leq 0.05$ and **$p \leq 0.005$ (T-test) are significant differences between susceptible and resistant strains at the same oxygen level.

The results of the effect of different phosphine concentrations on the respiration (CO_2 production) of the susceptible and resistant populations after 8, 16 and 24 hours exposure periods are shown in Figures. 2.2 to 2.4. Mostly, the respiration rate of the susceptible strain was higher than the resistant strain in all the exposure periods. At all exposure period, significant differences were recorded between the susceptible and resistant strains. Such as at combinations of 5% oxygen and 5 ppm phosphine of 8 hours exposure period (Figure 2.2),10% oxygen and 10 ppm phosphine of 16 hours exposure period (Figure 2.3) and 5% oxygen and 10 ppm phosphine of 24 hours exposure period (Figure 2.4).

The effect of different concentrations of phosphine on the CO_2 production was varied (Figures. 2.2 to 2.4), but there was mostly a reduction in the respiration of both the susceptible and resistant strains particularly at the higher phosphine concentrations (10 and 20 ppm). For example, at 8 hours exposure period in Figure 2.2, at 20.9% oxygen level, a significant reduction effect of phosphine on the respiration of the resistant and susceptible strains was recorded at 10 ppm phosphine in comparison with untreated control (without phosphine treatment). At 16 h exposure and 10% oxygen, high reduction of CO_2 production was recorded from resistant strain at 10-ppm phosphine in comparison with control (Figure 2.3). Likewise, the CO_2 production by resistant strain was reduced significantly after exposure to a combination of 20.9% oxygen and 10-ppm phosphine for 24 hours in comparison with 20.9% oxygen and control (Figure 2.4). A similar response pattern by susceptible strain at 16 and 24 hours exposure was also observed despite the high mortality of susceptible insects was obtained at 5, 10 and 20 ppm, respectively (Table 2.2).

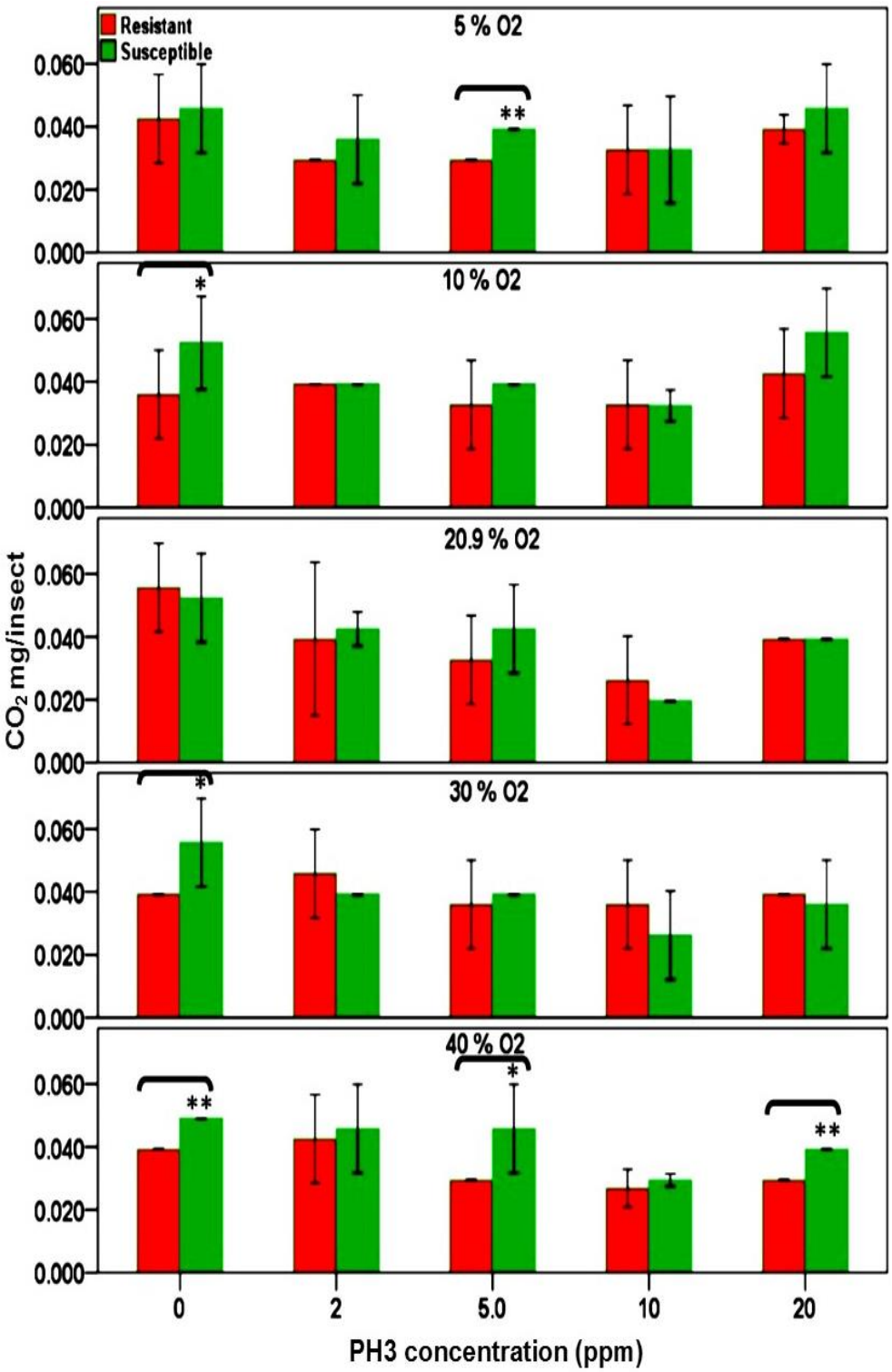

Figure 2.2. Respiration rates of *T. castaneum* after eight hours of exposure under different combination of oxygen and phosphine concentrations, Values represent the mean CO_2 production; error bars are confidence intervals of 95% (n=3). *$p \leq 0.05$ and **$p \leq 0.005$ (T-test) are significant differences between susceptible and resistant strains at the same PH_3 level.

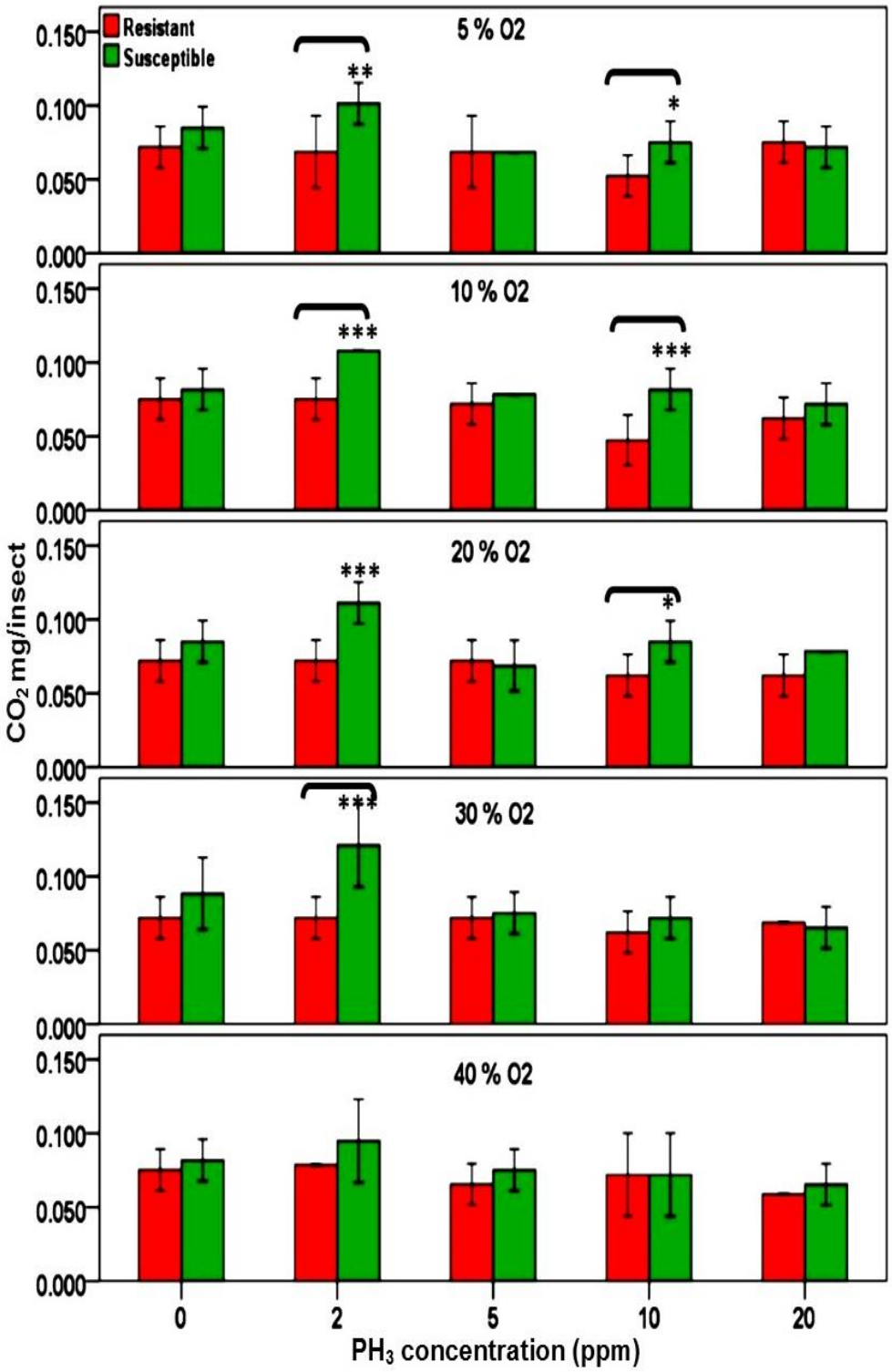

Figure 2.3. Respiration rates of *T. castaneum* after sixteen hours of exposure under different combination of oxygen and phosphine concentrations, Values represent the mean CO_2 production; error bars are confidence intervals of 95% (n=3). *$p \leq 0.05$, **$p \leq 0.005$ and ***$p \leq 0.0005$ (T-test) are significant differences between susceptible and resistant strains at the same PH_3 level.

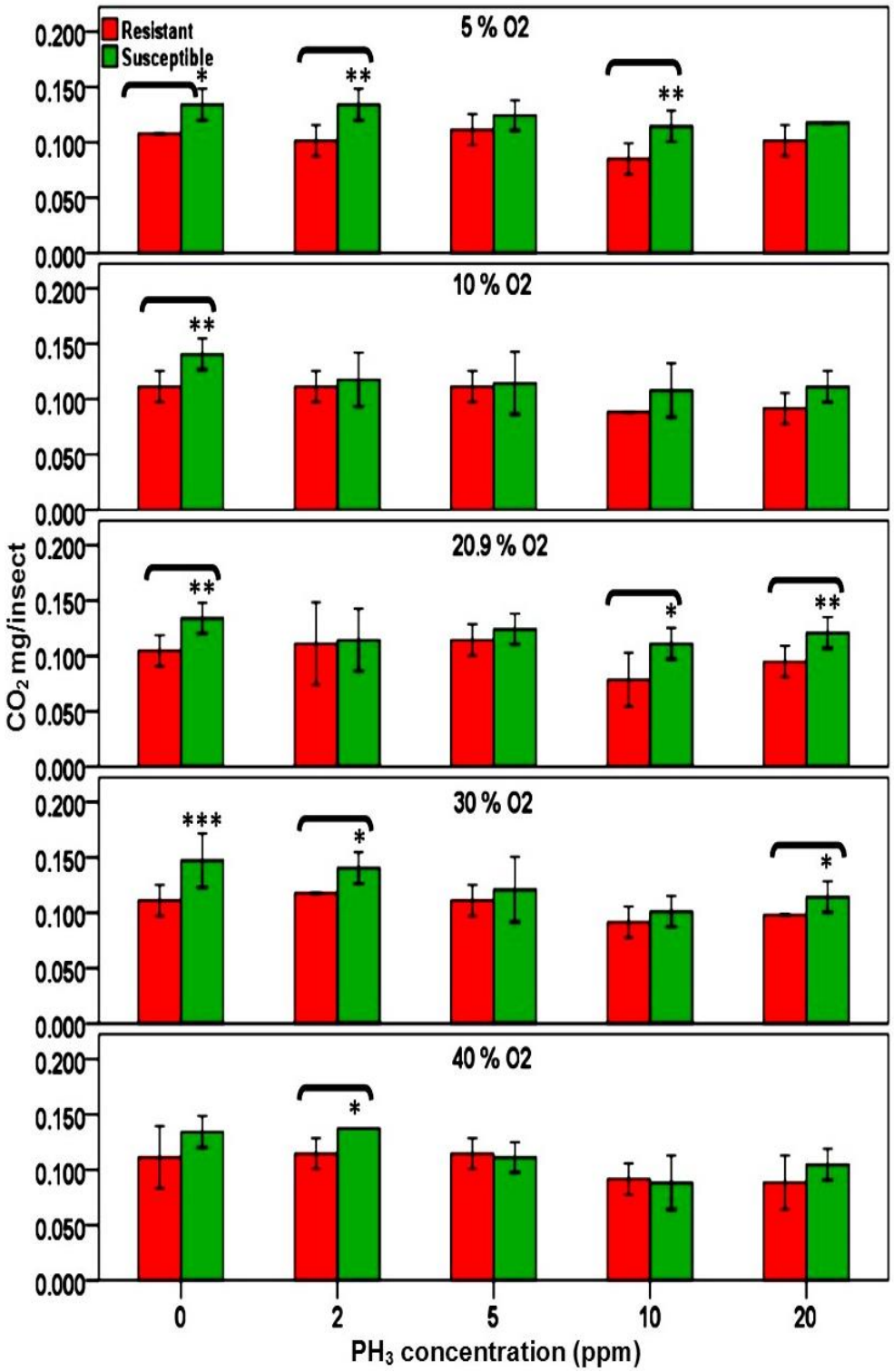

Figure 2.4. Respiration rates of *T. castaneum* after twenty-four hours of exposure under different combinations of oxygen and phosphine concentrations, Values represent the mean CO_2 production; error bars are confidence interval of 95% (n=3).*$p \leq 0.05$, **$p \leq 0.005$ (T-test) are significant differences between susceptible and resistant strains at the same PH3 level.

The results of the response of resistant insects to higher concentrations of phosphine are compared in Figure 2.5. A similar respiration pattern was observed in the three exposure periods. The respiration rate unexpectedly increased at the higher phosphine concentrations, specifically at 160 and 320 ppm. The highest increase of respiration in 8 and 16 h were recorded at 320 ppm. For example at 8 hours exposure and 20.9% oxygen, CO_2 production was raised significantly from 0.036 to 0.056 mg/insect after exposure to 320 ppm phosphine in comparison with 40-ppm phosphine. Similarly, at 16 hours exposure and 20.9% oxygen, the CO_2 production was increased by resistant strain significantly from 0.059 to 0.092 mg/insect after exposure to 320 ppm in comparison with 40-ppm of phosphine. At the 24 h exposure, the highest respiration rate mostly recorded at 320 ppm, such as at 20.9% oxygen and 320 ppm phosphine (0.137 mg/insect) in comparison with 20.9% oxygen and 40 ppm phosphine (0.085) (Figure 2.5), despite the high mortality of the insects recorded at this concentration (Table 2.2).

These unanticipated results led to the implementation of an additional experiment. The resistant strain had been exposed to a high level of phosphine of 292 ppm for 20 hours. The long term effect of this high concentration of phosphine on the respiration of surviving insects was investigated. The results (Figure 2.6) show the CO_2 production and O2 consumption of the resistant strain exposed to the high concentration of phosphine. Carbon dioxide production and oxygen consumption of the surviving insects were significantly higher than the respiration of untreated insects and that pattern remained constant until 30 days after fumigation (no further period was tested).

Table 2.2. Mortality (%) of the adults of *T. castaneum* during the experiment conduction.

PH_3 ppm	Exposure Time (h)																													
	8										16										24									
	Susceptible					Resistant					Susceptible					Resistant					Susceptible					Resistant				
	O_2%					O_2%					O_2%					O_2%					O_2%					O_2%				
	5	10	20.9	30	40	5	10	20.9	30	40	5	10	20.9	30	40	5	10	20.9	30	40	5	10	20.9	30	40	5	10	20.9	30	40
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	72	64	44	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	82	78	69	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	95	95	97	90	90	0	0	0	0	0	90	90	100	98	98	0	0	0	0	0
40	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0
80	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0
160	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	0	0	0	0	0
320	-	-	-	-	-	0	0	0	0	0	-	-	-	-	-	72	72	70	67	70	-	-	-	-	-	99	96	94	99	96

The carbon dioxide production of resistant insects treated with 292 ppm of phosphine were 0.133, 0.141, 0.144, 0.142, 0.155, 0.146, 0.155 and 0.152 mg/insect, and for the resistant untreated insects 0.110, 0.100, 0.101, 0.101, 0.110, 0.105, 0,121 and 0.115 mg/insect after 1, 3, 5, 7, 10, 15, 21 and 30 days, respectively. While the oxygen consumption of resistant insects treated with 292 ppm of phosphine were 0.106, 0.119, 0.118, 0.117, 0.128, 0.121, 0.121 and 0.126 mg/insect and for the resistant untreated insects 0.065, 0.079, 0.076, 0.079, 0.081, 0.086, 0.083 and 0.096 mg/insect after 1, 3, 5, 7, 10, 15, 21 and 30 days, respectively.

The result in Figure 2.7 shows the VOCs from the resistant strain after ten days of surviving from the 292 ppm concentration of phosphine in comparison with the resistant insects without phosphine treatment. There were no changes regarding the number of the peaks that were obtained from both the treated and untreated insects. Nevertheless, the GC response of the peaks that represent VOCs obtained from the treated resistant insects was in abundance compared to untreated insects (Figure 2.7).

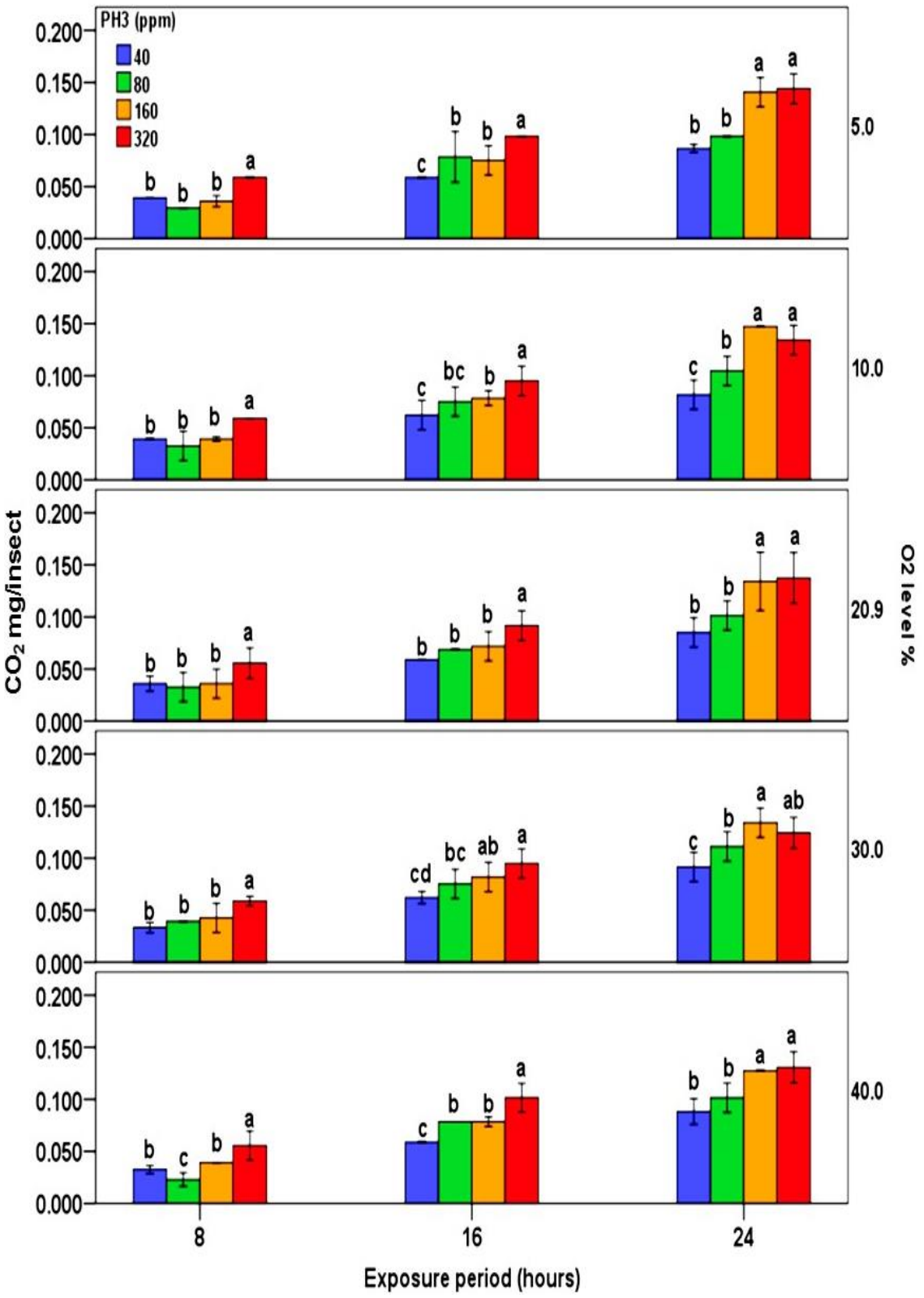

Figure 2.5. Effects of high phosphine concentrations on the respiration rate (CO_2 production) of a resistant strain of *T. castaneum*, each value represents the mean of CO_2 production, error bars are confidence intervals (n=3), different letters are significant differences in the same exposure period (Tukey HSD).

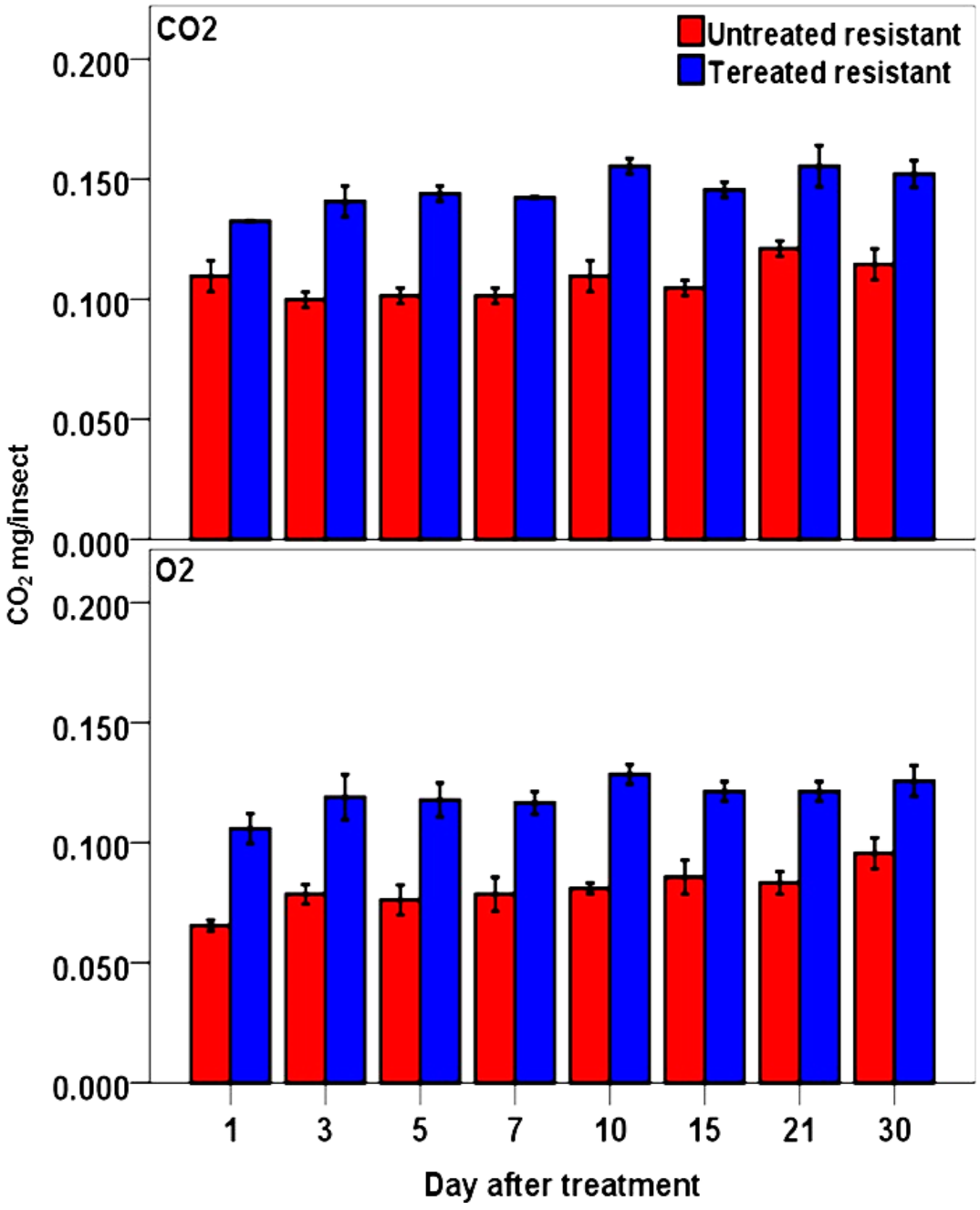

Figure 2.6. Comparison of respiration (CO_2 production and O_2 consumption) rate between non-treated resistant beetles and resistant insects survived from 20h fumigation of 292 ppm phosphine after different days of fumigation; error bars are confidence intervals 95% (n=3).

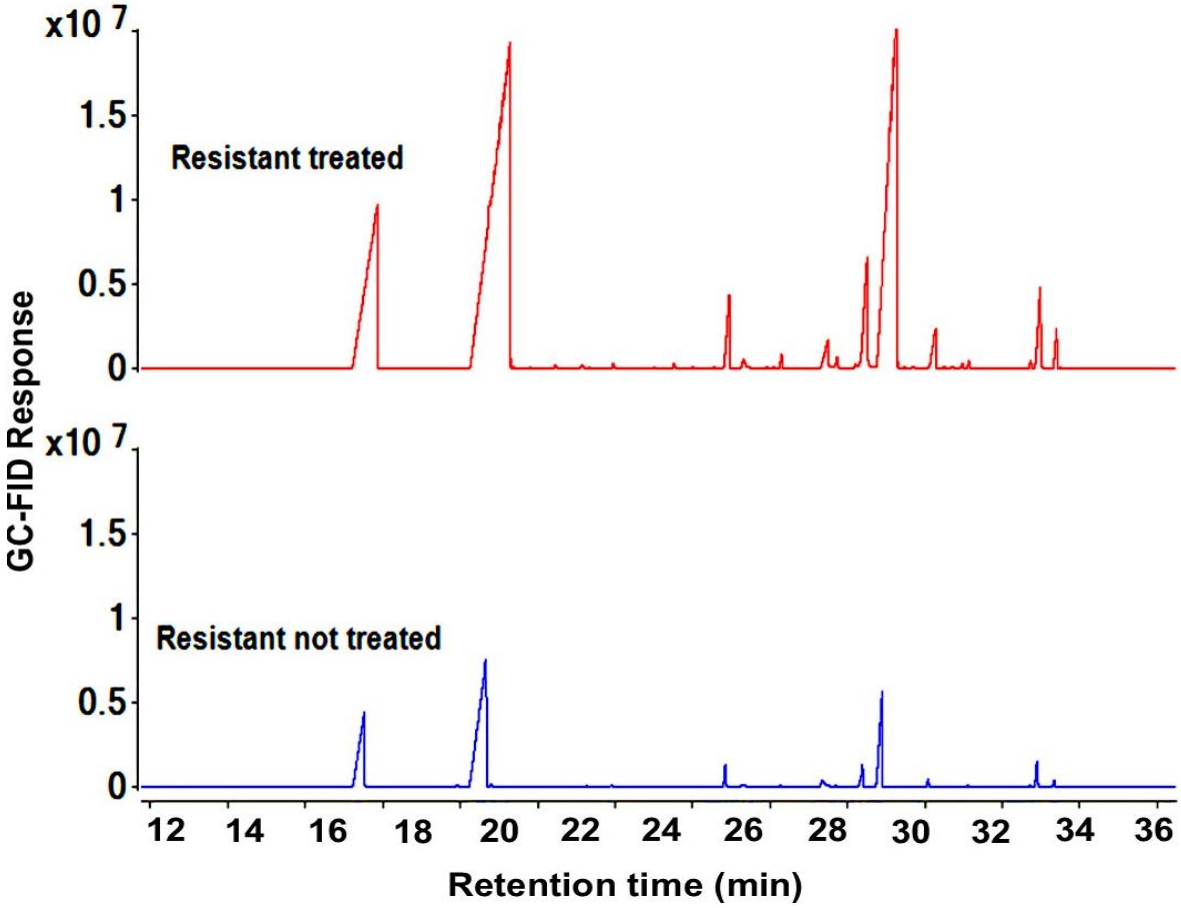

Figure 2.7. GC-FID chromatograms compare the metabolic changes (using volatile organic compounds as an indicator between resistant beetles treated with 0 phosphine and resistant insects survived from 20h fumigation of 292 ppm phosphine after 10 days of fumigation.

2.4 Discussion

The susceptibility of *T. castaneum* obtained from the populations which were selected as the susceptible insects was very close to the average that was observed by FAO (1975) standard method (FAO LC_{50} 0.008 mg/L for 20 hours exposure). These resistance ratios were in a similar range that was reported *T. castaneum*. (Jagadeesan et al, 2012).

No effect on the carbon dioxide production at different oxygen concentrations in both susceptible and resistant *T. castaneum* was observed. The results differ from previous studies that confirmed the effect of reduced oxygen levels that produced a higher respiration rate in *T. castaneum* than normal air for adults (Emekci et al., 2002; Emekci et al., 2001). However, the hourly change of carbon dioxide production for the susceptible and resistant strains indicated

that there is an increase in the rate of the respiration when the insects were exposed to a low oxygen level (5%) for an extended period.

The differences in the hourly rate between the susceptible and resistant strains might suggest different strains of different phosphine resistance level respond differently to oxygen, and this may explain why the hourly respiration change pattern of the resistant strain at 10, 20.9 and 30% oxygen was increased. On the other hand, the hourly change of carbon dioxide production for the susceptible strain at the same oxygen levels were opposite to the resistant strain respiration trend.

The result of the effect of phosphine on the respiration rate in this study proved that phosphine had slightly affected both the susceptible and resistant strains. This is therefore consistent with previous studies that confirmed the negative role of phosphine on respiration by reducing the respiration of the insects such as *R. dominica* (Price, 1980) and nematodes such as *Caenorhabditis elegans* (Zuryn, 2008).

The resistant population in this study produced less carbon dioxide than the susceptible insects, proving that there is a difference in metabolism between the two strains used in this study. These results agree with previous studies that confirmed the low respiration rate of the resistant populations compared to the susceptible population (Pimentel et al., 2007; Pimentel et al., 2008; Zuryn et al., 2008). In addition, the results support a previous suggestion that the resistance toward phosphine correlates with the low uptake of this fumigant by resistant populations (Chaudhry & Price, 1990; Price, 1984; Price, 1981).

The prevailing belief that the low rate of respiration of resistant populations in comparison with the susceptible populations might not suitably explain the resistance in all the organism species and strains. That comes from the fact that the mortality of resistant insects that absorbed an equal or much higher amount of phosphine is usually much lower than the mortality of susceptible insects that uptake much lower amount of phosphine (Chaudhry & Price, 1990). The results that were obtained in this study indicated that resistant insects produced more carbon dioxide when they are treated with a higher level of phosphine might support the thought that lower respiration rate by resistant insects is not enough by itself to explain the resistance phenomenon. That because the high rate of respiration in the resistant strain that was obtained after the exposure to the high concentration of phosphine means higher uptake of this fumigant, which disagrees with the concept that low respiration is equal to high resistance. Moreover, the low rate of respiration of the resistant insects that were used in this study may

be considered only as a consequence of resistance. That is similar to what was previously confirmed by studies about the fitness consequences of resistance to phosphine (Pimentel et al., 2007; Pimentel et al., 2012). In resistant insects, it was reported that there is a cost correlated with resistance status in the presence or absence of phosphine, such as a reduction in insect walking activity and a lower reproduction rate (Pimentel et al., 2007; Pimentel et al., 2012). This reduction of bioactivities of resistant insects might decrease energy demand compared to susceptible insects. Moreover, phosphine interrupts the metabolism of energy through its effect on mitochondrial respiration (Nath et al., 2011), which shows that energy deficiency is one of the reasons for death due to exposure to phosphine (Nath et al., 2011; Price, 1980b). Therefore, the increase of the respiration rate of resistant strains when they were treated with a high level of phosphine might be to compensate for the decreasing of energy caused by phosphine. This might, in turn, explains the higher VOCs produced by the resistant strain survived from a high level of phosphine.

2.5 Conclusion

To conclude, two strains of *T. castaneum* were tested to determine the bio-response to different levels of oxygen and phosphine. The respiration rate of the susceptible strain was higher than the respiration rate of the resistant strain. Oxygen level did not affect the respiration rate of both strains. There was a reduction in the respiration of both strains in low levels of phosphine, though; we observed an increase in the respiration of resistant insects after treatment with a high concentration of phosphine. We also compared metabolic variations after treatment with phosphine using VOCs as indicators. There were no changes with regard to the number of VOCs that were emitted. However, the amount of VOCs that was obtained from resistant insects treated with a high level of phosphine was much higher in comparison with untreated insects. Additionally, this study serves as a prelude for future experimental studies relating to the understanding of the respiration rate of phosphine-resistant and -susceptible strains of *T. castaneum*.

Statement of Contribution

Title of Paper	A novel Method for Analysis of Volatile Organic Compounds (VOCs *Tribolium castaneum* (H.) Using Headspace-SPME Technology
Publication Status	(•) Published ( ) Accepted for Publication ( ) Submitted for Publication ( ) Unpublished and Unsubmitted work written in a manuscript style
Publication Details	Published Current Analytical Chemistry, DOI: 10.2174/1573411015666190117125920

Principal Author

Name of Principal Author (Candidate)	Ihab alnajim
Contribution to the Paper	Concept, laboratory works, data analysis and writing manuscript
Overall percentage (%)	65%
Signature	[redacted] Date: 25/ 7/ 2019

Co-Author Contributions

By signing the Statement of Contribution, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all the co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	Tao Liu
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	YongLin Ren
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	15%
Signature	[redacted] Date: 26/ 7/ 2019

Please cut and paste additional co-author panels here as required.

Chapter 3

A novel Method for Analysis of Volatile Organic Compounds (VOCs) from *Tribolium castaneum* (H.) Using Headspace-SPME Technology

Abstract

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is one of the world's most serious stored grain insect pests. A method of early and rapid identification of red flour beetle in stored products is urgently required to improve control options. Specific chemical signals identified as volatile organic compounds (VOCs) that are released by the beetle can serve as biomarkers.

The headspace solid phase microextraction (HS-SPME) technique and the analytical conditions with GC and GC-MS were optimised and validated for determination of VOCs released from *T. castaneum*.

The 50/30 µm DVB/CAR/PDMS SPME fibre was selected for extraction of VOCs from *T. castaneum*. The efficiency of extraction of VOCs was significantly affected by the extraction time, temperature, insect density and type of SPME fibre. Twenty-three VOCs were extracted from insects in 4 mL flask at 35±1°C for four hours of extraction and separated and identificated with gas chromatography-mass spectroscopy. The major VOCs or chemical signals from *T. castaneum* were 1-pentadecene, p-Benzoquinone, 2-methyl- and p-Benzoquinone, 2-ethyl.

This study showed that HS-SPME GC-MS technology is a robust and cost-effective method for extraction and identification of the unique VOCs produced by *T. castaneum*. Therefore, this technology could lead to a new approach in the timely detection of *T. castaneum* and its subsequent treatment.

3.1 Introduction

Damage caused by insects decreases quantity and quality of grain by consuming, contaminating and producing the ideal conditions for growing the microorganism in grain (Neethirajan et al., 2007). The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is a vital pest infecting a broad range of stored products including grains (Arbogast, 1991; Lorini & Ferreira Filho, 2004). The economic importance of this species derives from damaging and reducing the grain quality by contaminating the products by insect parts, ecdysis skins and individuals of each life stages in addition to off-odour (Hameed et al., 2012; Trematerra et al., 2000). These insects can be very difficult to detect in large volumes of stored grains, especially when infestation rates are low. Many detection techniques used in stored products are not practical because of their high cost and low sensitivity (Neethirajan et al., 2007).

Volatile organic compounds (VOCs) are carbon compounds that evaporate at room temperature (Bennett & Inamdar, 2015). In an organism, they are released products of metabolic activities or tissue damage, and the profile of VOCs may be unique to each species. Hence, analysing VOCs released into the airspace surrounding stored products is a potential method of diagnosis and species identification (Laopongsit et al., 2014; Laothawornkitkul et al., 2010; Niu et al., 2015; Qiu et al., 2014b). A range of commercial solid phase micro-extraction (SPME) fibres were used to extract VOCs from the headspace (Balasubramanian & Panigrahi, 2011). The VOCs were analysed and identified using GC and GC-MS methods (Laopongsit et al., 2014). However, this method requires further optimisation to enhance the efficacy of the VOCs extraction (Niu et al., 2012).

This study aimed to optimise the parameters for detection of *T. castaneum* specific VOCs in the headspace of stored grains using the headspace SPME method, and various conditions like insect density, temperature, age, type of SPME fibre and time of equilibrium extraction were optimised to get a maximum number of VOC's to enhance the sensitivity of the detection.

3.2 Materials and Methods

3.2.1 Insects

Adult *T. castaneum* were obtained from the Department of Primary Industries and Regional Development (DPIRD), Australia. Insects of similar age were produced by incubating 3000 adult insects of *T. castaneum* with 500 g of wheat flour and yeast in a 12:1 ratio in 2000 mL jars sealed with meshed lids. Parent's insects were removed after four days. Cultures were incubated at 27±2 and 70% RH. As new insects emerged, the cultures were sieved and all adult insects removed and transferred to a new container with food to get adults of similar age. The insects used in the experiments were one month old. The flour was made from freshly harvested wheat (Australian Standard Wheat 1). Before using the wheat was sterilised by storing in a 60 L container at -20°C for seven days and then storing at 4°C until further use. The grain was milled using a Wonder Mill (Model WM2000, Korea), and the flour was also kept at 4°C until further use.

3.2.2 Analysis and identification of VOCs

3.2.2.1 Glassware, reagents and SPME fibres

A screw top 4 mL amber vial coupled with a cap equipped with septa (SUPELCO, USA) was used as a chamber for collection of VOCs released from insects. All chemical reagents used

were analytical grade and supplied by Sigma-Aldrich (Castle Hill, NSW, Australia). The 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) and 85 µm polyacrylate SPME fibres (SUPELCO, Bellefonte, PA, USA USA) were used according to manufacturer's recommendations.

3.2.2.2 Gas chromatography-flame ionisation detector (GC-FID)

Desorption and separation of extracted VOCs were achieved with gas chromatography-flame ionisation detector (GC-FID); Hewlett Packard 5890 (series II USA) coupled with mid-polarity phase column (RESTIK Rxi-5ms 30 m x 0.25 mm x 0.25 µm) (Cat no. 13423; serial no. 978690). Hydrogen was used as a carrier gas at a constant flow of 40 mL/min. The temperature of the GC inlet (split-less mode) and the detector were 250 and 290°C respectively. The oven temperature was programmed at 40°C for 5 min increased at a rate of 5°C/min to 250°C and held for 5 min with a total running time of 52 min. The oven temperature program was designed to volatilize most of the VOCs released by the insects in addition to providing good separation among the compounds. High inlet temperature was used to ensure that all the VOCs were desorbed from the SPME fibre (Niu et al., 2012).

3.2.2.3 Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry (GC-MS) was used for identification of the VOCs released by the insects. The instrument used was an Agilent technologies 7820A (serial# CN 14272038, USA) equipped with an Agilent 5977E Mass Spectrometry Detector (MSD) (serial# US1425R204, USA) fitted with an Agilent J&W mid-polarity column (HP-35 ms; 30 m x 0.25 mm x 0.25 µm). The GC inlet (split-less mode) operated under an SPME splitless mode injector. Helium was used as a carrier gas at a constant airflow of 1.1 mL/min. The oven temperature was programmed at 50°C for 5 min increased at 5°C/min to 250°C with a total operating time of 45 min. while the inlet and detector temperature were kept at (250 and 290°C respectively).

3.2.2.4 Optimization and validation studies

This study was conducted to determine the effect of some parameters on the extraction of VOCs from the red flour beetle *T. castaneum*. The extraction procedure involved cleaning the SPME fibre by heating it at 270°C for 15 min. The optimisation was started by evolution two commercial fibre types different in polarity coating of 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) and 85 µm polyacrylate (PA) fibres. The aim of comparing the performance of 85 µm polyacrylate fibre with three-

phase fibre was to determine if there are specific compounds that can be extracted only by 85 µm polyacrylate, as it has only one polar phase. The experiment was implemented using 10 adult insects for 2 h extraction period at 27±1°C and 70% RH with three biological replicates.

The screw-top 4 mL amber vials containing 1, 5, 10, 20 and 30 individual insects respectively and sealed with cap equipped with septa for sampling were used to evaluate the effect of the insect density on extraction of VOCs. The extraction was conducted at 27±1°C and 70% RH for a 2 h.

For Optimization of VOCs extraction, the clean fibre was inserted into the headspace of the vial containing the insects, combinations of two extraction temperatures of 27±1°C and 35±1°C at 70% RH for four extraction times (2, 4, 6 and 8 h) were evaluated. After the extraction had finished, the SPME fibre was withdrawn and inserted directly into the GC inlet for 5 min to desorb the collected VOCs at 250°C.

The VOCs identification was implemented using GC-MS by applying the optimal conditions that were obtained from the Optimization studies. Each of the above test was replicated three times.

3.2.3 Evaluation of the limit of detection of the analytical method

The limit of detection (LOD) was determined by analysing two external standards including toluene and acetophenone. Diluted standards were prepared with a range from 0 to 100 ng/mL by adding a specific amount to extraction vials. The standard samples were extracted and analysed with optimized procedures and conditions. Each test was replicated for three times.

3.2.4 Statistical analysis and compound identification

The area that represents each peak was extracted using MassHunter Quantitative Analysis software for GC (Agilent Technologies). The treatment groups were loaded to the software as one patch, and the same parameters were applied to the whole patch. Reports of a compound of peak areas were generated to Excel. Each peak area was given a metabolic identification indicates the GC flame ionisation detector and the retention time of each peak. The averages of compound areas were statistically analysed by Metaboanalyst 3.0 using Partial Least Squares - Discriminant Analysis (PLS-DA) (Xia & Wishart, 2016). The qualitative identification of VOCs was made by Automatic Mass Spectral Deconvolution and Identification System (AMDIS-32) software coupled with NIST 2.2 mass spectra library. Three criteria were taken into consideration when identifying compounds: similar mass spectra, high match factor and

the comparison of retention Kovat's index with retention index obtained from NIST (Table 3.S1).

3.3 Results and Discussion

3.3.1 Effect of SPME fibre type on the efficacy of extraction VOCs

Four factors were optimised and investigated, including SPME fibre type, extraction time, extraction temperature and sample size. The Figure 3.1 shows the different performance between two types of SPME fibres (50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) and 85 µm polyacrylate (PA)) on the extraction of volatile organic compounds (VOCs) from the adults of red flour beetle insects. The three-phase DVB/CAR/PDMS SPME fibre has extracted more broad VOCs than the PA SPME fibre. However, the PA SPME fibre could extract some compounds from *T. castaneum* like compounds at RT=14.45 and 25.27 min, but no new compounds different from the peak numbers and intensities compared to DVB/CAR/PDMS SPME that produced higher peak abundances for most of the VOCs chromatogram.

Therefore, the DVB/CAR/PDMS SPME fibre was selected as the most appropriate fibre to extract VOCs for evaluation of the other optimisation parameters. In this context, Jelen´ et al. (2000) reported that fibre coating is a vital factor in development appropriate SPME method, confirming that DVB/CAR/PDMS SPME is the best fibre for high efficient extraction VOCs and the further increasing the method sensitivity. Balasubramanian & Panigrahi (2011) indicated that using mid polarity fibre coating like DVB/CAR/PDMS is more efficient to extract a number of the volatiles. Similar results to our finding were obtained in a study by Qiu et al. (2014) who confirmed that the DVB/CAR/PDMS SPME fibre was the most efficient fibre among six commercial fibres including PA SPME fibre to extract VOCs from *Phytophthora cinnamomi*.

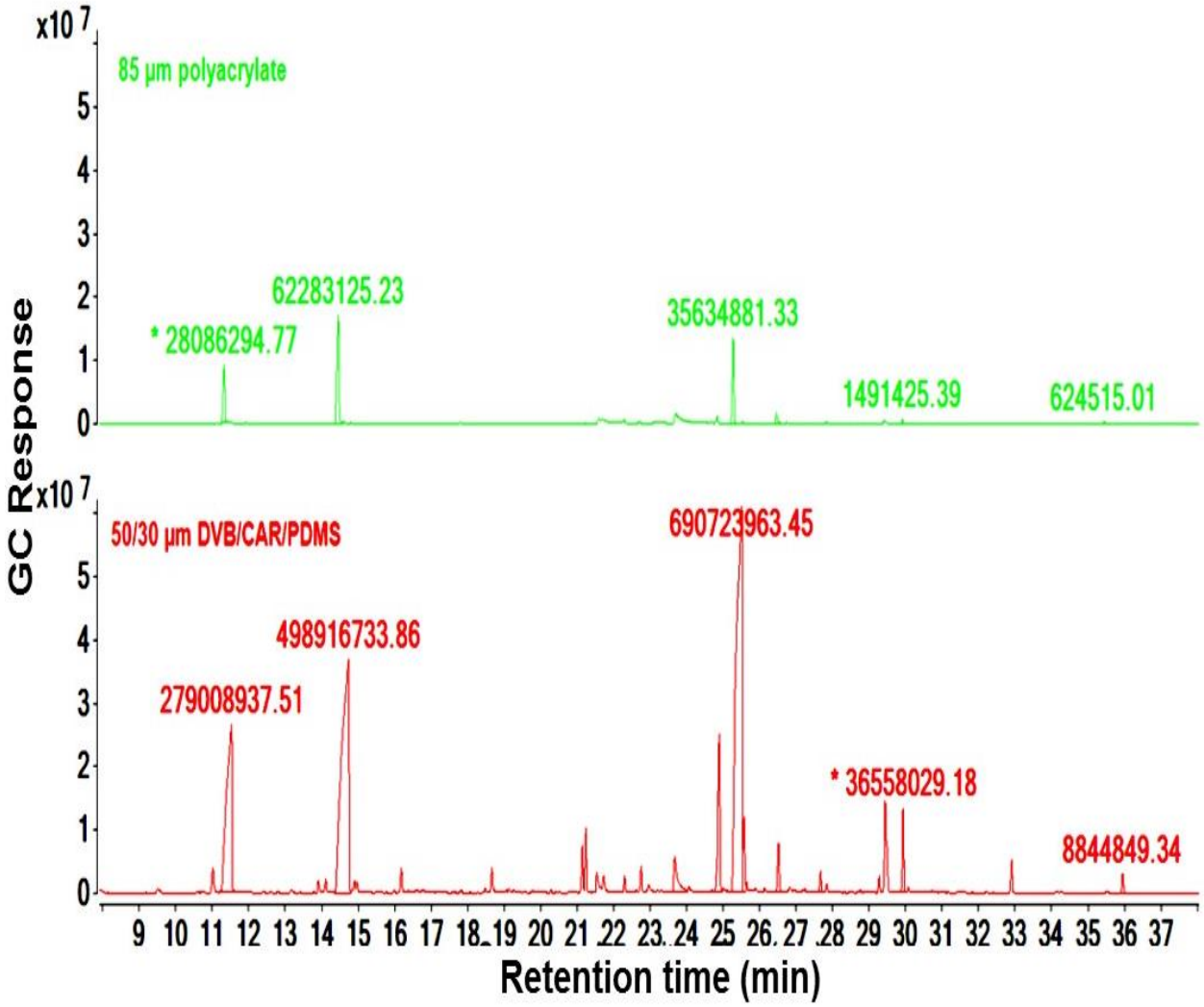

Figure 3.1. Comparison between two types coating SPME fibres in the total number of GC signal

The superior performance of DVB/CAR/PDMS SPME was also reported in many studies in comparison with other fibre coatings (Davoli et al., 2003; Garcia-Esteban et al., 2004; Gianelli et al., 2002; Jeleń et al., 2000; Marco et al., 2004; Tuduri et al., 2001). The polarity of the SPME strongly influences the extraction capacity, as well as the polarity of volatile compounds, also affects the SPME ability (Balasubramanian & Panigrahi, 2011). PA SPME was characterized for attracting polar semi-volatiles (Jeleń et al., 2000; Matisová et al., 1999; Sostaric et al., 2000), whereas DVB/CAR/PDMS fibre can absorb more extensive range of VOCs that have different physical and chemical properties (Jeleń et al., 2000; Risticevic et al., 2010). Since that the PA fibre was reported to have a little capacity to extract VOCs from a waste gas (Kleeberg et al., 2005).

3.3.2 Effect of insect densities on the efficacy of extraction VOCs

Once the DVB/CAR/PDMS was selected as the optimal fibre, different insect densities (1, 5, 10, 20 and 30) per vial were tested to investigate the optimal density of insects that should be used to improve the recovery of volatile organic compounds from *T. castaneum* adult insects. The average of the peak areas was analysed using Partial Least Squares – Discriminant Analysis (PLS-DA).

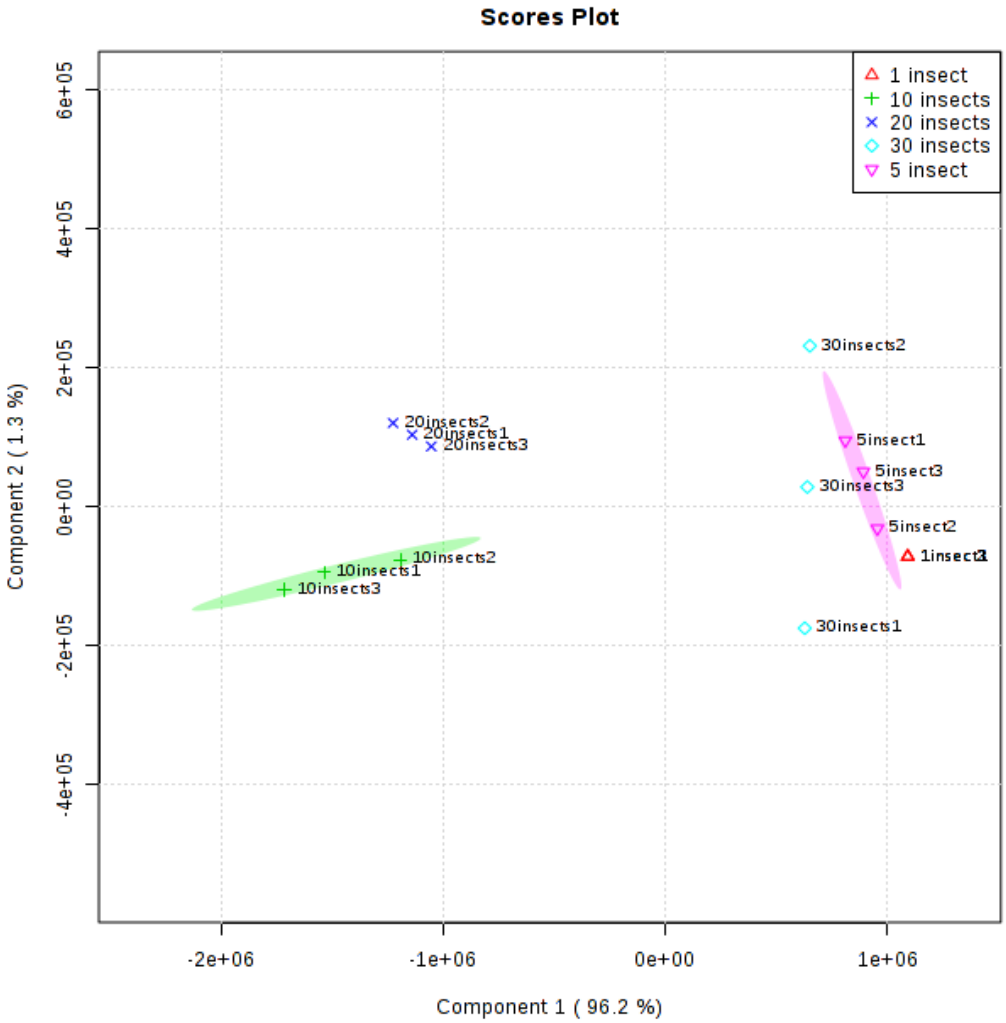

Figure 3.2. PLS-DA score plot shows the data obtained from *T. castaneum* in different insect densities based on VOCs with three biological replicates.

The result shows the scores plot between the selected PCs (Figure 3.2). The model showed a good separation among the tested treatment groups in this experiment, demonstrating the impact of the insect population density on the ability to emit the VOCs.

The density of 10 insects had the highest abundance of most metabolic products, including those which had high intensity in the GC-FID chromatogram like FID-25.01, 24.78, 20.74, 29.44 and 25.54 (Figure 3.3). In all cases, most of the VOCs increased steadily from one insect to 10 insects and then decreased from 20 and 30 insects.

This decreasing can be attributed to the overcrowding in the small vial (4 mL). The overcrowding might have caused a reduction in the metabolism of insects due to increase in the CO_2 quantity which has a critical effect on the biological and physiological processes of insects (Guerenstein & Hildebrand, 2008; Nicolas & Sillans, 1989). Jelen´ et al. (2000) indicated that the amount of sample strongly affects the amount of the extracted analyte. The higher concentration of VOCs extracted from ten insects proved that this number of insects is the optimal density to produce an abundant amount of VOCs that can be collected by SPME. Ten insects in 4 mL bottle (2.5 insects for 1 cm^3) were chosen as the optimum insect density to implement further optimization.

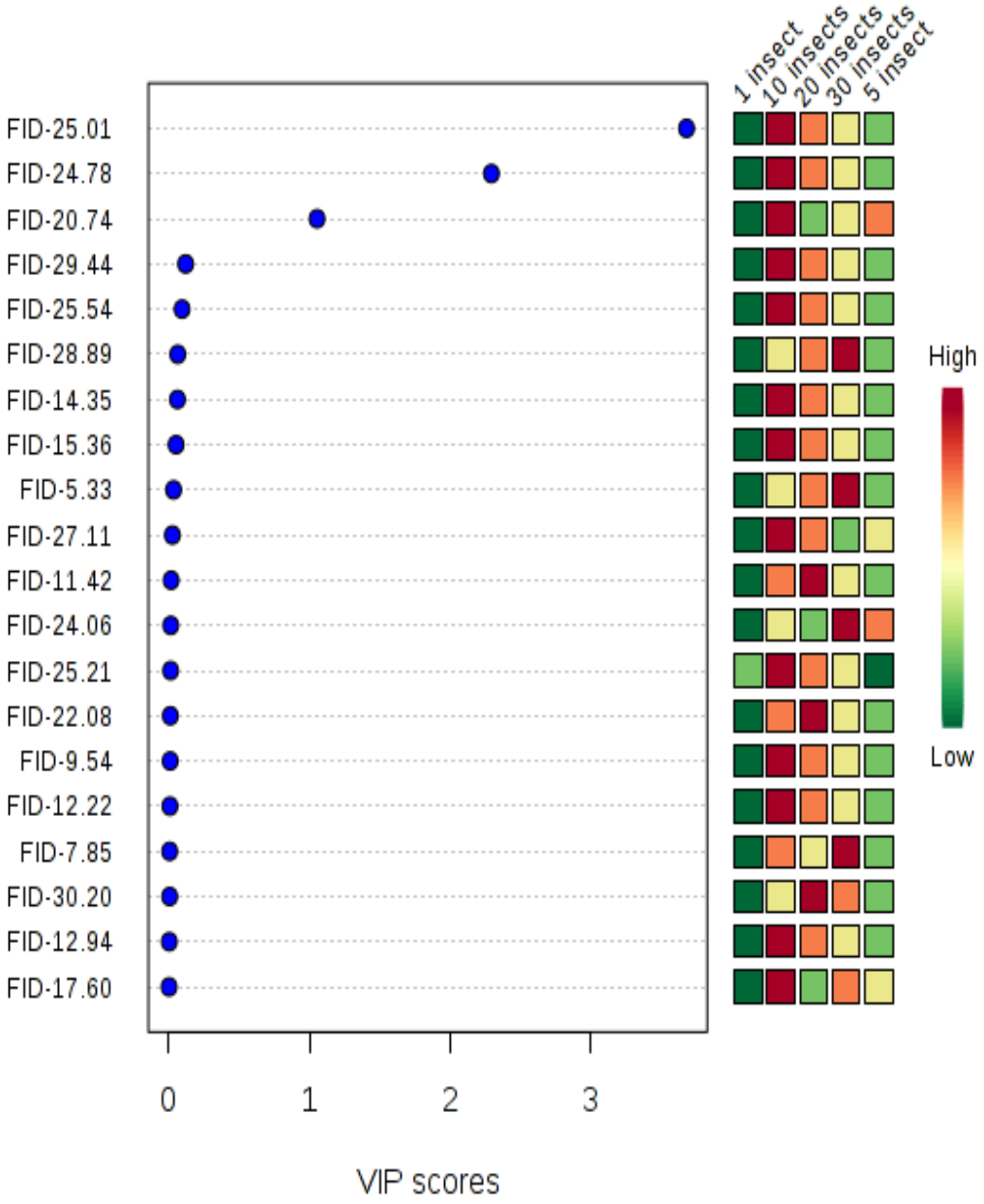

Figure 3.3. Variable Importance in Projection (VIP) shows important features identified by PLS-DA from data of different insect densities based on VOCs with three biological replicates. The greyest boxes on the right indicate the high relative abundance of the corresponding metabolites in each group under study. Codes in left side are the metabolic ID (FID indicates GC-FID detector and numbers indicate the retention times min).

3.3.3 Effect of fibre exposure period and temperature on the efficacy of extraction VOCs

Four extraction periods (2, 4, 6 and 8 h) were applied combined with two different extraction temperatures (27 and 35°C). The areas of the identical main peaks have been used to determine the optimal extraction period. The segregation of the results in the score plot model of the PLS-DA proves that extraction period and temperature have a significant effect on the VOCs emission from the adult of *T castaneum* (Figure 3.4). Variable Importance in Projection (VIP) scores (Figure 3.5) shows the importance of the metabolites that contributed in the PLS-DA model.

At 27°C, no change was observed in the number of compounds peaks over the four extraction periods. However, the intensities of chromatogram peaks increased significantly after increasing the extraction time from 2 to 4, 6 and 8 h (Figure 3.5). However, at 2 h, some of the early compounds had higher abundance than other extraction periods like a compound at FID-5.49 and FID-12.22. While at longer extraction time some of the later compounds like FID-27.19, FID-28.80 and FID-24.60 had higher peak abundance.

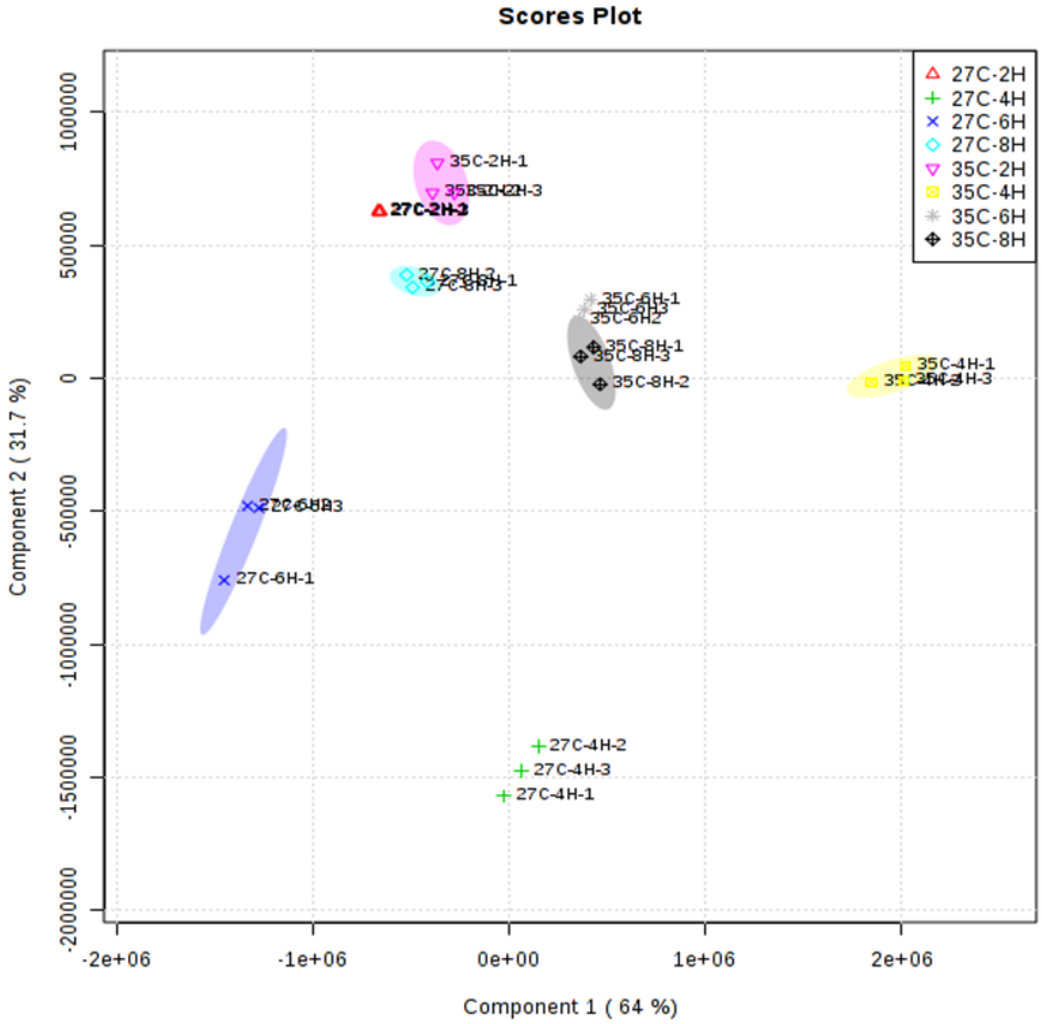

Figure 3.4. PLS-DA score plot shows the data obtained from *T. castaneum* in different extraction time and temperature based on VOCs with three biological replicates.

That is probably because at shorter extraction times the highly volatile compounds were absorbed faster in comparison with the intermediate and low volatility compounds that need more time to be collected. This explains the large area of the later peaks in longer extraction time (Laopongsit et al., 2014). In this regard, other studies focused on the importance of extraction time, finding it as a crucial factor in recovering VOCs from a range of sample types (Laopongsit et al., 2014; Niu et al., 2012).

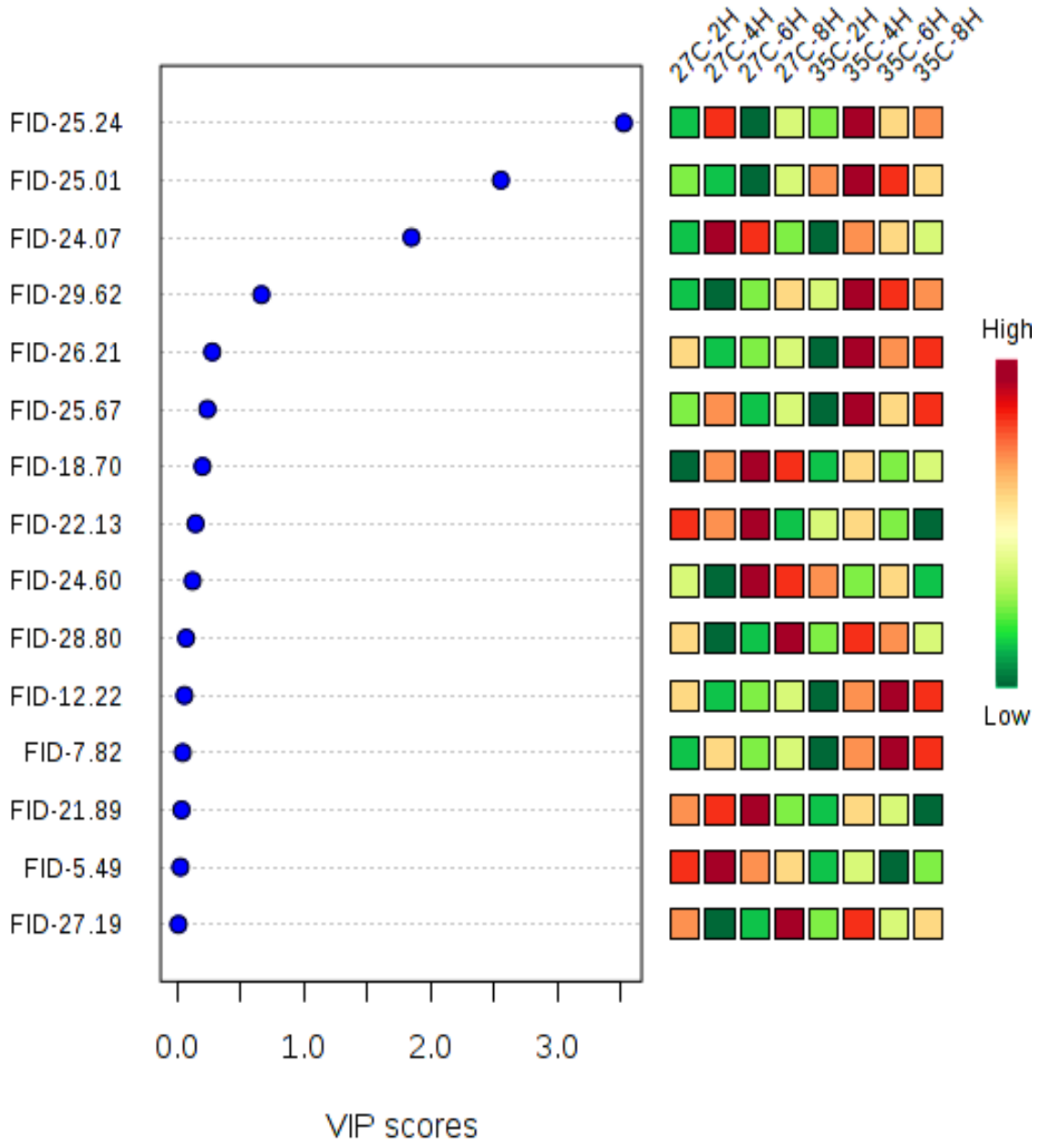

Figure 3.5. Variable Importance in Projection (VIP) shows important features identified by PLS-DA from data of different extraction time and temperature based on VOCs with three biological replicates. The greyest boxes on the right indicate the higher relative abundance of the corresponding metabolites in each group under study. Codes in left side are the metabolic ID (FID indicates GC-FID detector and numbers indicate the retention times min).

At 35°C, the extraction of the VOCs was mostly enhanced in comparison with 27°C. The abundant metabolic compounds (main compounds) in VIP score like FID-25.24, FID-25.01, FID-29.63, FID-26.21 and FID-25.67 were found to be higher at the 4 h extraction period than other the extraction periods (Figure 3.5). The decrease of these peak areas at 6 h and 8 h might

be because of accumulation of CO_2 in the extraction container, which would adversely affect role on the insects bioactivities (Guerenstein & Hildebrand, 2008; Nicolas & Sillans, 1989), although this was not measured. The results of the extraction time are consistent with a result from Niu et al. (2012) who confirmed that 4 hours are required to extract the VOCs from *T. castaneum*.

3.3.4 Validation study on *T. castaneum*

As a validation to the method developed in this study, VOCs compounds released from *T. castaneum* were extracted using a three-phase DVB/CAR/PDMS fibre at 35ºC for 4 h extraction of 10 insects in 4 mL bottle and identified using gas chromatograph mass spectrometry. The identification was according to specific criteria, including a high match factor, similar mass spectra and similar retention index; Twenty-three VOCs were identified from the beetles (Figure 3.6). The most abundant compound identified in this study was 1-Pentadecene (area= 34.99%). This compound was also reported as a common compound released by *Tribolium SPP*. in many previous studies (Arnaud et al., 2002; Keville & Kannowski, 1975; Niu et al., 2015; Villaverde et al., 2007). This pheromone can be secreted by both male and female and can stimulus both gender (Keville & Kannowski, 1975). However, it can also act as a spacing pheromone for *Tribolium spp*. and a defensive secretion against their enemies (Arnaud et al., 2002). Another compound that was detected in this study was 1,3-benzenediol, 4-ethyl-, this compound was also reported for the first time by (Niu et al., 2015).

Other main compounds identified were p-Benzoquinone, 2-methyl- (area= 14.8%); p-Benzoquinone, 2-ethyl- (area= 29.7%), consistent with previous studies that quinones are the major group of VOCs from *Tribolium spp* such as red flour beetle (Hodges et al., 1996; Unruh et al., 1998; Villaverde et al., 2007). In addition to a variety of traces of different chemicals some of them to our knowledge detected for the first time like E-9-Tetradecenol. Methyl-1, 4-benzoquinone and ethyl-1, 4-benzoquinone were detected in this study; these compounds were previously identified for their function as defensive secretions in *Tribolium spp* (Villaverde et al., 2007).

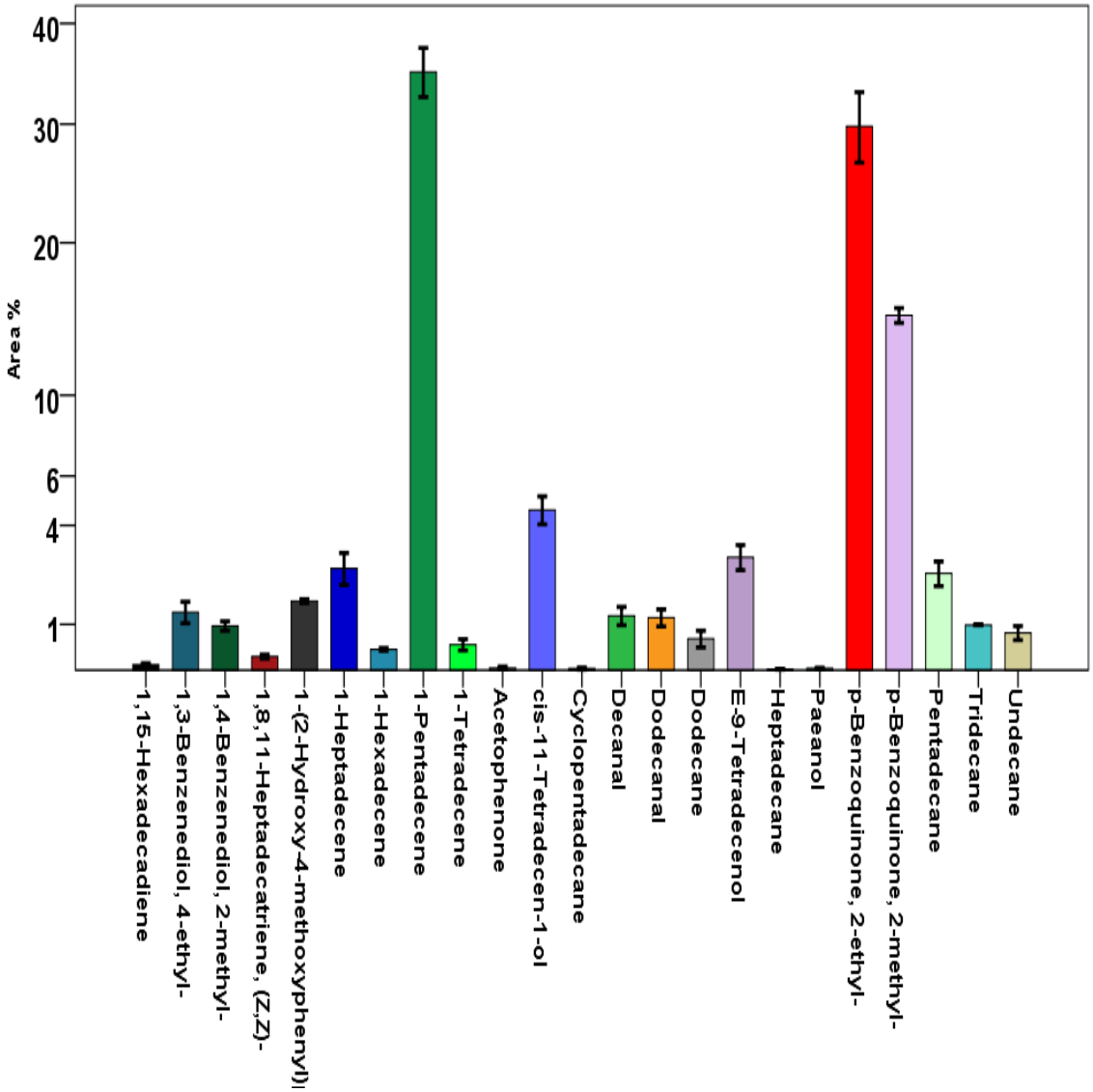

Figure 3.6. Area percentages of VOCs that were identified from *T. castaneum* using GC-MS.

3.3.5 The limit of detection and quantification

The LOD was used to determine the efficiency of extraction and analysis method along with method sensitivity. The low detection and quantification value to ng level (Table 3.1) obtained for the external standards after applying the optimal conditions of the extraction and analysis demonstrate high efficient and sensitive method which can be applied in early detection of infestation in sealed storage.

Table 3.1. Limits of detection (LOD) and limits of quatification (LOQ) obtained from analyse external standards using the optimised method.

Compounds	RT (min)	R^2	LOD (ng/mL)	LOQ (ng/mL)
Toluene	3.34	0.982	0.215	0.653
Acetophenone	13.18	0.970	0.347	1.051

RT = Retention time

R^2 = Regression coefficient

3.4 Conclusion

Optimal parameters to extract the VOCs from *T. castaneum* adult insects were use of 50/30 µm DVB/CAR/PDMS at 35±1°C with 10 insects in 4 mL vial for 4 h extraction. The method was validated on identification of many volatile organic compounds from the insects using GC-MS. The unique signature VOCs specific to *Tribolium* species (e.g. p-Benzoquinone, 2-methyl-; p-Benzoquinone, 2-ethyl- and 1-Pentadecene) which can further be explored to develop a sensitive method for early and timely detection of infestation or development of lures.

Supplimantary result

Table 3.S1. Volatile organic compounds detected from *T. castaneum* insects using headspace microextraction technique and gas chromatography mass spectrometry detector

RT	Volatile organic compounds	NRI[a]	Kovat's index [b]	MF[c] (%)
11.53	p-Benzoquinone, 2-methyl-	1018	1023.6	89
13.21	Acetophenone	1029	1033.1	85
14.28	Undecane	1100	1061.2	86
14.73	p-Benzoquinone, 2-ethyl-	1215	1116.7	86
16.18	Dodecane	1200	1150.8	88
18.63	Decanal	1204	1195.4	86
20.01	Tridecane	1300	1241.2	87
21.21	Dodecanal	1386	1330.9	75
21.60	1,4-Benzenediol, 2-methyl-	1225	1353.5	89
22.74	1-Tetradecene	1392	1387.8	76
23.67	1,3-Benzenediol, 4-ethyl-	1334	1434.1	89
24.04	Paeanol	1439	1448.1	88
24.84	cis-11-Tetradecen-1-ol	1664	1470.0	78
25.51	1-Pentadecene	1502	1503.9	75
25.57	Pentadecane	1512	1506.8	75
25.83	Cyclopentadecane	1536	1510.5	80
26.52	1-(2-Hydroxy-4-methoxyphenyl)propan-1-one	1538	1545.9	85
27.18	1,15-Hexadecadiene	1579	1568.9	77
27.66	1-Hexadecene	1592	1589.0	88
29.26	1,8,11-Heptadecatriene, (Z,Z)-	1665	1661.6	84
29.44	E-9-Tetradecenol	1668	1672.2	72
29.94	1-Heptadecene	1692	1692.7	87
30.19	Heptadecane	1700	1701.5	72

a. Retention indices obtained from NIST data base.

b. Retention indices obtained from running n-alkane external standards.

c. Match factors (%) obtained from NIST data base.

Statement of Contribution

Title of Paper	Investigation of metabolism differences of phosphine-resistant and volatile organic compounds (VOCs) as indicators
Publication Status	○ Published ○ Accepted for Publication ○ Submitted for Publication ◉ Unpublished and Unsubmitted work written in a manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Ihab alnajim
Contribution to the Paper	Concept, laboratory works, data analysis and writing manuscript
Overall percentage (%)	65%
Signature	[redacted] Date: 25/ 7/ 2019

Co-Author Contributions

By signing the Statement of Contribution, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all the co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Tao Liu
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	YongLin Ren
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	15%
Signature	[redacted] Date: 26/ 7/2019

Please cut and paste additional co-author panels here as required.

Chapter 4

Investigation of Metabolic Differences of Phosphine-Resistant and Susceptible Strains Using Volatile Organic Compounds (VOCs) as Indicators

Abstract

The resistance toward phosphine is an increasing issue around the world. Studies have indicated that this issue correlated with genetic changes which can create physiological changes in different processes such as respiration and the emmision of volatile organic compound (VOCs).

In this research, susceptible and resistant strains of *R. dominica* and *T. castaneum* were tested to determine the effect of resistance level on insect metabolism using volatile organic compounds (VOCs) as indicator. DVB/CAR/PDMS SPME fibre was utilised to collect the VOCs from the headspace and GC-MS to analyse the samples. The GC-MS response for most of the VOCs compound peaks that were detected from susceptible strains were higher in abundance than VOCs detected from the resistant strains. However, 2,3-Butanediol, [R-(R*, R*)] was significantly reported with high abundance in the resistant strain samples of *R. dominica*. While dodecanal was only detected from the susceptible strain of *T. castaneum*. The higher VOCs release by the susceptible strains correlated with high respiration rate in comparison with resistant strains.

To conclude, emission of VOCs of *R. dominica* and *T. castaneum* were significantly affected by the resistance level. The susceptible insects produced a higher amount of the VOCs. The study suggested using 2,3-Butanediol, [R-(R*, R*)] and dodecanal to differentiate resistant and susceptible strains of *R. dominica* and *T. castaneum*, respectively.

4.1 Introduction

The lesser grain borer, *Rhyzopertha dominica* (Fabricius) and the red flour beetle, *Tribolium castaneum* (Herbst) are serious pests that damage various of stored commodities (Arbogast, 1991; Nguyen, 2006). Phosphine was used worldwide for more than four decades as the main method to control stored grain insect pests (Kaur et al., 2015). It is the most important fumigant for the control of pest insects in stored produces but genetic resistance by pest insects have been developed (Schlipalius et al., 2008). Resistance to phosphine was reported in the lesser grain borer, *R. dominica* (F.) and the rust-red flour beetle, *T. castaneum* (H.) in different countries around the world (Ansell et al., 1990; Collins, 1998; Zeng, 1999). The little effect of phosphine on the resistant insects with respect to susceptible insects indicated that there are metabolic factors contribute to resistance to phosphine (Chaudhry & Price, 1990). A study on *Caenorhabditis elegans* indicated that phosphine affects both structure and function of

mitochondria; however, resistant *C. elegans* to phosphine had a substantial increase in the mitochondrial membrane potential and less consumption of oxygen (Zuryn et al., 2008). Constant with that, reduced levels of respiration were acquired for resistant strains with high resistance ratios from different insect species such as *T. castaneum, R. dominica and O. surinamensis* (Pimentel et al., 2007; Pimentel et al., 2008).

Biological volatile organic compounds (VOCs) are released by biological samples and reflect to biological metabolisms (Zini et al., 2001). Possessing special structures and providing crucial role such as very essential bio-information required in the communication between plants and insects (Inui & Itioka, 2007; Verheggen et al., 2008). Some VOCs secretions such as 1-heptadecene; 1, 8-heptadecadiene; 1-tetradecene; 1-hexadecene; 1, 6-pentadecadiene and heptadecatriene that released by *Tribolium spp*. can be used as strong repellents for individuals from the same or other species (SUZUKI et al., 1988; Suzuki et al., 1975). The presence of some biological VOCs released by some species of insects like tenebrionids such as methyl-1, 4-benzoquinone (MBQ) and ethyl-1, 4-benzoquinone (EBQ) in a commodity such as grain or flour can be used as an indicator of insect infestation (Unruh et al., 1998). Therefore, studies have been focused on using biological VOCs as biomarkers to detect particular species of fungus and insect (Laopongsit et al., 2014; Laothawornkitkul et al., 2010; Niu et al., 2015; Niu et al., 2012).

Genetic studies on phosphine resistance confirmed the inherited changes in resistant strains according to the resistance level (Kocak et al., 2015; Nguyen et al., 2015). Consequently, physiological changes of the resistant insects like respiration rate and fitness sequences were reported (Pimentel et al., 2008; Pimentel et al., 2007). Therefore, the study hypothesises that the heritable changes that lead to phosphine resistance can affect the bioprocesses of the resistant insects. Therefore, the objective of this study is to determine the effect of the resistance level on the metabolism of *R. dominica* and *T. castaneum* using the emission of VOCs as indicator and its relationship with strains respiration of different resistance level. Moreover, to determine the potential of using VOCs that release from resistant and susceptible insects to differentiate the insects in different resistance level.

4.2 Materials and Methods

4.2.1 The insect cultures

Adult insects, susceptible and resistant strains of *T. castaneum* and *R. dominica* were obtained from the Department of Primary Industries and Regional Development (DPIRD), Australia. The narrow aged insects (2-3 days) were cultured by incubating 3000 adult insects with 1000 g of food, broken wheat for *R. dominica*, susceptble (MUWRD-7) and resistant (MUWRDSR-675) wheat flour/yeast 12:1 ratio for *T. castaneum*, susceptble (MUWTC-6000) and resistant (MUWTCSR) in 2-L jars sealed with meshed lids. The Parents' insects were removed after three days and remaining cultural medium were incubated at 28±1°C and 70±2% RH. Newly emerged adults were narrow aged and transferred to the jar containing new food. The insects used in the experiments were one month old. The flour was made from freshly harvested wheat (Australian Standard Wheat). Before using, the wheat was sterilised by keeping at -20°C freezer for seven days followed by storing at 4°C until use. The grain was milled using a Wonder Mill (Model WM2000, Korea), and the flour was kept at 4°C.

4.2.2 Apparatuses

A screw top four mL amber vial coupled with a cap equipped with septa (SUPELCO, USA) was used to extract the VOCs. The 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fibre with 2 cm coating was supplied by Sigma-Aldrich, Bellefonte, USA. Prior to use, the fibre was activated according to manufacturer's recommendations. The supplementary respiration experiments were implemented using collection bottles of 250 mL clear glass equipped with screw caps (24 mm Mininert Valves) (Cat No. 24904; RESTEK, USA). The volume of the flasks was calculated by measuring of water required to fill it.

4.2.3 Instruments and analytical conditions

GC-MS analyses was implemented with Agilent 7820A gas chromatography coupled with an Agilent 5977B MSD mass spectrometer. Gas chromatographic system was fitted with an Agilent DB mid-polarity capillary column (35ms; 30 m x 0.25 mm x 0.25 µm), and equipped with a split/splitless injector and an SPME inlet (Supelco, Bellefonte, PA, USA), which operated under splitless mode during the analysis. The injection temperature of the GC inlet was 250°C. Helium was used as a carrier gas at a constant flow of 1.1 mL/min. The oven temperature program for analysis *T. castaneum* was: 50°C for 5 min increased at 5°C/min to 250°C with a total run time of 45 min. While program for *R. dominica* was 50°C for 5 min

increased at 5°C/min to 200°C and was held for 5 min with a total run time of 40 min. The MSD transfer line, ion source and quad-pole temperatures were 300, 230 and 150°C, respectively. Ionization energy was 70eV; scan acquisition mode was performed at scan ranged from 50 to 600 m/z at scan speed 10,000 µ/s.

The headspace gas analyser oxybaby Witt-Gasetechnik D-58454 (WITT Gasetechnik GmbH & Co, Germany) was used for Oxygen and Carbon dioxide measuring.

4.2.4 Phosphine generation and resistance factor determination

Phosphine was generated by dissolving commercial tablets (Quickphos, United Phosphorus Limited (UPL), NSW, Australia) of aluminium phosphide in 10% sulphuric acid solution, and the final purity of phosphine was 86% (FAO, 1975). To determine the resistance factor, bioassays were employed by using different concentrations of phosphine (control (air), 0.005, 0.01, 0.02, 0.03, 0.04, 0.1, 0.3, 0.6, 1, 2, 3 and 4 mg/L) with three replicates each concentration. Fifty adult insects in 1000 mL flasks for each replicate were fumigated with phosphine for 20 h for the susceptible and resistant strains, followed by a one-week recovery period at 25±1°C and 65±5% RH.

4.2.4 The extraction and analytical procedures

4.2.4.1 Volatile organic compounds

The headspace micro-extraction technique was used to extract the VOCs from the samples. DVB/CAR/PDMS SPME fibre was inserted into the headspace of the flasks that contained the sample for extraction period of four hours at 35°C and 70% RH (Alnajim et al., 2019). After the extraction time had finished, the SPME fibre was withdrawn and injected directly into the injection port of the GC-MS for five minutes to desorb the VOCs (Niu et al., 2015).

4.2.4.2 Respiration rate

Eighty adult insects of *R. dominica* and 50 insects of *T. castaneum* in 250 mL in flasks for each sample of the respiration. Oxygen consumption and carbon dioxide production were measured under different exposure periods of 8, 16 and 24 hours at 30±1°C and 70±5% RH by using Witt-Gasetechnik Oxybaby D-58454.

4.2.5 Data processing and analysis

The resistance factor was evaluated based on the LD_{50} of susceptible and resistant strains. After assessing the insect's mortality, probit analysis was carried out to determine LD_{50} using Polo

plus Probit and Logit Analysis (version 0.03). The means of three replicates of respiration data were analysed and compared by IPM SPSS statistics 24 using T-test. The GC-MS signals were collected by MassHunter Acquisition software (Agilent Technologies, USA). Automatic Mass Spectral Deconvolution and Identification System (AMDIS-32) software and NIST 2.2 mass spectra library were used to identify chemical compounds. The Kovat's retention indices that were calculated by running a C7-C30 alkane standard were also assisted the identification. The averages of compound areas were statistically analysed by Metaboanalyst 4.0 (http://www.metaboanalyst.ca/faces/upload/StatUploadView.xhtml) by using Volcano plot (Chong et al., 2018). Samples were uploaded to Metaboanalyst 4.0 as columns (unpaired); data filtering were characterized using mean intensity value. Sample normalization, data transformation and data scaling were specified as a "NONE" mode. Volcano plot was analysed at fold change ≥2 and t-test threshold of 0.05.

4.3 Results and Discussion

4.3.1 Resistance factor

The susceptibility of *R. dominica* and *T. castaneum* that obtained from the populations which were chosen as susceptible insects was very close to FAO (1975) standard method (FAO LC_{50}=0.008 mg/L for 20 hours exposure). A probit model concentration-mortality analyses showed that the LC_{50} of the susceptible strains of both species used in this study was 0.009 mg/L. While LC_{50} for resistant strains were 1.042 and 0.26 mg/L for *R. dominica* and *T. castaneum*, respectively. Consequently, resistance ratio has been calculated according to LC_{50} of the susceptible insects (RR = 115.77 fold for *R. dominica*) and (RR = 28.8 fold for *T. castaneum*). This resistance ratios were in the similar range that reported for *R. dominica and T. castaneum* from different areas (Jagadeesan et al., 2012; Mau et al., 2012).

4.3.2 Volatile organic compounds

The volatile organic compounds (VOCs) were investigated from different resistance level strains of *R. dominica* and *T. castaneum*. Mostly, VOCs that emitted by the susceptible strains of both species were in abundance compared to the resistant strains (Figures. 4.1 and 4.2). Variety of different group compounds were released by *R. dominica* such as alcohols and aldehydes (Supplementary results Table 4.S1).

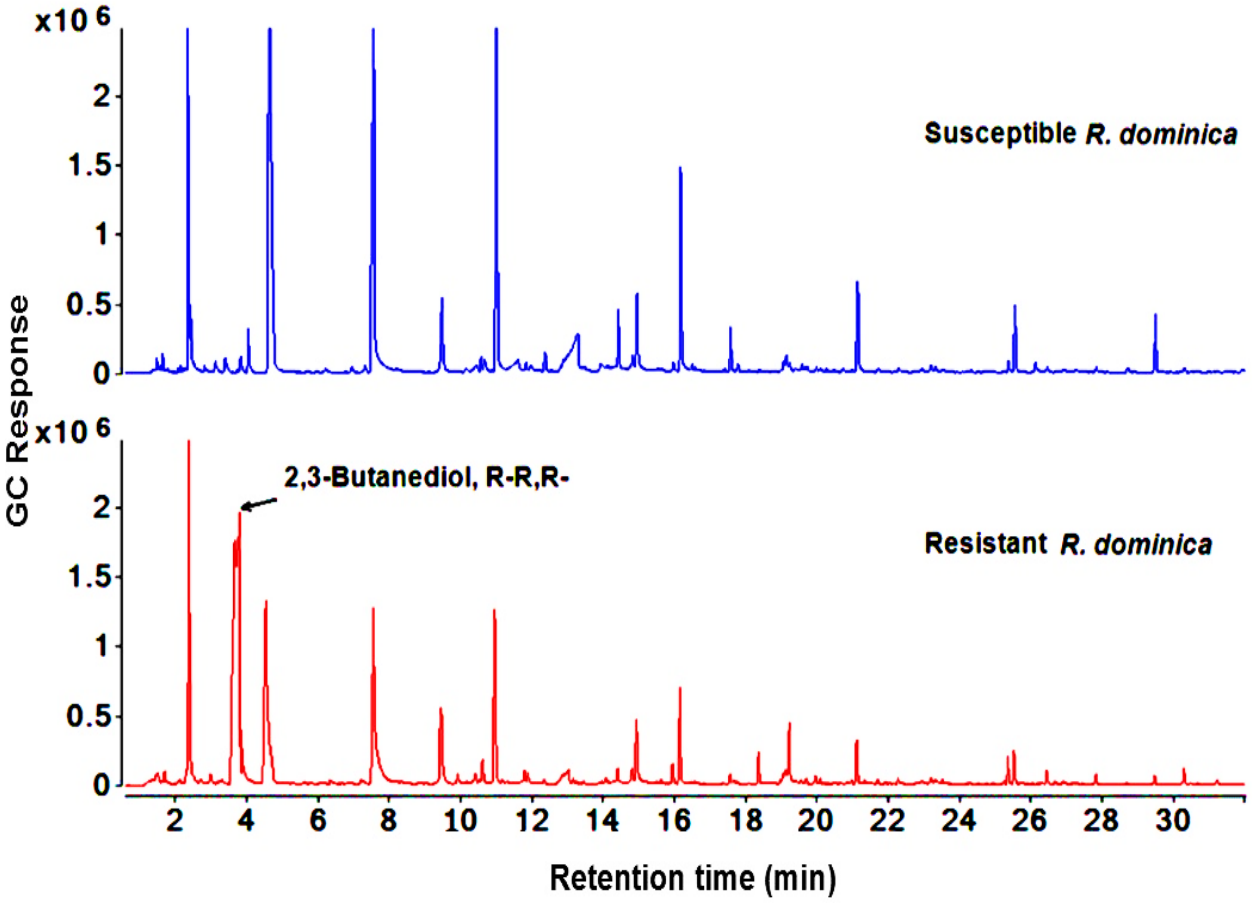

Figure 4.1. Total signal chromatograms show VOCs from susceptible and resistant strains of *R. dominica*.

Some compounds, which were detected from *R. dominica* in this study, were also reported in previous studies. Niu et al (2015) reported Nonanal; Hexanal; Hexanoic acid; Furan, 2-pentyl- and Decanal from the adults *R. dominica*. Whereas, 3-Pentanone, 2-methyl-; 5-Hepten-2-one, 6-methyl; Hexanal; 5-Hepten-2-one, 6-methyl-; 5-Hepten-2-ol, 6-methyl and Nonanal were detected from lesser grain borer infested grains (Seitz & Ram, 2004). While in the case of *T. castaneum*, the main compounds identified in both the susceptible and resistant insects were the quinone compounds like 2-Methylhydroquinone and 2-Methylquinone. Consistent with studies, which confirmed that the major volatile organic compounds from *Tribolium spp* such as red flour beetle are quinones (Hodges et al., 1996; Unruh et al., 1998; Villaverde et al., 2007). Another common compound (1-Pentadecene) which was also identified as one of the major peaks. This pheromone can stimulate both gender in addition to acting as a defensive secretion against *Tribolium spp* enemies (Arnaud et al., 2002; Keville & Kannowski, 1975).

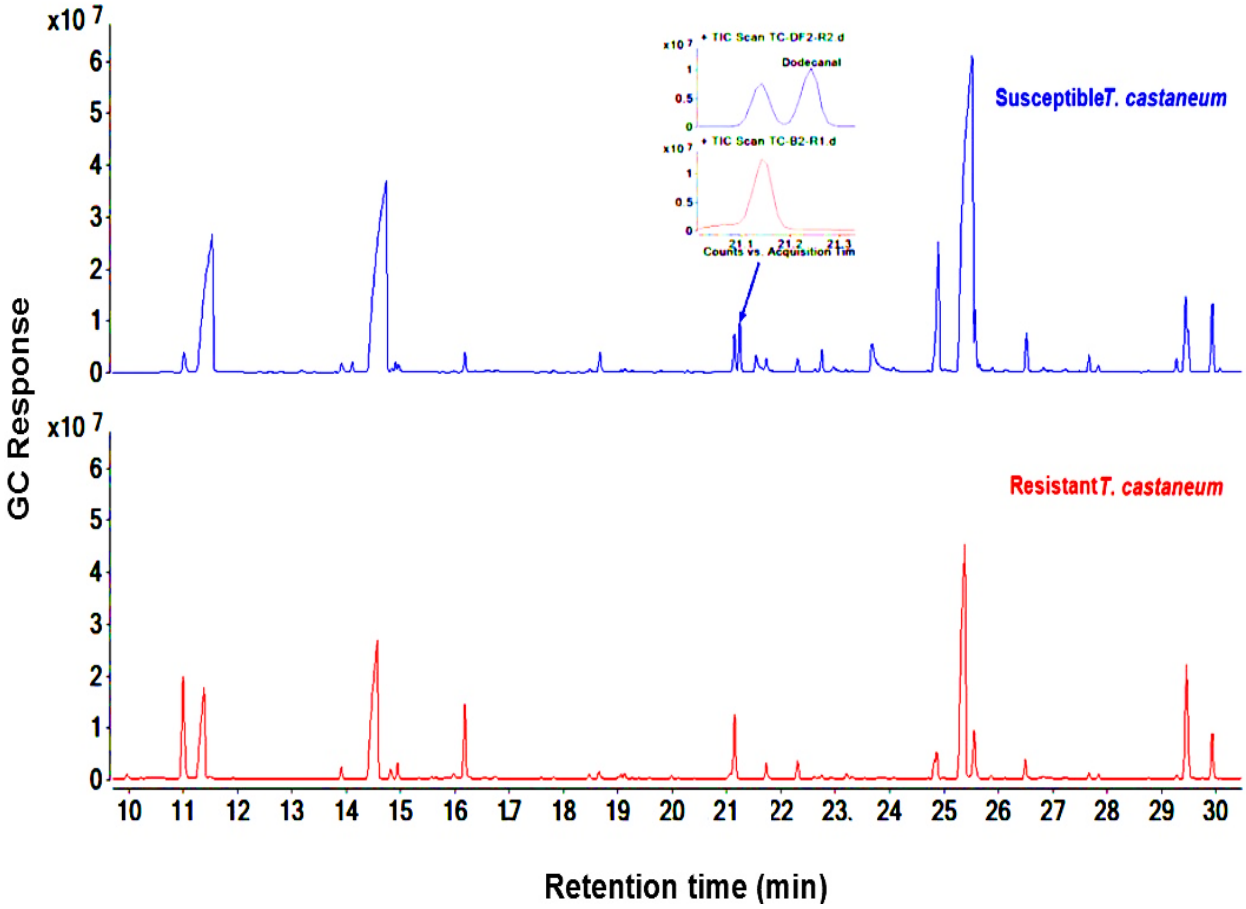

Figure 4.2. Total signal chromatograms show VOCs from susceptible and resistant strains of *T. castaneum*.

The higher release of the VOCs by the susceptible strains in comparison with the resistant strains correlated with a higher respiration rate (oxygen consumption and carbon dioxide production) (Figures. 4.3 and 4.4). significant differences of O_2 consumption after 8 and 16 hours sealing periods ($p \leq 0.0005$) were obtained from *R. dominica* (0.037, 0.060 mg/insect respectively for susceptible insects) and (0.026, 0.035 mg/insect respectively for resistant insects). Also, there was a significant difference ($p \leq 0.05$) of *T. castaneum* O_2 consumption after 24 h (0.102 and 0.090 mg O_2/insect for susceptible and resistant, respectively). Additionally, the susceptible strains of both *R. dominica* and *T. castaneum* produced significantly more CO_2 than the resistant strain (Figures. 4.3 and 4.4) at all of the sealing time. The carbon dioxide production of the susceptible of *R. dominica* insects after 8, 16, and 24 h were (0.046, 0.066 and 0.084 mg/insect respectively) and *T. castaneum* (0.051, 0.084 and 0.138 mg/insect respectively). While the carbon dioxide production after 8, 16, and 24 h of the resistant of *R. dominica* were 0.039, 0.052 and 0.77 mg/insect respectively and for the resistant

of *T. castaneum* were 0.042, 0.058 and 0.119 mg/insect respectively. The results of the respiration is consistent with previous researches which confirmed that the populations with lowest respiration rate (carbon dioxide production) had high resistance ratio (Pimentel et al., 2008; Pimentel et al., 2007), which agrees with the physiological mechanism of phosphine resistance that influences the uptake of phosphine to lower level which means in part of it lower consumption of oxygen. The lower uptake of phosphine by resistant strain in comparison with a susceptible strain was previously confirmed on *R. dominica* (Price, 1984; Price, 1981).

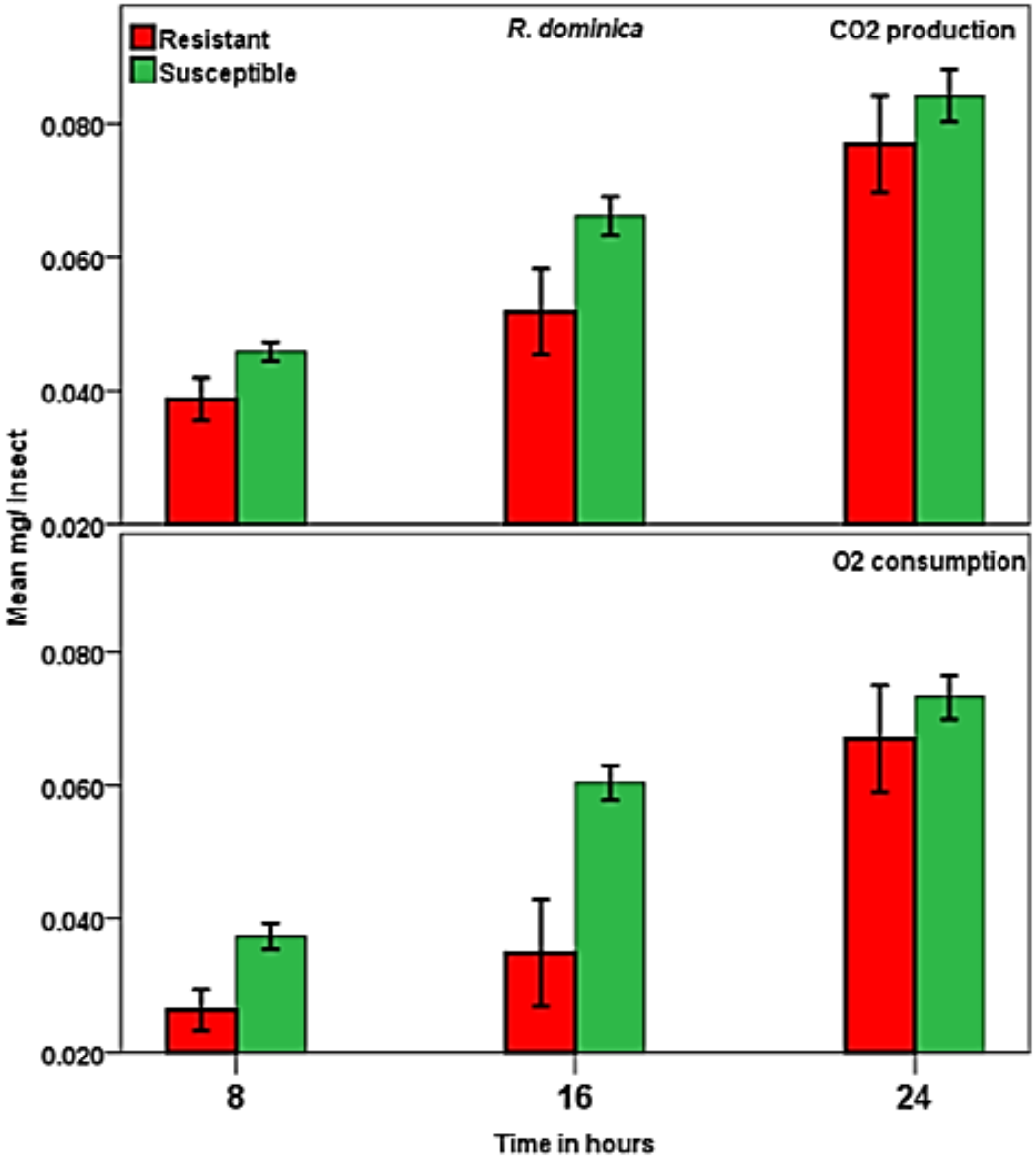

Figure 4.3. Comparison of oxygen consumption and carbon dioxide production between susceptible and resistant strains of *R. dominica* after different exposure periods. Error bars are confidence intervals (n=3).

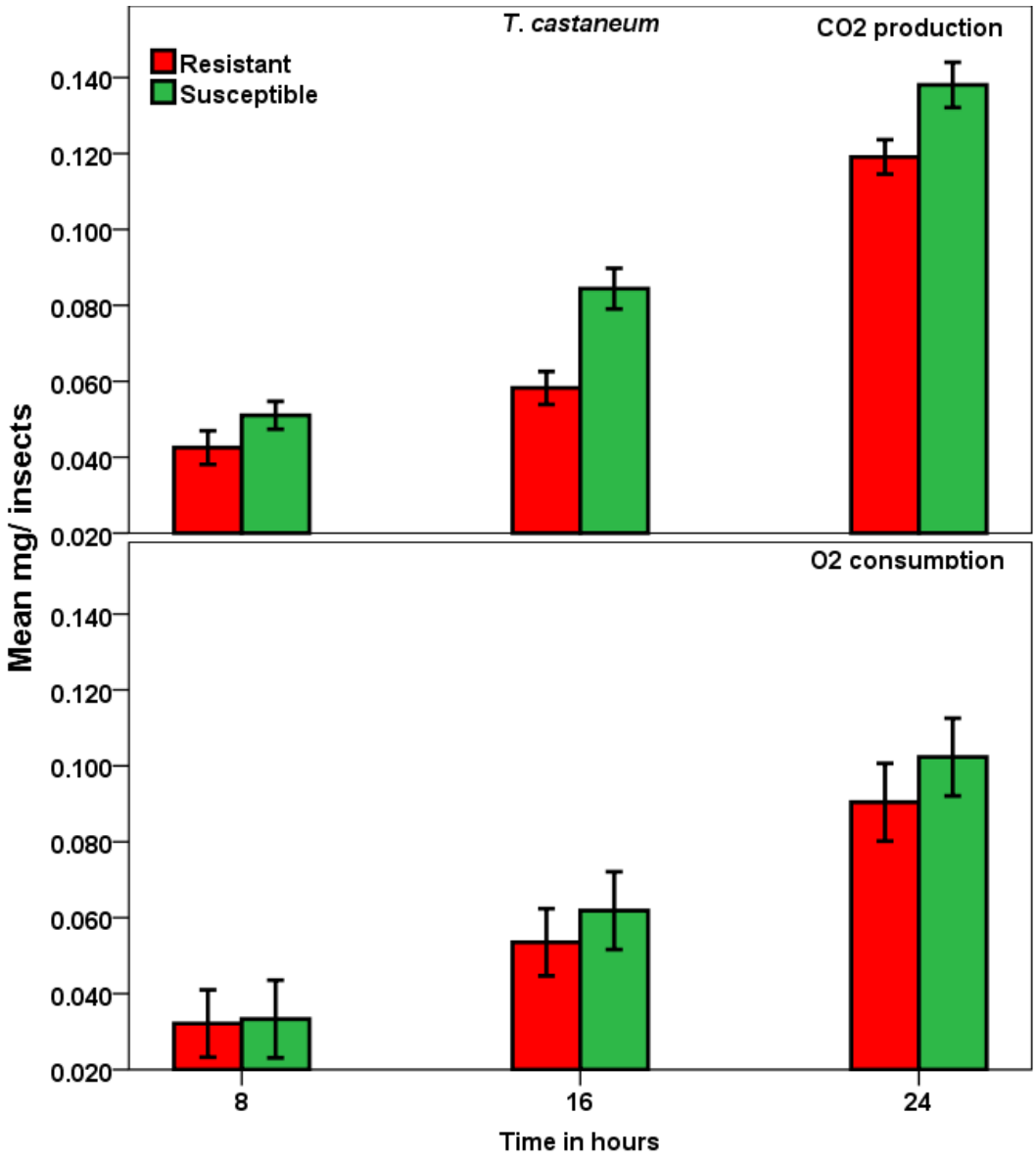

Figure 4.4. Comparison of oxygen consumption and carbon dioxide production between susceptible and resistant strains of *T. castaneum* after different exposure periods. Error bars are confidence intervals (n=3).

Despite there were no enormous differences regarding the number of the VOCs components detected from the susceptible and resistant strains of both species. Yet, Volcano plot data analysis detected significant differences from eleven chemicals of *R. dominica* and twelve VOCs of *T. castaneum* regarding the GC-MS response (peak area) (Figures 4.5 and 4.6). Among the twelve *T. castaneum* compounds, Paeonol (Ethanone, 1-2-hydroxy-4-methoxyphenyl-) was found to be significantly lower in abundance in the resistant strain as compared to the susceptible strain. A study confirmed that this compound could affect the

activity level of cytochrome oxidase of rats (Zhou et al., 2011). Since phosphine is a respiratory inhibitor (Chaudhry, 2000; Chefurka et al., 1976), this compound could be crucial for understanding the mechanism of phosphine resistance in the specific species. The results of this research prove that strains in different phosphine resistance level have various metabolism, which may explain the ability to phase out phosphine by resistant insects. The metabolism of insects in different resistance levels might be due to the genetic variations that were previously proved in many studies that focused on susceptible and the resistant strains (Kocak et al., 2015; Nguyen et al., 2015). Comparison of metabolism of resistant and susceptible strains is an important characteristic to understand the mechanism of phosphine resistance. Therefore, the mechanism of resistance to phosphine involved not only the reduction of uptake of phosphine in resistant strains (Price, 1980a; Price, 1980b). This because insects in different resistance level have different metabolism response to same amount uptake of phosphine, using the mortalities of insects as an indicator (Chaudhry & Price, 1990). This indicates that resistant insects resist phosphine through metabolic processes to reduce the toxicity of toxicant, in addition to the low absorption of phosphine (Chaudhry & Price, 1990). Therefore, studying insects in different resistance level showed a different biochemical response to a sub-lethal dose of phosphine. For example, susceptible and resistant Khapra beetle strains (*Trogoderma granarium*) had different metabolism of glycogen, trehalose, glucose and total lipids and enzymatic activities such as ChE, ICDH, LDH, Amylase and AcP (Naeem, 2016).

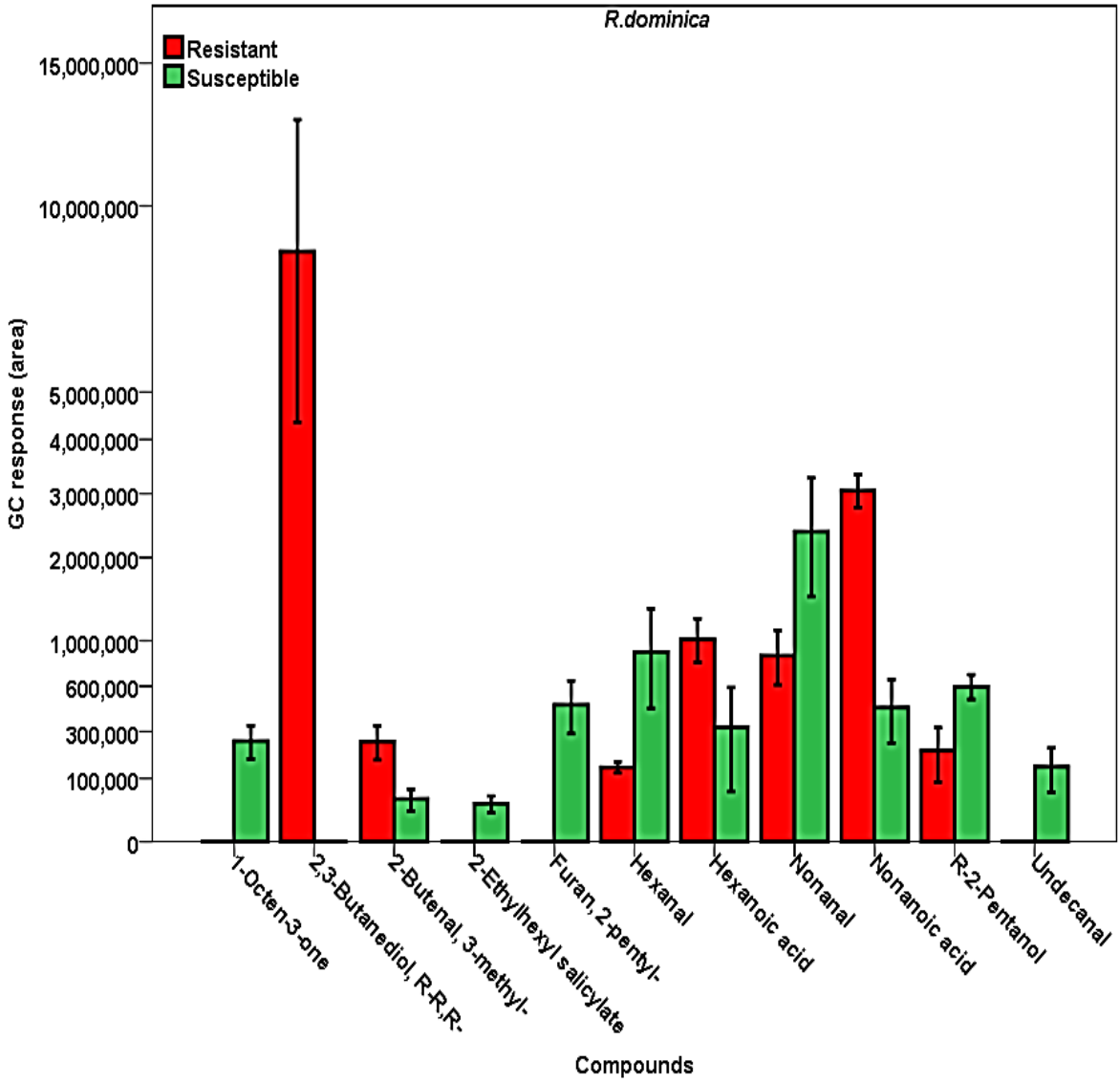

Figure 4.5. Compounds were selected by Volcano plot analysis with a significant difference between the susceptible and resistant strains of *R. dominica*.

The results of the VOCs of this study confirmed that there are metabolic differences according to the resistance level of the insects. Such results can be developed to be bio tools to diagnose the resistance level for *R. dominica* and *T. castaneum* species using VOCs from phosphine susceptible and resistant strains. That because using VOCs as biomarkers is potential method to diagnose the insect species infect stored products such as red flour beetle *T. castaneum* (Niu et al., 2015; Villaverde et al., 2007), peanut beetle *Ulomoides dermestoides* (Villaverde et al., 2009), and lesser grain borer *R. dominica* (Seitz & Sauer, 1996; Seitz & Ram, 2004). The (2, 3-Butanediol, [R-(R*,R*)]-) compound which was also reported in another study (Niu et al., 2015) was detected with high intensity in all resistant insects samples while the amount of this

this compound was almost non-existent in the susceptible samples (Figure 4.1). While in the case of *T. castaneum* dodecanal was only detected in susceptible beetles (Figure 4.2). This aldehydes compound was also reported in some previous studies as a volatile compound from different sources like insects and plants (Goh et al., 2016; Niu et al., 2015). Subsequently, these compounds may be utilized as metabolite pattern to differentiate the susceptible and resistant populations of *R. dominica* and *T. castaneum*.

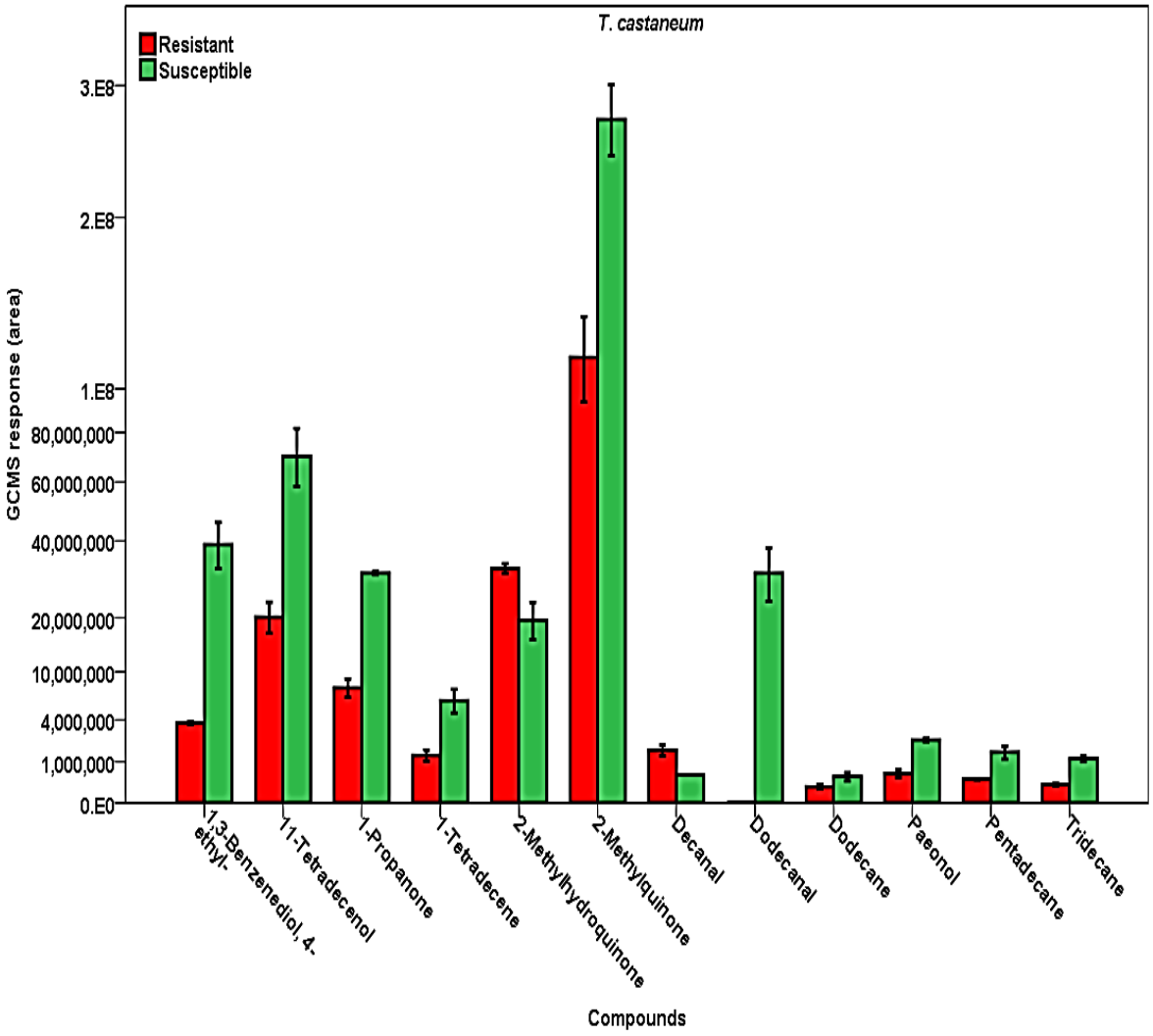

Figure 4.6. Compounds were selected by Volcano plot analysis with a significant difference between the susceptible and resistant strains of *T. castaneum*.

4.4 Conclusion

The effect of the resistance level toward phosphine on the metabolism using VOCs of two stored product species as indicators were investigated. Results of VOCs showed differences in the intensities of volatile compounds depend on the resistance level of the insects. These results could be useful to show an obvious picture of the resistance mechanism and can be exploited as a potential diagnostic. The study suggested using compounds like 2,3-Butanediol, [R-(R*, R*)]- from resistant of *R. dominica* and dodecanal from susceptible of *T. castaneum* as biomarkers to differentiate the resistant from the susceptible strains.

Supplementary results

Table 4.S1. Volatile organic compounds detected from *R. dominica* using headspace microextraction technique and gas chromatography-mass spectrometry detector

No.	RT	Compound name	NRI	RI	MF (%)
1	2.0	Butanal, 3-methyl-	652	655	86.5
2	2.3	2-Butenal, 3-methyl-	692	696	84.5
3	2.4	(R)-(-)-2-Pentanol	681	702	80.0
4	2.8	3-Buten-1-ol, 3-methyl-	730	728	82.5
5	3.1	3-Pentanone, 2-methyl-	745	746	85.0
6	3.3	Toluene	757	757	89.0
7	3.4	Butane, 1-chloro-3-methyl-	758	763	78.0
8	3.6	2,3-Butanediol, [R-(R*,R*)]-	743	778	82.0
9	3.8	1-Octene	785	791	88.5
10	4.0	Hexanal	800	802	88.5
11	9.8	1-Octen-3-one	979	983	77.0
12	10.4	Hexanoic acid	990	991	84.0
13	10.6	5-Hepten-2-one, 6-methyl-	986	992	83.0
14	10.8	Furan, 2-pentyl-	993	996	86.5
15	11.0	5-Hepten-2-ol, 6-methyl-	994	998	85.0
16	11.8	D-Limonene	1018	1032	84.5
17	12.0	1-Hexanol, 2-ethyl-	995	1035	87.5
18	12.4	Benzeneacetaldehyde	1045	1047	90.0
19	13.1	2-Pentenoic acid, 2,3-dimethyl-	1036	1067	78.0
20	13.4	1-Octanol	1071	1075	87.0
21	13.9	Heptanoic acid	1078	1091	82.0
22	14.4	Nonanal	1104	1106	90.0
23	14.7	Phenylethyl Alcohol	1116	1115	87.0
24	15.1	Hexanoic acid, 2-ethyl-	1124	1126	90.0
25	17.6	Decanal	1206	1207	90.0
26	19.5	Nonanoic acid	1273	1274	86.5
27	20.5	Undecanal	1307	1308	86.5
28	26.1	Benzoic acid, 4-ethoxy-, ethyl ester	1529	1528	86.5

29	27.8	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	1591	1601	84.5
30	31.4	Tetradecanoic acid	1768	1762	82.5
31	31.9	Benzenesulfonamide, N-butyl-	1794	1787	87.0
32	32.4	2-Ethylhexyl salicylate	1811	1810	85.0
33	32.6	Hexadecanal	1817	1818	81.0
34	33.7	Phthalic acid, diisobutyl ester	1870	1872	88.0
35	35.5	n-Hexadecanoic acid	1954	1961	87.0

RT= retention time; NRI= retention index obtained from NIST database; RI= retention index obtained from running n-Alkane standard; MF= Match factors were generated from NIST database.

Table 4.S2. Volatile organic compounds detected from *T. castaneum* insects using headspace microextraction technique and gas chromatography-mass spectrometry detector

No.	RT	Compound name	NRI	RI	MF (%)
1	11.53	p-Benzoquinone, 2-methyl-	1018	1023	89
2	13.21	Acetophenone	1029	1033	85
3	14.28	Undecane	1100	1061	86
4	14.73	p-Benzoquinone, 2-ethyl-	1215	1116	86
5	16.18	Dodecane	1200	1150	88
6	18.63	Decanal	1204	1195	86
7	20.01	Tridecane	1300	1241	87
8	21.21	Dodecanal	1386	1330	75
9	21.60	1,4-Benzenediol, 2-methyl-	1225	1353	89
10	22.74	1-Tetradecene	1392	1387	76
11	23.67	1,3-Benzenediol, 4-ethyl-	1334	1434	89
12	24.04	Paeanol	1439	1448	88
13	24.84	cis-11-Tetradecen-1-ol	1664	1470	78
14	25.51	1-Pentadecene	1502	1503	75
15	25.57	Pentadecane	1512	1506	75
16	25.83	Cyclopentadecane	1536	1510	80
17	26.52	1-(2-Hydroxy-4-methoxyphenyl)propan-1-one	1538	1545	85

18	27.18	1,15-Hexadecadiene	1579	1568	77
19	27.66	1-Hexadecene	1592	1589	88
20	29.26	1,8,11-Heptadecatriene, (Z,Z)-	1665	1661	84
21	29.44	E-9-Tetradecenol	1668	1672	72
22	29.94	1-Heptadecene	1692	1692	87
23	30.19	Heptadecane	1700	1701	72

RT= retention time; NRI= retention index obtained from NIST database; RI= retention index obtained from running n-Alkane standard; MF= Match factors were generated from NIST database.

Statement of Contribution

Title of Paper	New Method of Analysis of Lipids in *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Fabricius) Insects by Direct Immersion Solid-Phase Microextraction (DI-SPME) Coupled with GC–MS
Publication Status	(•) Published ( ) Accepted for Publication ( ) Submitted for Publication ( ) Unpublished and Unsubmitted work written in a manuscript style
Publication Details	Published in "INSECTS"

Principal Author

Name of Principal Author (Candidate)	Ihab alnajim
Contribution to the Paper	Concept, laboratory works, data analysis and writing manuscript
Overall percentage (%)	65%
Signature	[redacted] Date: 25/12/2019

Co-Author Contributions

By signing the Statement of Contribution, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all the co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Xin Du
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	Byungho Lee
Contribution to the Paper	Editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	Tao Liu
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	YongLin Ren
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 25/12/2019

Chapter 5

New Method of Analysis of Lipids in *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Fabricius) Insects by Direct Immersion Solid-Phase Microextraction (DI-SPME) Coupled with GC–MS

Abstract

Lipids play an essential role in providing energy and other physiological functions for insects. Therefore, it is important to determine the composition of insect lipids from cuticular and internal tissues for a better understanding of insect biology and physiology. A novel non-derivatization method for the analysis of lipids including fatty acids, hydrocarbon waxes, sterols in *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Fabricius) was explored using the direct immersion solid-phase microextraction (DI-SPME) coupled with gas chromatography–mass spectrometry (GC–MS). Nine extraction solvents, acetonitrile, methanol, hexane, ethanol, chloroform, acetonitrile and ethanol (1:1 *v/v*), acetonitrile and water (1:1 *v/v*), ethanol and water (1:1 *v/v*) and acetonitrile and ethanol and water (2:2:1 *v/v/v*) were selected and evaluated for the extraction of insect lipids with DI-SPME fiber. Acetonitrile extraction offered the best qualitative, quantitative, and number of lipids extracted from insects samples results. Acetonitrile extracted high-boiling point compounds from both species of tested insects. The range of hydrocarbons was C25 (pentacosane) to C32 (dotriacontane) for *T. castaneum* and C26 (11-methylpentacosane) to C34 (tetratriacontane) for *R. dominica*. The major compounds extracted from the cuticular surface of *T. castaneum* were 11-methylheptacosane (20.71%) and 3-methylheptacosane (12.37%), and from *R. dominica* were 10-methyldotriacontane (14.0%), and 15-methyltritriacontane (9.93%). The limit of detection (LOD) for the n-alkane compounds ranged between 0.08 (nonacosane) and 0.26 (dotriacontane) µg/g and for the fatty acids between 0.65 (arachidic acid) to 0.89 (oleic acid) µg/g. The study indicated that DI-SPME GC–MS is a highly efficient extraction and a sensitive analytical method for the determination of non-derivatized insect lipids in cuticular and homogenized body tissues.

5.1 Introduction

Lipids are the main biological compounds in animals and plants (Cerkowniak et al., 2013), including fatty acids and hydrocarbon waxes on and in insect bodies (Cohen & Moussian, 2016). Fatty acids are the most basic form of biological lipids and are usually bound with other compounds to build more composite lipids, such as triglycerides, which are energy stores (Desbois & Smith, 2010). The cuticular lipid layer of the insect consists of different chemicals such as a long chain of hydrocarbons and fatty acids (Gibbs, 1998). An analysis of the cuticular lipids of Acanthoscelides obtectus showed that adults contain a variety of chemicals such as hydrocarbons, triacylglycerols, fatty acid esters, free fatty acids, sterols, aldehydes, ketones

and alcohols (Gołębiowski et al., 2008b). Hydrocarbons are the major lipid category in the cuticle of insects, including straight-chain saturated and unsaturated hydrocarbons (Gołębiowski et al., 2011), which in some species reach to more than 90% of cuticular lipids and are usually a mixture of components including n-alkanes, branched methyl-alkanes and ethyl-alkanes (Cohen & Moussian, 2016). An analysis of cuticular lipids from adults of *Zygogramma exclamationis* showed that large amounts of lipids in the cuticle of males and females were hydrocarbons ranging from C23 (tricosene) to C56 (trimethyltripentacontane) (Nelson & Charlet, 2003). The functions of insect cuticular hydrocarbons have evolved to not only keep water in the organism but to also play an essential role in communication among the population individuals and between individuals of different sexes (Berson & Simmons, 2019; Cohen & Moussian, 2016; Snellings et al., 2018). A study on the adults of *Drosophila melanogaster* demonstrated that increased desiccation resistance is linked with the total amount of cuticular hydrocarbons (Ferveur et al., 2018). Cuticular lipids have also been reported to protect insects from harmful pathogens (Cohen & Moussian, 2016; Gołębiowski et al., 2008a). The composition of cuticular lipids varies among the insect species, and it is a reflection of the genetic structure and changes induced by ecological circumstances (Toolson & Kuper-Simbrón, 1989). Therefore, an analysis of insect lipids is fundamental to understand insect metabolism and physiology.

The extraction of lipids from insects is the first critical step that leads to a reliable qualitative and quantitative analysis of insect lipids (Cerkowniak et al., 2013). Numerous methods have been used to extract insect lipids; however, extraction solvents and methods are the core of a reliable extraction process that transfers lipids from a matrix to the liquid phase (Buckner et al., 1999; Cerkowniak et al., 2013; Saïd et al., 2005; Ye et al., 2007), thus enabling coupling with various analytical methods (Cerkowniak et al., 2013). Examples include using a single solvent like hexane to extract cuticular hydrocarbons from six species of flies and an analysis with gas chromatography–mass spectrometry (GC–MS) (Ye et al., 2007), extraction with chloroform and an analysis with high-performance thin-layer chromatography (HPTLC) to determine surface lipids of *Bemisia argentifolii* (Buckner et al., 1999), a, and extraction with dichloromethane and an analysis with gas chromatography–Flame ionization detector (GC–FID) for profiling the cuticular hydrocarbons of four *Periplaneta* species including *Periplaneta brunnea*, *Periplaneta fuliginosa*, *Periplaneta australasiae* and *Periplaneta americana* (Saïd et al., 2005). Commonly, the use of two solvents, such as hexane/chloroform, have been used for extraction of the cuticular lipids from the *Osmia lignaria* and *Megachile rotundata* bees and the *Aleurodicus dugesii* giant whitefly, which were then analyzed with GC–

FID and GC–MS (Buckner et al., 2009).Solid-phase microextraction (SPME) has been used to extract insect lipids, especially cuticular lipids (Cerkowniak et al., 2013). This extraction is done either by rubbing the SPME fiber onto the surface of the insect cuticle (Ginzel et al., 2006; Roux et al., 2002) or by using the headspace solid-phase microextraction (HS-SPME) method (De Pasquale et al., 2007). However, these procedures are not suitable for the analysis of semi-volatile compounds. Keeping this in mind, a novel method of direct immersion solid-phase microextraction (DI-SPME) in different solvents coupled with GC–MS has been used. Therefore, this study aimed to evaluate the use of the DI-SPME technique coupled with GC–MS to extract and analyze insect lipids like fatty acids and hydrocarbon waxes from the surface and whole adult body of two main stored products insects, *Tribolium castaneum* and *Rhyzopertha dominica*. This technique involves direct immersion in the solvents, so the appropriate solvent selection is critical. Therefore, a range of solvents including acetonitrile, methanol, hexane, ethanol, chloroform, acetonitrile and ethanol (1:1 v/v), acetonitrile and water (1:1 v/v), ethanol and water (1:1 v/v), and acetonitrile and ethanol and water (2:2:1 v/v/v) were evaluated in this study in order to determine their ability to enhance the extraction of the analytes.

5.2. Material and Methods

5.2.1 The insect culture

Adult insects, *Tribolium castaneum* and *Rhyzopertha dominica*, were obtained from the Department of Primary Industries and Regional Development (DPIRD), Australia. The work with the two species of insects was approved by Murdoch University (Approval number: WBM-18-249), as there was no ethical concern about using *T. castaneum* and *R. dominica*. The narrow aged insects (2–3 days) were cultured by incubating 3000 adult insects with 1000 g of food—broken wheat for *R. dominica* (strain MUWRD -7), and wheat flour/yeast (12:1) ratio for *T. castaneum* ((strain MUWTC -6000) —in 2-L jars sealed with meshed lids. The parents' insects were removed after three days, and the remaining culture was incubated at 28 ± 1 °C and 70 ± 2% relative humidity (RH). Newly emerged adults were narrowly aged and transferred to the jar containing new food.

The insects used in the experiments were one month old. The flour was made from freshly harvested wheat (Australian Standard Wheat). Before use, the wheat was sterilized by keeping it at −20 °C for seven days, followed by storage at 4 °C until use. The grain was milled using a Wonder Mill (Model WM2000 , WonderMill, Korea), and the flour was kept at 4 °C.

5.2.2 Chemical reagents and apparatuses

The solvents used were acetonitrile ≥99.9% *v/v* (Fisher Scientific, Glee, Belgium), methanol ≥99.9% *v/v*, hexane ≥95% *v/v*, ethanol ≥99.9% *v/v*, and chloroform ≥99.9% *v/v* (Sigma-Aldrich, Bellefonte, PA, USA). Deionized water (DI) was purified through a Milli-Q Biocel system (Millipore, Burlington, MA, USA).

Various combinations of mixed solvents were prepared and used, such as acetonitrile and ethanol (1:1 *v/v*), acetonitrile and water (1:1 *v/v*), ethanol and water (1:1 *v/v*), and acetonitrile and ethanol and water (2:2:1 *v/v/v*).

Individual external standards were purchased from Merck-Sigma Aldrich Co. and included palmitic acid ≥99% *w/w*, linolenic acid ≥99% *w/w*, linoleic acid ≥99% *w/w*, oleic acid ≥99% *w/w*, arachidic acid ≥99% *w/w*, cholesterol ≥99% *w/w*, p-benzoquinone ≥98% *w/w*, 2-methyl-p-benzoquinone ≥98% *w/w* and 1-pentadecene ≥98% *v/v*. In addition, n-alkane, standard C7–C40 (1000 mg/mL in hexane) was purchased from (Supelco, Bellefonte, PA, USA).

The 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber with a 2 cm coating was supplied by Sigma-Aldrich, Bellefonte, PA, USA. Prior to use, the fiber was activated according to the manufacturer's recommendations by exposing the fiber's coating to 270 °C for half an hour.

5.2.3 GC-MS instrument and analytical conditions

All GC–MS analyses were performed with an Agilent GC 7890B gas chromatography coupled with an Agilent 5977B mass spectrometer detector (MSD). In the gas chromatographic system, an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm, (Agilent Technologies, Santa Clara, CA, USA) was used. The GC was equipped with a split/splitless injector and an SPME inlet (Supelco, Bellefonte, PA, USA), which operated under the splitless mode during the analysis. The injection temperature of the GC inlet was 270 °C. Helium was used as a carrier gas at a constant flow of 1.2 mL/min. The oven temperature program was 60 °C for 2 min and increased at a rate of 7 °C/min to 200 °C, 5 °C/min to 300 °C, and finally at a rate of 50 °C/min to 320 °C before being held for 3 min with a total run time of 45.4 min. The MSD transfer line, ion

source and quad-pole temperatures were 300, 230 and 150 °C, respectively. Ionization energy was 70 eV, and scan acquisition mode was performed at a scan ranged from 50 to 600 m/z at a scan speed of 10,000 amu/s.

5.2.4 The extraction and analytical procedures

5.2.4.1 Preparation of diluted standards

All the fatty acid external standard chemicals were prepared by dilution with acetonitrile to 1 mg (standards)/g (acetonitrile) in 20 mL clear glass vials. The final fatty acid standard was prepared by mixing the diluted individual standards. This included a range of fatty acids (palmitic acid, linolenic acid, linoleic acid, oleic acid and arachidic acid), p-benzoquinone, 2-methyl-p-benzoquinone, and cholesterol with final concentrations of 0.1, 0.5, 1, 10 and 100 µg/g, respectively. The second standard mixture was prepared by mixing n-alkane standards C7 to C40 (1000 mg/mL in hexane, Supelco, Bellefonte, PA, USA) to obtain concentrations of 0.1, 0.5, 1, 5 and 10 µg/g. Meanwhile, acetonitrile was used as a blank.

5.2.4.2 Evaluation of effect of different solvents on DI-SPME for extraction of lipids from *T. castaneum*

Prior to extraction, all the insects used in this research were cleaned by letting them move on a wet tissue paper for 15 min and then transferring them onto clean, dry tissue paper for 10 min. The cleaned insects were frozen with liquid nitrogen. To evaluate the effect of the solvent type on the extraction, twenty frozen dead adults *T. castaneum* were transferred into a 2-mL plastic microtube, to which 1.6 mL of solvent was added. Three milling balls were added, and the microtube was closed for homogenization using a Beadbug homogenizer for 1 min at 4000 revolutions per minute (rpm). The extracts were centrifuged at 8150× g for 3 min using the Dynamica mini centrifuge, and then 1.5 mL supernatant was transferred to a 2 mL HPLC clear vial using a 1000 µL micropipette. Each of the nine solvents was replicated four times.
The SPME fiber was inserted into the extract for 14 h at 25 ± 2 °C. Immediately after completing the extraction, the fibre was withdrawn and injected directly into the GC–MS injector for the determination of lipids.

5.2.4.3 Comparison of lipid compositions between two insect species in acetonitrile

The adult insects were cleaned as described above. For the extraction of cuticular lipids, the cleaned insects (20 *T. castaneum* and 25 *R. dominica*) were separately transferred into 2 mL microtubes containing 1.6 mL of acetonitrile (HPLC grade, Fisher Scientific, Glee,

BelgiumFisher Chemical, USA) using a small clean brush and then sealed with screw cap. The microtubes were shaken gently by hand for 3 min, and then the extract was transferred into a 2 mL amber GC vial with septa using a micropipette.

The lipids in the remaining extracted insect bodies were homogenized using a Beadbug homogenizer in a 2 mL BeadBug ™ microtube containing 1.6 mL of HPLC grade acetonitrile for 1 min at 4000 rpm before being then centrifuged at 8150× g for 3 min using the Dynamica mini centrifuge. The supernatant (1.5 mL) was transferred into a 2 mL amber GC vial with septa.

Each extraction with acetonitrile was repeated four times. The SPME fiber was inserted into the extract for 14 h at 25 ± 2 °C for DI-SPME extraction and the determination of the lipids by GC–MS.

5.2.5 Data processing and analysis

The GC–MS signals were collected by the MassHunter Acquisition software (Agilent Technologies, Santa Clara, CA, USA). The Automatic Mass Spectral Deconvolution and Identification System (AMDIS-32) software and the National Institute of Standards and Technology (NIST) 2.2 mass spectra library (version 2.2) were used to identify chemical compounds. The Kovat's retention index was used to assist identification. Data sorting and linear regression were processed by Microsoft Excel 2016. The averages of the compound areas were statistically analyzed by Metaboanalyst 4.0 (http://www.metaboanalyst.ca/faces/upload/StatUploadView.xhtml), using hierarchical cluster analyses (heatmap) (Chong et al., 2018). Samples were uploaded to Metaboanalyst 4.0 as columns (unpaired); data filtering was characterized using the mean intensity value. Sample normalization, data transformation and data scaling were specified as a "NONE" mode. Heatmap parameters were as follows: distance measure = Euclidean; clustering algorithm = ward; and standardization = auto scale feature. The heatmap statistical model was the t-test/ANOVA. The LOD was calculated by the linear regression method (Shrivastava & Gupta, 2011) using Equation (1):

$$LOD = 3S/b \quad (1)$$

Where S is the standard deviation of the linear response of the GC–MS and b is the slope of the calibration curve.

5.3 Results and Discussion

5.3.1 Effect of direct immersion on SPME extraction in solvent

This research reported a novel comprehensive DI-SPME method for the extraction of lipids such as fatty acids and hydrocarbon waxes from the cuticular surface and the whole body of insects (Figures 5.1 and 5.2). Since fiber coating was reported as a vital factor in the development of an appropriate SPME method (Alnajim et al., 2019; Balasubramanian & Panigrahi, 2011; Jeleń et al., 2000), a three phase combination DVB/CAR/PDMS fiber was used in this study to extract the lipid compounds from the samples. The selection was based on previous research (Niu et al., 2012; Qiu et al., 2014a) and also because the fiber coating of DVB/CAR/PDMS covers wide range of polarities from non-polar to polar compounds, which enables it to extract a wide range of compounds; as such, the extraction was strongly affected by the polarity of the SPME (Balasubramanian & Panigrahi, 2011). The lipid profile of *T. castaneum* showed the most GC peaks of the hydrocarbon wax components in the low boiling point waxes (LBWs) range. In contrast, for *R. dominica*, a limited level of hydrocarbon wax peaks were seen in the same zone; however, most of the hydrocarbon wax peaks were shown in the region of high boiling point waxes (HBWs) (>300 °C of the GC–MS oven program). Lockey, 1978 (Lockey, 1978) reported that various classes of n-alkanes and branched alkanes in *T. castaneum* were in the region of C25–C32 using conventional solvent method for lipid extraction, followed by an analysis with GC–MS. However, a similar result was simply achieved using DI-SPME fibers, e.g., alkanes and branched alkanes ranging between C25 to C32 from *T. castaneum* and C26–C34 from *R. dominica*. The DI-SPME method developed from this research preserved all the extracted insect lipids, as the extraction procedure was conducted at room temperature without the application of external heat. Since the method was direct immersion in the extract solution, there was no need to reduce the extract volume for pre-concentration, which significantly reduced the loss of some volatiles during vaporization. Moreover, an important innovation is that the DI-SPME enabled a lipid analysis without introducing additional reactions and chemicals for derivatizing lipids. This significantly led to reduced time consumption, cost, loss of lipids, and soaping. Soaping is a problem if the reaction is not carried out in very restrict conditions (Aldai et al., 2005). The DI-SPME allowed for the conduction of longer periods such as a 14 h extraction at room temperature, which resulted in

absorbing most of the analytes from the sample matrixes on the SPME fiber coating. However, a previous study used SPME to extract the lipids in the headspace of *Bagrada hilaris* at high extraction temperatures of 130 and 150 °C (De Pasquale et al., 2007), which significantly affected the distribution constant of the volatiles between the headspace and the matrix (Kataoka et al., 2000) and can also lead to the degradation of the long chains to small chains compounds. Roux et al., 2002, used SPME to directly rub the insect cuticle layer, but rubbing cannot extract all the compounds in insect bodies.

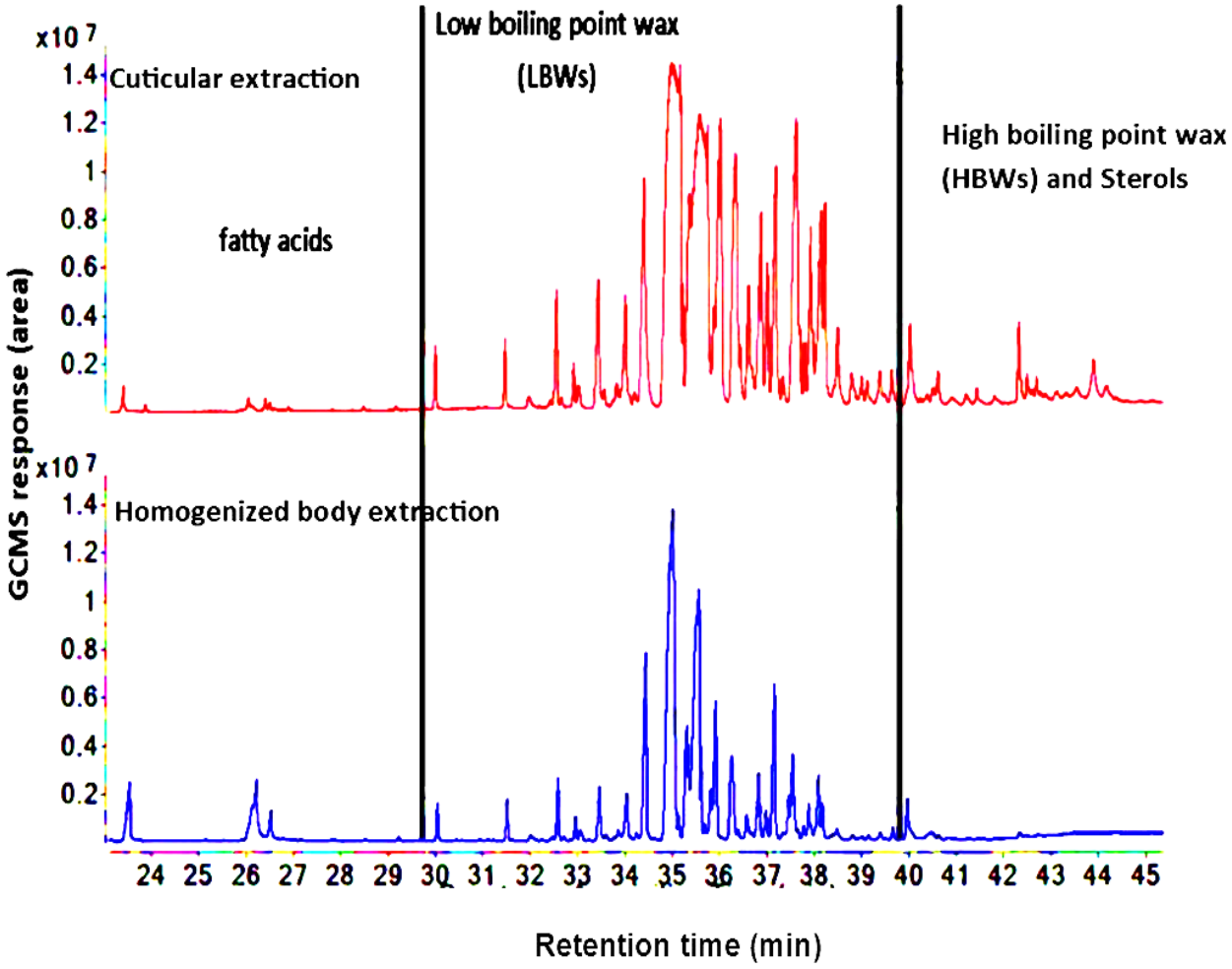

Figure 5.1. Total signal chromatogram of lipids of cuticular extraction followed by homogenized body extraction of same insects of *T. castaneum* using acetonitrile as a solvent.

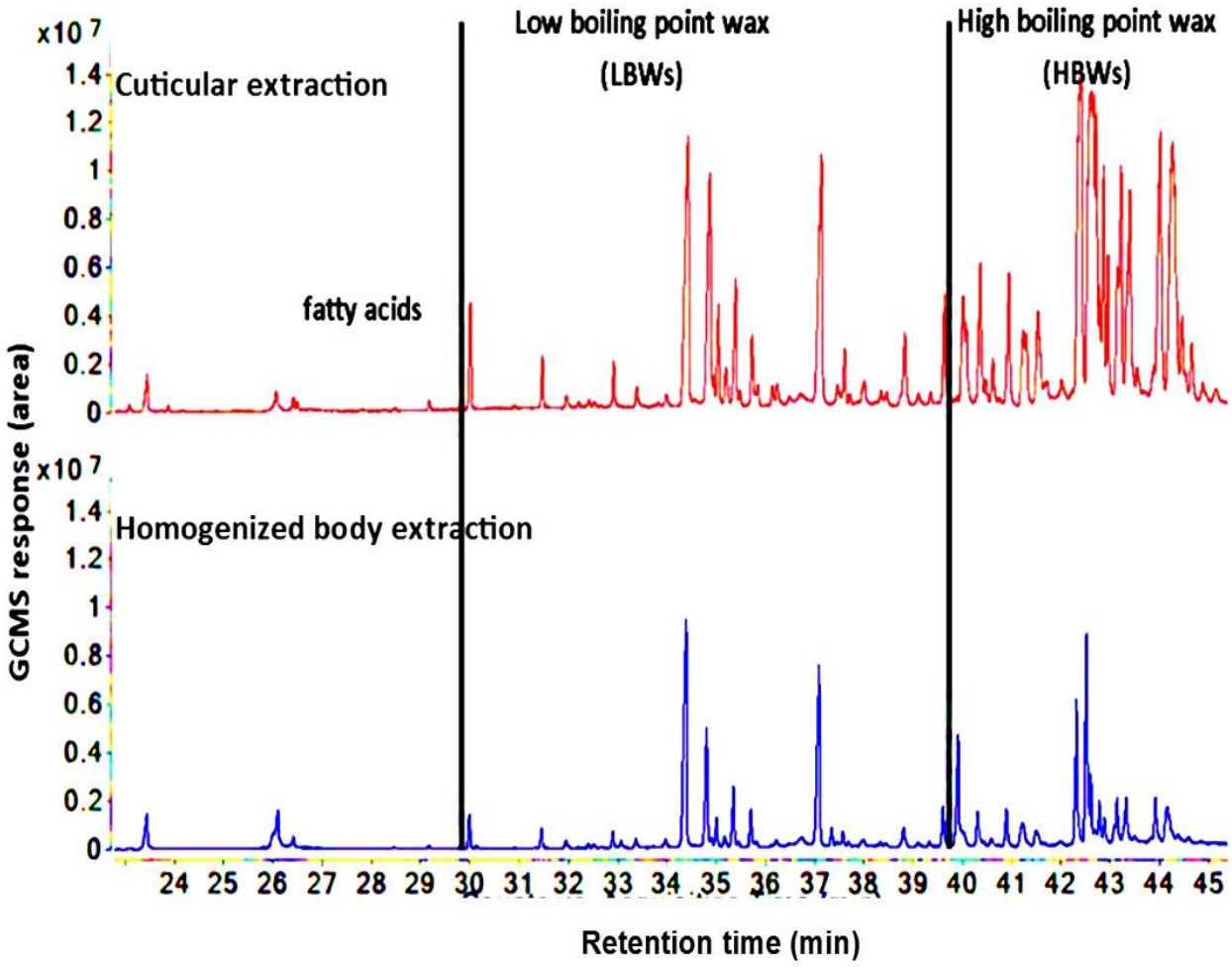

Figure 5.2. Total signal chromatogram of lipids of cuticular extraction followed by homogenized body extraction of same insects of *R. dominica* using acetonitrile as a solvent.

5.3.2 Effect of extraction solvents

Figure 5.3 shows the hierarchical cluster analysis (heatmap) in the form of a dendrogram. The heatmap indicates the relative intensity of four biological replicates which depended on the peak area of each compound in each solvent. Chloroform is not included in the figure because the SPME fiber coating was dissolved in this solvent. The highest relative GC response of the compound in comparison to the other was specified a "hot" color, while those that are lower in their values were given a "cold" color. The top of the dendrogram indicates the similarity among the solvents and the data variation among the replicates (Figure 5.3). The side arrows show a major compound with high area GC response in each solvent (Figure 5.3). The compounds obtained from the eight solvents consisted of an assortment of fatty acids, hydrocarbon waxes, and sterols in addition to some metabolic products, such as p-

benzoquinone and methyl-p-benzoquinone (Supplementary Material Table 5.S1). Fifty-three compounds were acquired from a total of eight tested solvents. Acetonitrile and the combination of acetonitrile and ethanol had the highest peak numbers with 41 and 34 compounds, respectively, including most of the fatty acids and hydrocarbon waxes, while the lowest number of compounds (22) (Table 5.1) was obtained from acetonitrile and water. It was observed that each compound had a different GC response according to the solvent used in the extraction process. Both acetonitrile and the combination of acetonitrile and ethanol showed a similar influence regarding the GC response of compounds. Most of the highest molecular weight compounds had higher intensities when acetonitrile and the combination of acetonitrile and ethanol were used as solvents. On the other hand, both the combination of acetonitrile and ethanol and water and the combination of acetonitrile and water had parallel effects (Figure 5.3). This could have been because of the similar polarity of the combined solvents. However, a higher GC response of some main fatty acids was detected in the combination of acetonitrile and water, such as palmitic acid (23.37_135), stearic acid (26.42_143) and linolenic acid (26.05_137) (Figure 5.3 and Supplementary Material Table 5.S1) because these are more polar compounds and the presence of water in a solvent increase the polarity of the solvent.

Table 5.1. The number of separated and identified compounds obtained in each solvent out of the total of 53 compounds from *T. castaneum* using acetonitrile as a solvent.

No.	Solvent	Compound Numbers	The Rate of Total Expected Compounds (%)	Number of Compounds Only Detected in Solvent
1	Acetonitrile	41	77.4	5
2	Hexane	23	43.4	1
3	Ethanol	25	47.2	0
4	Methanol	28	52.8	1
5	Acetonitrile and ethanol (1:1 *v/v*)	34	64.2	0
6	Acetonitrile and water (1:1 *v/v*)	22	41.5	0
7	Ethanol and water (1:1 *v/v*)	27	50.9	0
8	Ethanol and acetonitrile and water (2:2:1 *v/v/v*)	30	56.6	1

While the sterol compounds showed a higher GC response under the acetonitrile and ethanol and water solvent, methanol and ethanol were quite similar in their extraction abilities, although

methanol efficiently extracted most of the high boiling point hydrocarbon waxes. However, the use of methanol as an extraction solvent may form artificial methyl esters of fatty acids in the presence of some organic or inorganic materials (Brondz et al., 2007). As per Figure 5.3, with hexane, most of the compounds were between retention time (RT) = 6.65 and to 15.55 min and not at higher RT where C25–C34 compounds for *Tribolium* and *Rhyzopertha* could be seen, though hexane was commonly used as a solvent to extract the cuticular lipids from many insects (Caputo et al., 2007; Ye et al., 2007). However, a low yield of hydrocarbon waxes in this study could have be due to the fact of the incompatibility of fiber coating and the solvent as a result a lack of the efficient distribution of analytes between the fiber and the solvent. Chloroform is another solvent which has been successfully used as a single solvent (Buckner et al., 1996) or combined with another solvent such as hexane (Buckner et al., 2009) to extract insect lipids, producing a high yield (Cerkowniak et al., 2013). However, this was not appropriate to use for the DI-SPME technique as it can destroy the SPME fiber coating.

From the above results, acetonitrile was selected as the optimal solvent in the validation study. In previous studies, many solvents were used to extract either fatty acids or hydrocarbons, such as petroleum ether with dichloromethane (Gołębiowski et al., 2010; Nelson et al., 2000), hexane with chloroform (Nelson et al., 2000) and dichloromethane alone (Saïd et al., 2005). However, only acetonitrile demonstrated the ability to extract both fatty acids and hydrocarbon waxes from insects.

The results obtained from insect samples were compared with external standards of n-alkane, fatty acids, sterol, p-benzoquinone, methyl-p-benzoquinone and 1-pentadecene. The chemical compounds were identified using the NIST database after comparison with the mass spectra and retention indexes (RI) by running the external standards. The results of the LOD in Table 5.2 indicate that the new method could detect quinones (p-benzoquinone, 2-methyl-), fatty acids (arachidic acid), cholesterol and alkanes (heptacosane) at levels of 0.36, 0.65, 034 and 0.08 µg/g respectively. Therefore, this method has been demonstrated to be a robust method to analyze a variety of lipids.

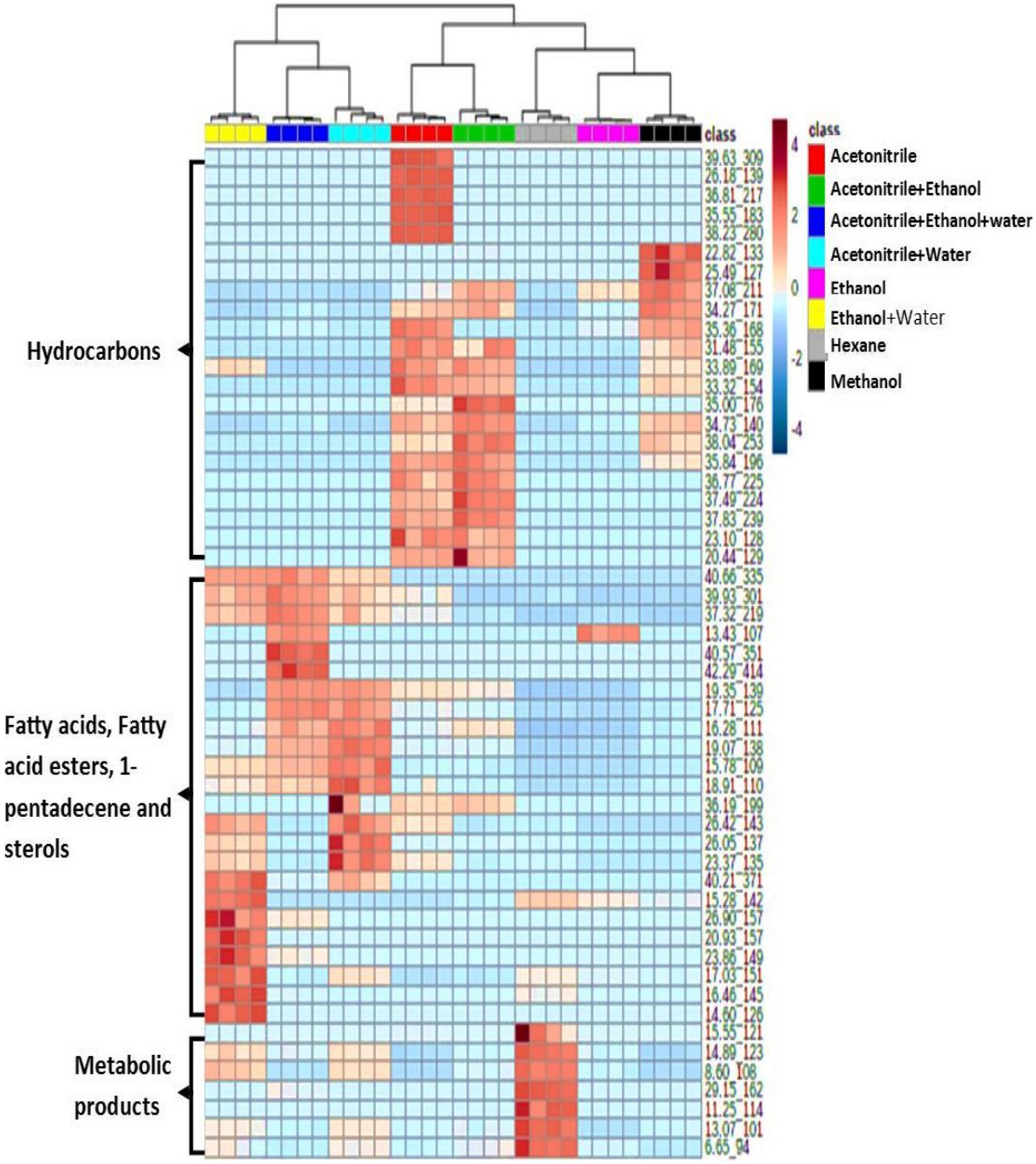

Figure 5.3. Hierarchical cluster analyses (heatmap) represents the data obtained from *T. castaneum* using eight solvents. Hot colour means high GC response based on peak areas. The top dendrogram indicates the similarity among the solvents as well as presenting the data variation among the replicates. Side arrows show the majority of compounds were detected with high GC response in each solvent.

Table 5.2. The limit of detection (LOD) of external reference standards of high boiling point n-alkanes, fatty acids, sterol, p-benzoquinone, methyl-p-benzoquinone and 1-pentadecene using acetonitrile as the solvent.

	Chemical Standards	Formula	RT (min)	R^2	LOD (µg/g)
1	p-benzoquinone	$C_6H_4O_2$	4.84	0.995	0.47
2	p-benzoquinone, 2-methyl-	$C_7H_6O_2$	6.72	0.976	0.36
3	1-pentadecene	$C_{15}H_{30}$	16.17	0.999	0.22
4	Palmitic acid	$C_{16}H_{32}O_2$	23.44	0.990	0.84
5	Henicosane	$C_{21}H_{44}$	25.35	0.939	0.21
6	Linolenic acid	$C_{18}H_{30}O_2$	25.99	0.937	0.87
7	Linoleic acid	$C_{18}H_{32}O_2$	26.13	0.989	0.87
8	Oleic acid	$C_{18}H_{34}O_2$	26.43	0.993	0.89
9	Docosane	$C_{22}H_{46}$	26.60	0.964	0.13
10	Arachidic acid	$C_{20}H_{40}O_2$	28.21	0.963	0.65
11	Tricosane	$C_{23}H_{48}$	31.30	0.961	0.13
12	Tetracosane	$C_{24}H_{50}$	32.24	0.920	0.21
13	Pentacosane	$C_{25}H_{52}$	33.06	0.923	0.24
14	Hexacosane	$C_{26}H_{54}$	34.41	0.961	0.14
15	Heptacosane	$C_{27}H_{56}$	35.27	0.988	0.08
16	Octacosane	$C_{28}H_{58}$	36.10	0.979	0.10
17	Nonacosane	$C_{29}H_{60}$	37.40	0.987	0.08
18	Triacontane	$C_{30}H_{62}$	39.52	0.988	0.08
19	Hentriacontane	$C_{31}H_{64}$	41.48	0.982	0.09
20	Cholesterol	$C_{27}H_{46}O$	39.98	0.948	0.34
21	Dotriacontane	$C_{32}H_{66}$	42.45	0.935	0.26

RT is retention time, LOD is the limit of detection, and R^2 is a linear regression coefficient. Based on solvent weight, the concentration of standards presents as µg/g.

5.3.3 Comparison of lipid compositions between two insect species

The GC responses for *T. castaneum* and *R. dominica* that were identified as compounds from the total GC responses were 91.93 ± 2.74% and 82.22 ± 3.06% from the homogenized body and 93.38 ± 3.93% and 81.32 ± 2.82% from the cuticular extractions, respectively. Thirty-eight and 39 compounds were obtained from the cuticular and homogenized body extraction of *T. castaneum*, whereas 30 and 26 compounds were identified from the cuticular and homogenized body extraction of *R. dominica*, respectively (Tables 5.3 and 5.4). The carbon chain lengths of *T. castaneum* varied from 25 (pentacosane) to 32 (dotriacontane) carbons, and *R. dominica* had a range of compounds from 26 (11-methylpentacosane) to 34 (tetratriacontane) carbons. According to the cuticular and homogenized body extractions, n-alkanes and methyl-branched alkanes were the major compounds identified from *R. dominica* and *T. castaneum*. A previous study by Lockey, 1978 also reported the same classes of n-alkanes and branched alkanes in *T. castaneum* in the region of C25–C32. In this study, the two major lipid compounds from *T. castaneum* cuticular extraction were 11-methylheptacosane (20.71%) and 3-methylheptacosane (12.37%), and the two major lipid compounds from the homogenized body were 1-pentadecene (22.70%) and 11-methylheptacosane (16.50%). The lipid compounds 10-methyldotriacontane (14.0%) and 15-methyltritriacontane (9.93%) were the two major compounds in the cuticular extraction from *R. dominica*, and 13-methylnonacosane and 13-methylheptacosane had the highest peak areas (20.30% and 18.10%, respectively) in the homogenized body. These results demonstrate that this method could extract and identity specific hydrocarbons from different insect species. This might indicate that the method can be used as a tool for the identification of insect species, which further supports previously reported studies that used cuticular hydrocarbons as chemotaxonomic tools for the identification of insect species (Barbosa et al., 2017; Braga et al., 2013).

The results from both insect species showed that the majority of hydrocarbon waxes were in abundance in the cuticular extraction in comparison to the homogenized body extraction (RT = 31.5 to 39.6 min for *T. castaneum* and RT = 31.5 to 45.2 min for *R. dominica*, Figures 5.1 and 5.2) including the major compounds such as 11-methylheptacosane and 3-methylheptacosane from *T. castaneum*. This is evident from the distribution coefficient between the homogenized body and the cuticular extraction (Tables 5.3 and 5.4), where the total peak areas were 29.11% and 24.17%, respectively, for the two major compounds of 11-methylheptacosane and 3-methylheptacosane in the homogenized body as compared to the cuticular extraction, suggesting the fact that these compounds are more in abundance in the

cuticular extraction. A similar result for *R. dominica* was also observed for the two major compounds 10-methyldotriacontane and 15-methyltritriacontane, which showed distribution coefficients of 10.61% and 7.11% in the homogenized extraction compared to the cuticular extraction. However, the fatty acids peak areas were opposite in the GC response. The peak areas of most of fatty acids were higher in the homogenized body extraction in comparison to the cuticular extraction. Linolenic acid showed the highest distribution coefficients of 96.01% and 88.72% for *T. castaneum* and *R. dominica*, respectively, in the homogenized body extraction. Thus, this research provides a robust tool not only to analyze cuticular and whole body lipids but to also assist in understanding the cuticular lipid compositions in comparison to internal lipid composition. This, in turn, may provide information to deduce the essential roles of lipids in many chemical and biological processes such as protecting insect bodies from dryness and pathogens (Cerkowniak et al., 2013; Cohen & Moussian, 2016; Wang & Leger, 2005).

Table 5.3. Extracted and identified compounds from the cuticle layer and homogenized body of *T. castaneum* in acetonitrile.

Compounds	RT (min)	NIST RI	Calculated RI	Qualitative *m/z*	GC Response (10^5) ± SD, n = 4		Relative GC Response		Distribution Coefficient B/(A + B) × 100
					Cuticular Extraction (a)	Homogenized Body Extraction (b)	Cuticular	Homogenized Body	
2-methylbenzoquinone	6.73	1116	1117	122	14 ± 1	29 ± 39	0.13	0.49	66.64
2-ethyl-p-benzoquinone	8.66	1215	1212	108	47 ± 8	46 ± 4	0.41	0.78	49.27
1,4-benzenediol, 2-methyl-	13.55	1223	1234	124	16 ± 3	65 ± 2	0.14	1.1	80.52
1,2-benzenediol, 4-ethyl-	15.07	1392	1388	138	30 ± 3	105 ± 2	0.26	1.78	77.65
7-dodecenol	15.83	1465	1468	165	72 ± 12	84 ± 4	0.63	1.43	53.86
1-pentadecene	16.26	1502	1504	154	1250 ± 172	1336 ± 4	10.98	22.70	51.66
Benzene, 1-ethoxy-4-isothiocyanato-	16.54	1527	1528	166	2 ± 0.3	2 ± 0.1	0.02	0.03	43.78
1-(2-hydroxy-4-methoxyphenyl)propan-1-one	17.07	1538 *	1558	151	47 ± 2	33 ± 3	0.40	0.56	41.42
7-hexadecene, (Z)-	17.82	1620	1605	152	39 ± 1	28 ± 3	0.34	0.47	41.75
1,8,11-heptadecatriene, (Z,Z)-	18.98	1655	1653	149	ND	11 ± 0.4	ND	0.19	100
cis-7-tetradecen-1-ol	19.14	1660	1661	179	515 ± 60	357 ± 31	4.52	6.06	40.9
2-hexadecanol	19.50	1702	1705	182	453 ± 36	374 ± 17	3.98	6.37	45.23
Myristic acid	20.44	1752	1755	185	ND	0.82 ± 0.09	ND	0.01	100
Palmitoleic acid	23.10	1936	1938	192	4 ± 0.3	ND	0.04	ND	0
Palmitic acid	23.60	1954	1956	199	17 ± 2	143 ± 12	0.15	2.44	89.54
Linolenic acid	26.15	2115	2119	222	3 ± 0.2	72 ± 5	0.03	1.23	96.01

Oleic acid	26.25	2134	2125	220	ND	92 ± 4	ND	1.57	100
Stearic acid	26.57	2153	2157	227	5 ± 0.6	45 ± 5	0.05	0.77	89.68
Unknown	31.52	-	2505	-	12 ± 2	4 ± 0.4	0.10	0.08	26.71
Pentacosane	33.30	2500	2515	238	33 ± 3	14 ± 0.7	0.29	0.24	30.49
Hexacosane	33.85	2600	2612	266	174 ± 27	59 ± 3	1.53	1.00	25.3
Unknown	34.06	-	2618	-	27 ± 2	38 ± 4	0.23	0.65	58.52
2-methylhexacosane	34.28	2661	2684	294	105 ± 10	5 ± 0.7	0.92	0.08	4.418
13-methylheptacosane	34.41	2731	2741	296	289 ± 22	274 ± 19	2.53	4.66	48.72
11-methylheptacosane	34.82	2734	2750	309	2358 ± 186	969 ± 85	20.71	16.5	29.11
2-methylheptacosane	35.02	2762	2766	336	635 ± 37	218 ± 14	5.57	3.71	25.57
3-methylheptacosane	35.58	2773	2771	337	1409 ± 147	449 ± 17	12.37	7.64	24.17
Octacosane	35.87	2800	2815	323	540 ± 35	118 ± 10	4.74	2.00	17.9
3-methyloctacosane	36.28	2872	2849	351	531 ± 76	133 ± 10	4.66	2.27	20.05
Nonacosane	36.60	2900	2902	365	177 ± 29	30 ± 3	1.55	0.51	14.58
Unknown	36.85	-	2908	-	165 ± 18	41 ± 3	1.44	0.70	19.99
Unknown	37.08	-	2911	-	437 ± 15	393 ± 31	3.83	6.68	47.36
Unknown	37.49	-	2917	-	457 ± 40	23 ± 2	4.01	0.40	4.86
13-methylnonacosane	37.58	2930	2927	379	766 ± 74	71 ± 7	6.73	1.21	8.52
11-methylnonacosane	37.92	2939	2950	393	31 ± 2	ND	0.27	ND	0
Nonacosane, 2-methyl-	38.13	2962	2961	421	229 ± 16	79 ± 2	2.42	1.34	25.56
3-methylnonacosane	38.44	2974	2973	395	275 ± 17	10 ± 1	2.41	0.16	3.351
Triacontane	39.64	3000	3003	239	141 ± 17	13 ± 1	1.24	0.22	8.425
Cholesterol	40.20	3087	3060	386	29 ± 1	75 ± 6	025	1.29	73.01
Desmosterol	40.51	3125	3133	364	15 ± 2	29 ± 1	0.13	0.49	66.06
Dotriacontane	42.37	3200	3203	449	36 ± 1	13 ± 1	0.32	0.22	26.27

The list contains only the compound that was identified properly; some compound may be present on the GC–MS chromatogram but are not on the list due to the lack of the identification. Compounds with matching RI differences more than 30 were reported as "Unknown." RT = retention time; NIST RI = retention indices obtained from National Institute of Standards and Technology database (NIST). * Estimated non-polar retention index (n-alkane scale NIST). Calculated RI = retention indices calculated using n-alkane standards C7–C40. Relative areas were calculated according to the total area of the listed compounds. *m*/*z* = mass to charge ratio. SD = standard deviation (n = 4). ND = not detected.

Table 5.4. Extracted and identified compounds from cuticle layer and homogenized body of *R. dominica* in acetonitrile.

Compounds	RT (min)	NIST RI	Calculated RI	Qualitative M/Z	GC Response (10^5) ± SD, n = 4		Relative GC Response		Distribution Coefficient B/(A + B) × 100
					Cuticular Extraction (A)	Homogenized Body Extraction (B)	Cuticular	Homogenized Body	
Palmitic acid	23.53	1954	1956	199	21 ± 3	ND	0.53	ND	0
Linolenic acid	26.06	2115	2119	222	6 ± 0.7	51 ± 5	0.17	4.39	88.72
Stearic acid	26.42	2153	2157	227	3 ± 0.3	13 ± 3	0.07	1.15	83.79
Octadecanamide, N-(2-hydroxyethyl)-	29.16	2347	2347	280	17 ± 0.7	9 ± 1	0.43	0.75	34.31
Unknown	31.46	-	2515	-	17 ± 3	ND	0.45	ND	0
11-methylpentacosane	31.94	2535	2555	281	5 ± 0.8	3 ± 0.2	0.12	0.28	41.64
Unknown	32.91	-	2628	-	17 ± 2	ND	0.45	ND	0
13-methylheptacosane	34.41	2731	2741	296	291 ± 56	209 ± 26	7.52	18.10	41.77
2-methylheptacosane	34.98	2762	2766	336	166 ± 13	31 ± 6	4.28	2.66	15.64
3-methylheptacosane	35.34	2773	2771	337	154 ± 28	7 ± 1	3.98	0.59	4.20
Octacosane	35.82	2800	2815	323	45 ± 4	33 ± 7	1.16	2.89	42.77
Unknown	37.10	-	2912	-	321 ± 25	15 ± 2	8.30	1.32	4.54

13-methylnonacosane	37.58	2930	2927	379	33 ± 3	234 ± 21	0.84	20.3	87.79
Triacontane	39.64	3000	3003	239	156 ± 30	16 ± 2	4.04	1.44	9.59
Cholesterol	40.00	3087	3060	386	12 ± 1	48 ± 8	0.32	4.21	79.67
Hentriacontane	41.22	3100	3117	435	65 ± 3	23 ± 3	1.69	1.98	25.93
2-methylhentriacontane	41.53	3162	3152	436	68 ± 5	28 ± 5	1.77	2.46	29.39
3-methylhentriacontane	42.01	3172	3182	424	29 ± 6	14 ± 3	0.75	1.21	32.50
Dotriacontane	42.37	3200	3203	449	271 ± 51	6 ± 0.9	7.00	0.52	2.16
10-methyldotriacontane	42.50	3235	3218	477	540 ± 57	64 ± 7	14.00	5.55	10.61
8-methyldotriacontane	42.60	3240	3221	450	92 ± 6	118 ± 9	2.37	10.20	56.17
Unknown	42.78	-	3231	-	181 ± 12	17 ± 2	4.69	1.46	8.53
Unknown	42.87	-	3237	-	66 ± 12	ND	1.70	ND	0
Unknown	42.96	-	3249	-	243 ± 36	24 ± 4	6.29	2.08	8.98
Dotriacontane, 2-methyl-	43.22	3263	3266	481	260 ± 38	30 ± 5	6.29	2.64	10.47
Unknown	43.40	-	3276	-	240 ± 20	42 ± 5	6.22	3.66	14.95
15-methyltritriacontane	44.03	3333	3323	463	384 ± 60	29 ± 5	9.93	2.55	7.11
Unknown	44.47	-	3351	-	58 ± 6	63 ± 12	1.50	5.47	52.17
Tetratriacontane	44.88	3400	3387	492	65 ± 12	11 ± 0.7	1.68	0.95	14.40

The list contains only the compound that was identified properly; some compound may be present on the GC–MS chromatogram but are not on the list due to the lack of the identification. Compounds with matching RI differences of more than 30 were reported as "Unknown." RT = retention time; NIST RI = retention indices obtained from National Institute of Standards and Technology database (NIST). Calculated RI = retention indices were calculated using n-alkane standards C7–C40. Relative areas were calculated according to the total area of the listed compounds; *m*/*z* = mass to charge ratio; SD = standard deviation (n = 4). ND = not detected.

5.4 Conclusion

The DI-SPME method coupled with GC–MS was explored for the first time to analyze insect cuticular and homogenized body lipids including hydrocarbons and fatty acids without derivatization. The four solos and their four combination solvents were evaluated, and acetonitrile was found to be the optimal solvent for the extraction of hydrocarbons and fatty acids from insects. The method was validated by analyzing the cuticular and internal lipids from two stored product insect species. The results indicate that the method is robust, reliable and sensitive for the extraction and identification of lipids from different species of insects.

Supplementary results

Table 5.S1. Extracted and identified compounds from homogenized whole *T. castaneum* in different solvents

Feature ID	Chemical name	NIST RI	Calculated RI	Qualitative M/Z	GC response (10^5)±SD, n=4							
					Acetonitrile	Hexane	Ethanol	Methanol	Acetonitrile + ethanol	Acetonitrile + water	Ethanol + water	Ethanol + Acetonitrile + water
6.65_94	2-methylbenzoquinone	1116	1117	122	14±1	242±29	25±3	5±0.4	41±5	49±3	43±3	10±2
8.60_108	2-ethyl-p-benzoquinone	1215	1212	108	18±3	425±24	60±6	20±1	86±10	171±9	227±26	34±6
11.25_114	3-undecanone	1283	1289	134	ND	8±3	ND	ND	ND	ND	ND	ND
13.07_101	Methoxytoluquinone	1281	1332	128	30±3	346±32	ND	29±2	43±17	83±4	70±6	20±0.3
13.43_107	1,4-benzenediol, 2-methyl-	1223	1234	124	7±0.05	ND	64±10	ND	ND	ND	ND	69±5
14.60_126	2,5-cyclohexadiene-1,4-dione, 2-ethoxy-5-methyl-	1381*	1373	132	ND	ND	ND	ND	ND	ND	5±0.6	ND
14.89_123	1,2-benzenediol, 4-ethyl-	1392	1388	138	51±10	584±41	120±7	44±3	113±9	217±18	257±39	137±13
15.28_142	Ethanone, 1-(2-hydroxy-4-methoxyphenyl)-4-	1438	1432	126	ND	32±0.3	16±0.6	11±1	4±0.2	ND	71.35±5.35	ND
15.55_121	3-hydroxybenzoic acid methyl ester	1417	1447	127	ND	204±48	ND	ND	ND	ND	ND	ND
15.78_109	7-dodecenol	1465	1468	165	73±8	17±2	25±3	56±7	70±11	565±82	245±18	358±35
16.28_111	1-pentadecene	1502	1504	154	815±72	207±21	326±27	604±39	1124±79	2429±244	792±86	1779±271
16.46_145	Benzene, 1-ethoxy-4-isothiocyanato-	1527	1528	166	1±0.1	16±0.7	2±0.1	ND	0.8±0.04	ND	139±23	5±0.3
17.03_151	1-(2-hydroxy-4-methoxyphenyl)propan-1-one	1538*	1558	151	41±3	296±9	219±3	164±30	78±1	391±51	1204±171	109±28

17.71_125	7-hexadecene, (Z)-	1620	1605	152	25±3	5±0.2	4.±0.6	12±1	20±2	82±13	14±2	94±8
18.91_110	1,8,11-heptadecatriene, (Z,Z)-	1655	1653	149	15±2	2±0.1	4±0.4	9±0.4	9±0.8	85±15	24±3	40±4
19.07_138	cis-7-tetradecen-1-ol	1660	1661	179	328±46	56±6	104±11	227±11	334±26	1451±92	312±27	945±24
19.35_139	2-hexadecanol	1702	1705	182	370±21	34±5	72±9	188±11	317±22	722±58	111±21	771±24
20.44_129	Myristic acid	1752	1755	185	2±0.1	ND	ND	ND	3±1	ND	ND	ND
20.93_157	Tetradecanoic acid, ethyl ester	1794	1780	213	ND	ND	ND	ND	ND	1.23±0.12	10±3	ND
22.82_133	Hexadecanoic acid, methyl ester	1926	1934	189	ND	ND	ND	14±4	0.5±0.08	ND	ND	ND
23.10_128	Palmitoleic acid	1936	1938	192	97±10	ND	ND	ND	55±8	8±2	ND	ND
23.37_135	Palmitic acid	1954	1951	199	356±59	106±5	12±0.7	15±1	77±13	1272±207	553.17±76	39±2
23.86_149	Palmitic acid, ethyl ester	1993	2002	201	ND	ND	5±0.9	ND	ND	ND	259±49	32±6
25.49_127	Z,Z-2,13-Octadecadien-1-ol	2078	2084	206	ND	ND	ND	11±2	ND	ND	ND	ND
26.05_137	Linolenic acid	2115	2119	222	390±42	209±13	10±2	15±2	287±42	4585±285	1754±203	49±9
26.18_139	Oleic acid	2134	2125	220	474±6	ND	4±0.4	4±0.2	ND	5±0.6	8±0.1	ND
26.42_143	Stearic acid	2153	2157	227	188±26	34±2	4±0.1	4±0.2	23±4	475±59	367.74±15	15±2
26.90_157	Stearic acid, ethyl ester	2195	2194	241	ND	ND	1±0.1	ND	ND	ND	80±14	14±2
29.15_162	Octadecanamide, N-(2-hydroxyethyl)-	2347	2355	238	ND	61±4	ND	ND	ND	ND	ND	4±0.6
31.48_155	Unknown	-	2505	-	15±2	ND	ND	7±1	11±3	ND	ND	ND
33.32_154	Pentacosane	2500	2515	238	55±5	ND	ND	24±3	35±3	ND	ND	ND
33.89_169	Hexacosane	2600	2612	266	48±7	ND	ND	20±2	44±9	ND	23±3	ND
34.27_171	2-methylhexacosane	2661	2684	294	69±8	3±0.1	24±2	121±16	84±8	11±1	6±0.6	16±2
34.73_140	11-methylheptacosane	2734	2750	309	385±50	8±1	89±12	501±42	864±84	ND	ND	71±11
35.00_176	2-methylheptacosane	2762	2766	336	1153±13	ND	13±1	18±2	1709±174	ND	ND	13±2
35.36_168	Unknoun	-	2771	337	343±46	ND	51±8	263±20	ND	ND	ND	27±5

35.55_183	3-methylheptacosane	2773	2771	337	636±18	ND	ND	ND	ND	ND	ND	ND
35.84_196	Octacosane	2800	2815	323	184±21	ND	ND	61±11	218±27	ND	ND	ND
36.19_199	3-methyloctacosane	2872	2849	351	174±22	ND	ND	ND	198±23	209±57	ND	ND
36.77_225	Nonacosane	2900	2902	365	33±8	ND	ND	ND	47±9	ND	ND	ND
36.81_217	Unknown	-	2908	-	33±0.7	ND	ND	ND	ND	ND	ND	ND
37.08_211	Unknown	-	2911	-	50±3	16±2	90±11	240±43	162±22	ND	ND	7±0.8
37.32_219	Unknown	-	2917	-	23±0.7	ND	5±0.5	ND	7±1	44±10	52±1	84±9
37.49_224	13-methylnonacosane	2930	2927	379	72±9	ND	ND	ND	138±31	ND	ND	ND
37.83_239	11-methylnonacosane	2939	2950	393	38±2	ND	ND	ND	57±9	ND	ND	ND
38.04_253	Nonacosane, 2-methyl-	2962	2961	421	40±4	ND	ND	49±7	114±12	ND	ND	ND
38.23_280	3-methylnonacosane	2974	2973	395	37±0.5	ND	ND	ND	ND	ND	ND	ND
39.63_309	Triacontane	3000	3003	239	21±2	ND	ND	ND	ND	ND	ND	ND
39.93_301	Cholesterol	3087	3060	386	88±10	28±3	11±0.7	15±1	12±2	142±18	189±37	255±36
40.21_371	Unknown	-	3075	-	ND	ND	ND	ND	ND	25±3	50±7	4±0.2
40.57_351	Unknown	-	3129	-	ND	ND	ND	ND	ND	ND	ND	8±2
40.66_335	Desmosterol	3125	3133	364	0.9±0.2	ND	ND	ND	ND	8±0.2	13±0.6	14±2
42.29_414	Dotriacontane	3200	3203	449	0.9±0.01	ND	ND	ND	ND	ND	ND	15±2

Feature ID includes retention time (min) and mass to charge ratio m/z. Compounds with match RI difference more than 30 were reported as "Unknown"; *Estimated non-polar retention index (n-alkane scale NIST); SD=standard deviation (n=4). ND=not detected.

Statement of Contribution

Title of Paper	Preliminary study on the differences in hydrocarbons between phosphine susceptible and resistant strains of *Rhyzopertha dominica* (Fabricius) and *Tribolium castaneum* (Herbst) using direct immersion solid-phase microextraction coupled with GC-MS
Publication Status	○ Published ○ Accepted for Publication ◉ Submitted for Publication ○ Unpublished and Unsubmitted work written in a manuscript style
Publication Details	Submitted to "Molecules"

Principal Author

Name of Principal Author (Candidate)	Ihab alnajim
Contribution to the Paper	Concept, laboratory works, data analysis and writing manuscript
Overall percentage (%)	65%
Signature	[redacted] Date: 25/12/2019

Co-Author Contributions

By signing the Statement of Contribution, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all the co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	Tao Liu
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	BeiBei Lid
Contribution to the Paper	Editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	Xin Du
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 25/12/2019

Name of Co-Author	YongLin Ren
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 25/12/2019

Chapter 6

Preliminary Study on the Differences in Hydrocarbons between Phosphine Susceptible and Resistant Strains of *Rhyzopertha dominica* (Fabricius) and *Tribolium castaneum* (Herbst) Using Direct Immersion Solid-Phase Microextraction Coupled with GC-MS

Abstract

Phosphine resistance is a worldwide issue threatening the grain industry. The cuticles of insects are covered with a layer of lipids, which protect insect bodies from the harmful effects of pesticides. The main components of the cuticular lipids are hydrocarbon compounds. In this research, phosphine-resistant and -susceptible strains of two main stored-grain insects, *T. castaneum* and *R. dominica*, were tested to determine the possible role of their cuticular hydrocarbons in phosphine resistance. Direct immersion solid-phase microextraction followed by gas chromatography-mass spectrometry (GC-MS) was applied to extract and analyse the cuticular hydrocarbons. The results showed significant differences between the resistant and susceptible strains regarding the cuticular hydrocarbons that were investigated. The resistant insects of both species contained higher amounts than the susceptible insects for the majority of the hydrocarbons, sixteen from cuticular extraction and nineteen from the homogenized body extraction for *T. castaneum* and eighteen from cuticular extraction and twenty-one from the homogenized body extraction for *R. dominica*. 3-methylnonacosane and 2-methylheptacosane had the highest significant difference between the susceptible and resistant strains of *T. castaneum* from the cuticle and the homogenized body respectively. Unknown5 from the cuticle and 3-methylhentriacontane from the homogenized body recorded the highest significant differences in *R. dominica*. The higher hydrocarbon content is a key factor in eliminating phosphine from entering resistant insect bodies, acting as a barrier between insects and the surrounding phosphine environment.

6.1 Introduction

The lesser grain borer *Rhyzopertha dominica* and the red flour beetle *Tribolium castaneum* are critical global pests that destroy various commodities including stored grains (Donahaye, 2007; Edde, 2012). Phosphine is the optimal fumigant to control pests in the store-product industry (Collins, 2006). However, resistance to phosphine is a serious issue that threatens the grain industry worldwide (Bell & Wilson, 1995). Long and ineffective applications are additional factors which have caused the development of resistance to this fumigant in most stored-grain insect species (Benhalima et al., 2004). The whole biochemical mechanism of resistance continues to be unclear despite a large number of studies on the subject (Chaudhry, 2000). However, the resistance mechanism involves the absorption of less phosphine by the resistant strains than by the susceptible strains. Elimination of phosphine from the respiratory system is one of the accepted mechanisms of insect resistance to phosphine (Chaudhry & Price, 1992;

Pimentel et al., 2007). Nevertheless, no evidence has been provided to explain the exclusion mechanism.

Insects normally contain a high content of lipids, making up 50-75% of the dry weight of some insects (Pino Moreno & Ganguly, 2016). The insect cuticle layer is a large part of the dry insect weight and plays an essential role in protecting insects from the surrounding environment (Cohen & Moussian, 2016). The cuticle layer is protected by complex mixtures of nonpolar and polar compounds, which vary in composition and quantity according to species and insect stage (Gołębiowski et al., 2011). Lipids are a significant part of the cuticle, proving that cuticular lipids are an essential part of insect lipid content (Lockey, 1978). Species and the developmental stage, in addition to genetic structure and ecological conditions, affect the cuticular lipid composition and quantity (Gołębiowski et al., 2011). The cuticular lipids of insects consist mostly of wax esters and hydrocarbons, in addition to fatty acid esters, triacylglycerols, aldehydes, alcohols, ketones and free fatty acids (Gołębiowski et al., 2011). The cuticular lipids of *Acanthoscelides obtectus* consist of hydrocarbons, methyl and ethyl esters of fatty acids sterols, triacylglycerols, free fatty acids, aldehydes and alcohols (Gołębiowski et al., 2008b). The cuticular lipids of larvae of *Calliphora vicina, Dendrolimus pini* and *Galleria mellonella* are composed of three lipid groups, hydrocarbons, triacylglycerols, and free fatty acids (Gołębiowski et al., 2008a), while the cuticular lipids of adults and larvae of *Frankliniella occidentalis* contain only hydrocarbons and free fatty acids (Gołębiowski et al., 2007). Thus insect surface hydrocarbons, the largest group of cuticular lipids, appear to be essential. They are usually a mixture of components consisting of n-alkanes and branched n-alkanes (Cohen & Moussian, 2016). A study on *T. castaneum* revealed the hydrocarbons n-alkanes (C25 to C31), 3-methylalkanes (C26 to C32), branched monomethylalkanes (C27 to C32) and dimethylalkanes (C29 to C31) (Lockey, 1978). The main purpose of insect cuticular hydrocarbons is to serve as a barrier between living insect bodies and their environment to protect insects from pathogens and dryness (Gołębiowski et al., 2008a).

Solid-phase microextraction (SPME) was successfully used to extract the cuticular lipids (Gołębiowski et al., 2013). This method was applied as an alternative to solvent extraction of insect cuticular hydrocarbons (Roux et al., 2002; Sledge et al., 2000), and produced similar results to those obtained by solvent extraction (Bland et al., 2001; Tentschert et al., 2002). The application of SPME was implemented either by rubbing the insect body (Ginzel et al., 2006) or by head-space microextraction (HS-SPME) (De Pasquale et al., 2007). With the rubbing of

insect body experiments, optimization of experimental parameters was not undertaken except for selection of the stationary phase of fibers (De Pasquale et al., 2007). HS-SPME might need more attention paid to some extraction conditions like extraction vial size and temperatures (De Pasquale et al., 2007). Bland et al. (2001) successfully used both HS-SPME and direct contact SPME coupled with GS-MS for extraction and identification of the major cuticular hydrocarbons of the subterranean termite *Coptotermes formosanus* Shiraki. Direct immersion solid-phase microextraction was another technique that produced a high yield of hydrocarbons from *R. dominica* and *T. castaneum* (Alnajim et al., 2019). The method uses both solvents and SPME by immersing the SPME coating directly into the extract (Alnajim et al., 2019).

From the point of view of genetics, Kocak et al. (2015) confirmed that the inherited changes in resistant strains of *T. castaneum* appeared according to the resistance level (Kocak et al., 2015), which reflect changes in certain biochemical processes that are important for the phosphine resistance mechanism (Schlipalius et al., 2012). The possibility of the existence of metabolic differences between resistant and susceptible strains led to this study on the proposition that there are differences in hydrocarbon quantities between susceptible and resistant strains of *R. dominica* and *T. castaneum* and that this difference may have a significant role in phosphine resistance. To provide more evidence about the difference between the hydrocarbon profiles of susceptible and resistant strains, the direct immersion solid-phase microextraction technique coupled with gas chromatography-mass spectrometry (GC-MS) was used to extract and analyze the cuticular hydrocarbons and the remaining hydrocarbons on or inside the bodies of the two species.

6.2 Material and Methods

6.2.1 The insect culture

Susceptible and resistant adult insects, *T. castaneum* (MUWTCSS-6000 and MUWTCSR) and *R. dominica* (MUWRDSS-7 and MUWRDSR-675) were provided by the Department of Primary Industries and Regional Development (DPIRD), Australia. Narrow aged insects (2-3 days) were reared by incubating 3000 adult insects with 1000 g of food, broken wheat for *R. dominica* and wheat flour/yeast 12:1 ratio for *T. castaneum* in 2-L jars sealed with meshed lids. The adult insects were removed after four days and the remaining cultural medium was incubated at 28±1°C and 70±2% RH. As new insects emerged, adults were transferred to new food to keep insects of the same age together. The insects used in the trials were one month old. The flour was made from newly collected wheat (Australian Standard Wheat). Before

using, the wheat was sterilized by keeping in freezer at -20°C for seven days, followed by storing at 4°C until use. The grain was prepared using a Wonder Mill (Model WM2000, Korea), and the flour was kept at 4°C.

6.2.2 Generation of phosphine gas and determination of resistance factor

Phosphine was generated by dissolving commercial tablets (Quickphos, United Phosphorus Limited (UPL), NSW, Australia) of aluminium phosphide in 10% (v/v) sulphuric acid solution. The final purity of phosphine was 86% (FAO, 1975)[28]. To determine the resistance factor, bioassays were implemented by using different concentrations of phosphine (control (air), 0.005, 0.01, 0.02, 0.03, 0.04, 0.1, 0.3, 0.6, 1, 2, 3 and 4 mg/L) with three replicates of each concentration. Fifty adult insects in 1000 mL flasks for each replicate were fumigated with phosphine for 20 h for both the susceptible and resistant strains followed by a one-week recovery period at 25±1°C and 65±5% RH.

6.2.3 Chemical reagents and apparatuses

The 50/30 µm fiber with combination coating of 2 cm of Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) was supplied by Sigma-Aldrich, Bellefonte, USA. Fiber was activated according to the manufacturer's recommendations by exposing its coating to 270°C for half an hour. The extraction was performed using acetonitrile ≥ 99.9% (*v/v*) (HPLC grade, Fisher Chemical, USA), 2 mL microtube (Benchmark Scientific, USA), 2 mL amber screw HPLC vials (Agilent Technology, USA), multiple volumes of micropipette (Dragon Lab, China), BeadBug microtube homogenizer (Benchmark Scientific, USA) and a Dynamica Velocity 13µ microcentrifuge (Dynamica Pty Ltd, United Kingdom).

6.2.4 GC-MS instrument and analytical conditions

All GC-MS analyses were performed with Agilent 7890B gas chromatography coupled with an Agilent 5977B mass spectrometer detector (MSD). The gas chromatographic system included a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm, RESTEK, Cat No 13423). The GC-MS was provided with a split/splitless injector and an SPME inlet (Supelco, Bellefonte, PA, USA), which operated under the splitless mode throughout the analysis. Helium was used as a carrier gas at a continuous flow of 1.2 mL/min. The injector temperature of the GC-MS was 270°C. The oven initial temperature was 60°C for 2 min, increased at a rate of 7°C/min to 200°C, at a rate of 5°C/min to 300°C and then finally at a rate of 50°C/min to

320°C and held for 3 min, with a total run time of 45.4 min. The ion source, MSD transfer line and quad-pole temperatures were 230, 300 and 150°C, respectively. Ionization energy was 70eV; scan acquisition mode was performed at scan ranged from 50 to 600 m/z at scan speed 10,000 μ/s.

6.2.5 The extraction and analytical procedures

Prior to extraction, all the insects used in this research were cleaned by allowing them to walk on wet tissue paper for 15 min and then transferred onto clean dry tissue paper for 10 min. For extraction, a direct immersion method as per (Alnajim et al., 2019) was used. The extraction of cuticular hydrocarbons was done with cleaned insects (20 insects of *T. castaneum* and 25 insects of *R. dominica*). The insects were transferred into 2 mL microtubes containing 1.6 mL of HPLC grade acetonitrile with a small clean brush and then the microtube was sealed with a screw cap. The microtubes were shaken gently by hand for 3 min and then the extract was moved into a 2 mL amber GC vial with septa using a micropipette. To provide more representative figures about the hydrocarbon content of the insect bodies, the hydrocarbons in the remaining insect bodies after extraction were homogenized using a Beadbug homogenizer in 2 mL BeadBug™ microtube containing 1.6 mL HPLC grade acetonitrile for 1 min at 4000 RPM and then centrifuged at 8150 xg for 3 min using the Dynamica mini centrifuge. The supernatant (1.5 mL) was transferred into a 2 mL amber GC vial with septa. The SPME fiber was inserted into the extract for 14 h at 25±2°C, immediately after completing the extraction and the fiber was withdrawn and injected directly into the GC-MS injector for identification of lipids.

6.2.6 Data processing and analysis

The GC-MS signals were collected by MassHunter Acquisition software (Agilent Technologies, USA). Automatic Mass Spectral Deconvolution and Identification System (AMDIS-32) software and NIST 2.2 mass spectra library were used to identify compounds. Kovat's retention index was used to support identification. Data arrangement and sorting were processed by Microsoft Excel 2016. The averages of the peak areas were statistically analysed by Metaboanalyst 4.0 (http://www.metaboanalyst.ca/faces/upload/StatUploadView.xhtml) by using T-test. Data were uploaded to Metaboanalyst 4.0 as columns (unpaired); data filtering was conducting using mean intensity value. Sample normalization, data scaling and data transformation were specified as a "NONE" mode. T-test was analysed at 95% confidence regions.

6.3 Results

6.3.1 Resistance factor

A probit model of concentration-mortality analyses showed that the LC50 of strains that were considered as susceptible in both targeted species was 0.009 mg/L while the LC50 for resistant strains were 1.042 and 0.26 mg/L for *R. dominica* and *T. castaneum* respectively. Consequently, the resistance ratio has been calculated according to the LC50 of the susceptible insects (RR = 115.77 fold for *R. dominica*) and (RR = 28.8 fold for *T. castaneum*).

6.3.2 Hydrocarbons profiles of susceptible and resistant strains

The total signal ions of the GC-MS chromatograms show the differences in the GC-MS response for hydrocarbon data (RT= 16.2 min and from RT= 31.4 to 39.63 min) obtained from the cuticular extraction and homogenized body extraction of susceptible and resistant strains of *T. castaneum* (Figures 6.1 and 6.2). Identification based on retention index values and the NIST database showed that the majority of hydrocarbon compounds detected in *T. castaneum* were long-chain n-alkanes and methyl-branched alkanes ranging from 25 (pentacosane) to 32 (dotriacontane) carbons (Supplementary materials Table 6.S1).

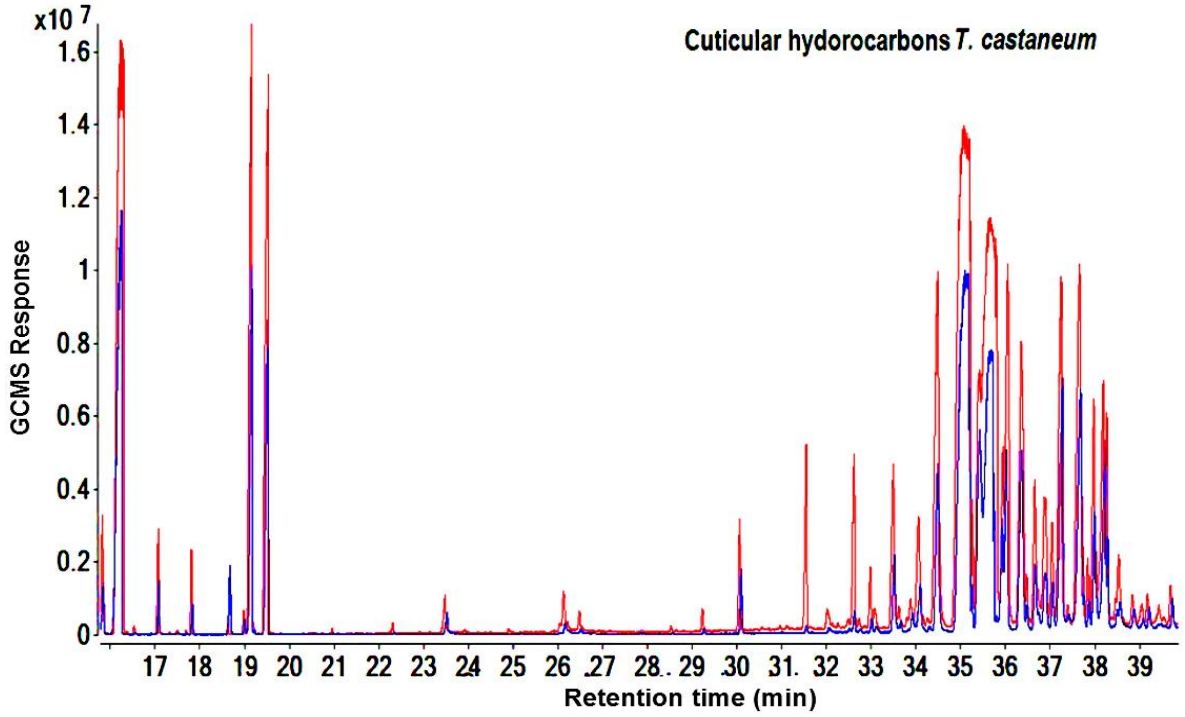

Figure 6.1. Total signal chromatograms show the differences of peak areas of cuticular hydrocarbons between resistant (red) and susceptible (blue) strains of *T. castaneum* using direct immersion solid-phase microextraction technique for extraction.

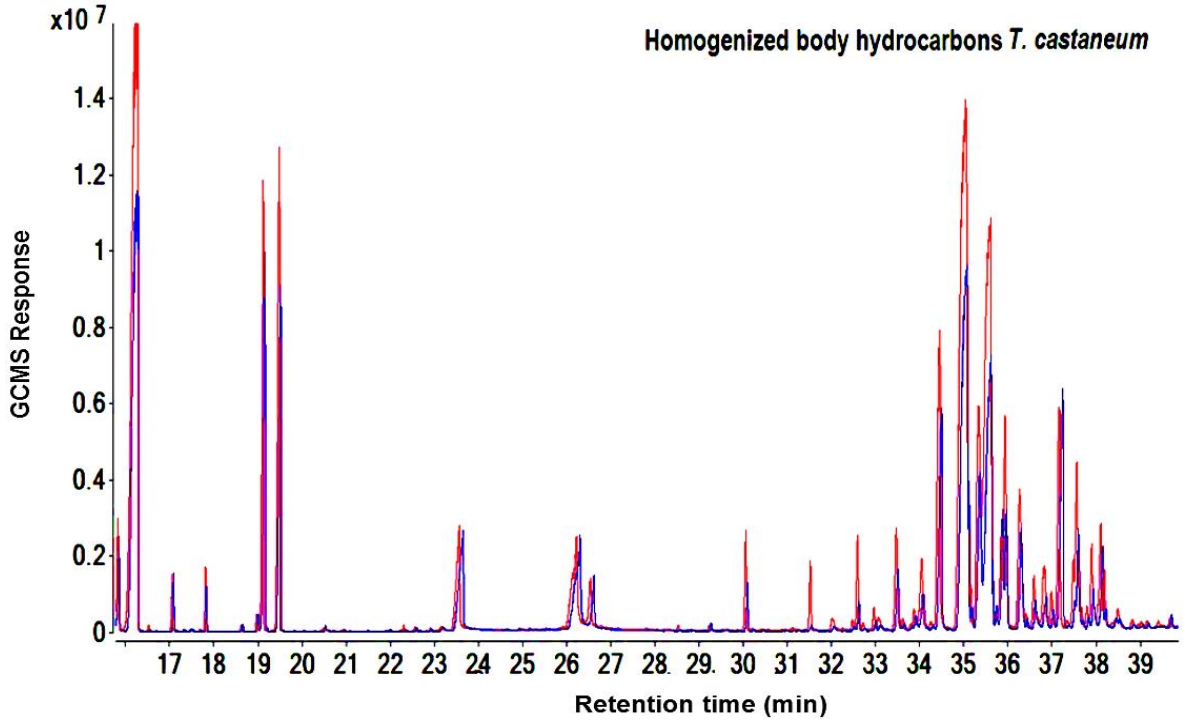

Figure 6.2. Total signal chromatograms show the differences of peak areas of homogenized body hydrocarbons between resistant (red) and susceptible (blue) strains of *T. castaneum* using direct immersion solid-phase microextraction technique for extraction.

Similar results for the compound categories of alkanes and methyl-branched and peak patterns were also seen in *R. dominica*. However, the hydrocarbons covered a larger area in the GC-MS chromatogram between RT: 31.4 to 45.1 min (Figures 6.3 and 6.4) and included higher molecular weight compounds that ranged from 26 (11-methylpentacosane) to 34 (tetratriacontane) carbons. (Supplementary materials Table 6.S2). Overall, GC-MS data from the cuticular extraction and homogenized body extraction of susceptible and resistant strains showed nearly the same GC-MS response regarding the number of hydrocarbon peaks and the fragmentation pattern. However, figures 6.1 to 6.4 clearly show variance in peak abundances.

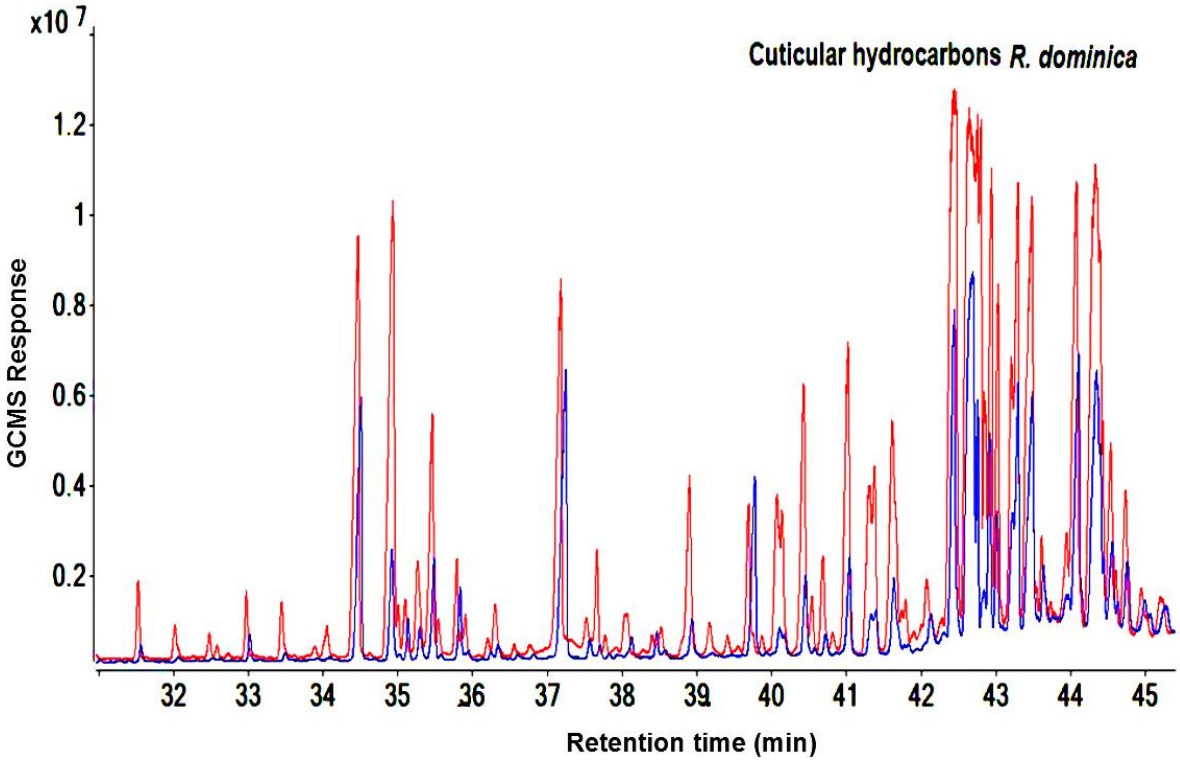

Figure 6.3. Total signal chromatograms show the differences of the peak areas of cuticular hydrocarbons between resistant (red) and susceptible (blue) strains of *R. dominica* using direct immersion solid-phase microextraction technique for extraction.

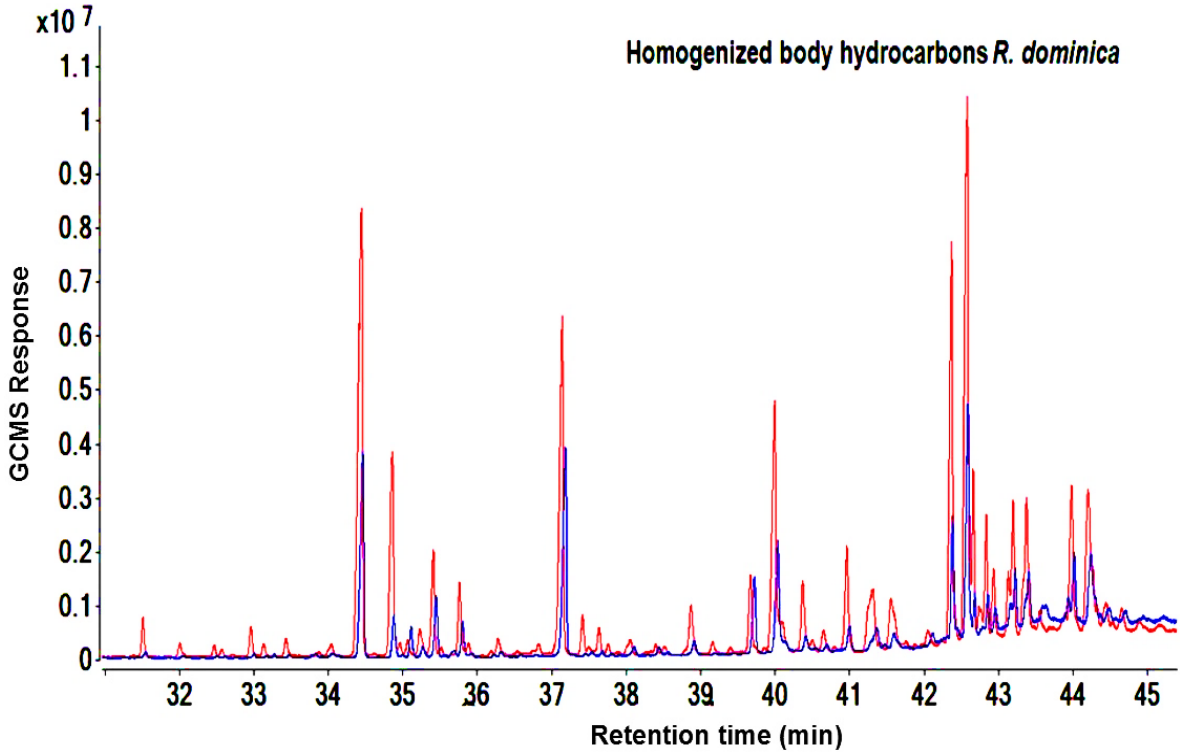

Figure 6.4. Total signal chromatograms show the differences of the GC-MS response (peak areas) of homogenized body hydrocarbons between resistant (red) and susceptible (blue) strains of *R. dominica* using direct immersion solid-phase microextraction technique for extraction.

To provide an inclusive indication of the differences in hydrocarbon quantities, statistical analysis was conducted using T-test. Results obtained from *T. castaneum* showed that, out of a total of twenty-two detected compounds, sixteen of cuticular hydrocarbon compounds and twenty-one from the homogenized body were found significantly different between the susceptible and resistant strains regarding their GC-MS response (Figure 6.5). The majority of the compounds (sixteen from cuticle and nineteen from the homogenized body) were found in significantly higher abundance in the resistant strain than in the susceptible strain (Supplementary materials Table 6.S1). In cuticular hydrocarbons of *T. castaneum*, 1-pentadecene, unknown1, pentacosane, unknown2, 13-methylheptacosane, 11-methylheptacosane, 2-methylheptacosane, 3-methylheptacosane, octacosane, 3-methyloctacosane, nonacosane, unknown3, unknown4, 2-methylnonacosane, 3-methylnonacosane and triacontane were all in significantly higher abundance in the resistant strain. 3-methylnonacosane exhibited the highest significant variance ($p=0.068\times10\text{-}3$) between the resistant strain and the susceptible strain, while 13-methylnonacosane, 11-methylnonacosane, 2-methylhexacosane, dotriacontane and hexacosane were similarly abundant in both susceptible and resistant strains. Statistical analysis of data from homogenized body of *T. castaneum* revealed that levels of 1-pentadecene, pentacosane, hexacosane, unknown2, 2-methylhexacosane, 11-methylheptacosane, 2-methylheptacosane, 3-methylheptacosane, octacosane, 3-methyloctacosane, nonacosane, unknown3, unknown4, 13-methylnonacosane, 11-methylnonacosane, 2-methylnonacosane, 3-methylnonacosane, triacontane and dotriacontane were all significantly higher in the resistant strain than in the susceptible strain. 2-methylheptacosane had the highest significant variance ($p=0.013\times10\text{-}3$). Abundance of 13-methylheptacosane was similar in both resistant and susceptible strains (Supplementary materials Table 6.S1).

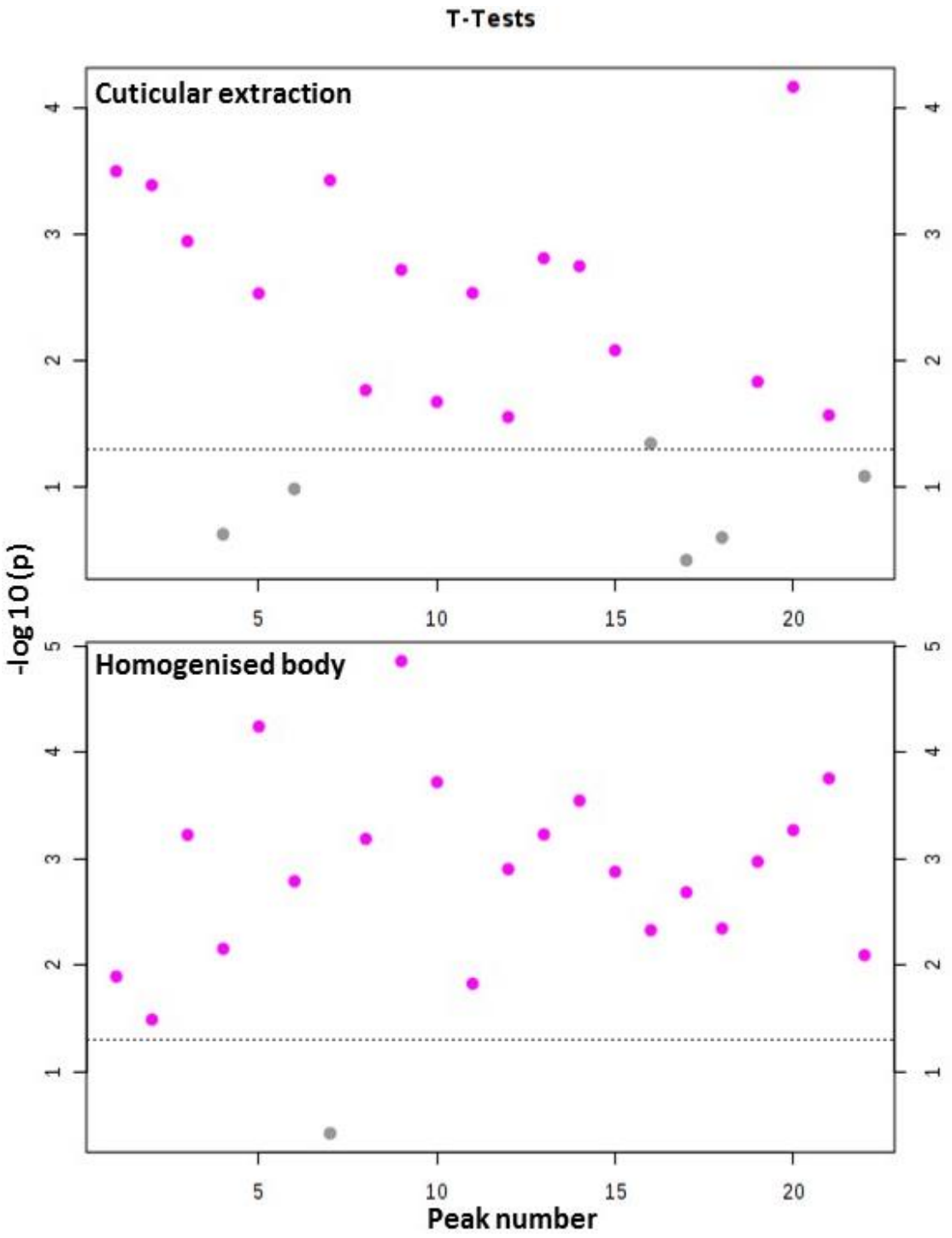

Figure 6.5. Number of hydrocarbons with significant difference obtained from analysis of cuticular and homogenized body hydrocarbons of phosphine resistant and susceptible strains of *T. castaneum* using t-tests with threshold 0.05. The red circles are features above the threshold; p values were transformed by -log10 to plot the more significant features (with smaller p values) higher on the graph.

In *R. dominica*, the GC-MS response of twenty cuticular hydrocarbons compounds, out of twenty-four compounds detected, was found to vary significantly between the two strains (Figure 6.6). Results for cuticular hydrocarbons revealed that the resistant strain produced significantly higher hydrocarbon levels (eighteen compounds) than the susceptible strain did. These compounds included unknown1, 11-methylpentacosane, 2-methylheptacosane, 3-methylheptacosane, 13-methylnonacosane, hentriacontane, 2-Methylhentriacontane, 3-Methylhentriacontane, dotriacontane, 10-Methyldotriacontane, 8-methyldotriacontane, unknown4, unknown5, unknown6, 2-methyldotriacontane, 15-methyltritriacontane, unknown8 and tetratriacontane. Results also showed that compound unknown5 had the lowest p-value ($p=0.00012\times10\text{-}3$) and, exhibited the highest statistical difference in abundance between the two strains. Statistical analysis also demonstrated that the compounds unknown2, 13-methylheptacosane, octacosane and unknown3 were not statistically different in their abundance between the susceptible and resistant strains with a p-value higher than the confidence region ($p\geq0.05$). The results from the homogenized body showed that twenty-two hydrocarbon compounds exhibited significantly different abundance between the susceptible and resistant strains (Figure 6.6). Twenty-one of these compounds were recorded to be in higher abundance in resistant insects than in the susceptible insects including unknown1, 11-methylpentacosane, 13-methylheptacosane, 2-methylheptacosane, 3-methylheptacosane, 13-methylnonacosane, triacontane, hentriacontane, 2-methylhentriacontane, 3-methylhentriacontane, dotriacontane, 10-methyldotriacontane, 8-methyldotriacontane, unknown4, unknown5, unknown6, 2-methyldotriacontane, unknown7, 15-methyltritriacontane, unknown8, and tetratriacontane. Moreover, 3-methylhentriacontane had the lowest p-value ($p=0.0027\times10\text{-}3$), exhibiting the greatest statistical difference between the two strains. Neither compound unknown2 or octacosane exhibited statistically different abundance in the resistant and the susceptible strains (Supplementary materials Table 6.S2).

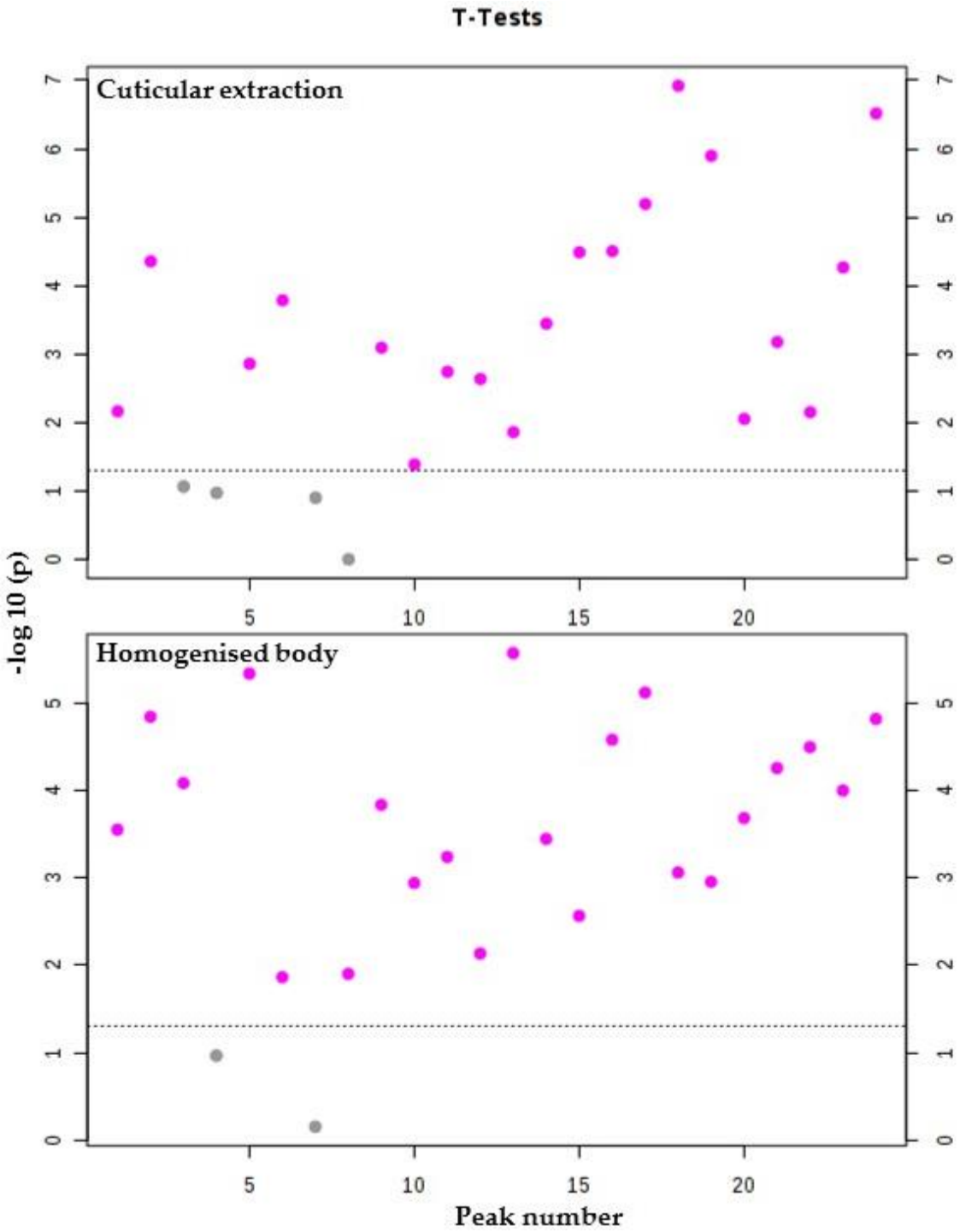

Figure 6.6 Number of hydrocarbons with significant difference obtained from analysis of cuticular and homogenized body hydrocarbons of phosphine resistant and susceptible strains of *R. dominica* using t-tests with threshold 0.05. The red circles are features above the threshold; p values were transformed by -log10 to plot the more significant features (with smaller p values) higher on the graph.

The fold change was another indicator taken into consideration to determine the difference in the GC-MS response (area) between the resistant and the susceptible strains. Results of the fold changes shown in Supplementary materials, Tables 6.S1 and 6.S2 reveal a higher quantity of hydrocarbons obtained from resistant strains than from susceptible insects in both species. High difference ratios were obtained for the majority of hydrocarbons obtained from *T. castaneum* ranging for cuticular hydrocarbons from 1.17 (13-methylnonacosane) to 8.24 fold (unknown1) and for homogenized body hydrocarbons from 1.24 (1-pentadecene) to 6.31 fold (unknown1) (Table 6.S1). However, the fold changes of cuticular hydrocarbons obtained from *R. dominica* were between 1.28 (octacosane) and 59.17 fold (11-methylpentacosane) and for the homogenised body hydrocarbons from 1.07 (octacosane) to 8.15-fold (13-methylnonacosane) (Supplementary materials Table 6.S2).

6.4 Discussion

6.4.1 Resistance factor

The susceptibility of *R. dominica* and *T. castaneum* obtained from populations which were selected as susceptible was very close to the FAO (1975) standard (FAO LC50 0.008 mg/L for 20 hours exposure). The resistance ratios were in a similar range as reported for *R. dominica* and *T. castaneum* from different areas (Jagadeesan et al., 2012; Mau et al., 2012).

6.4.2 Hydrocarbons of susceptible and resistant strains

The aim of studying the variance in quantities of hydrocarbons between the phosphine susceptible and resistant strains of *T. castaneum* and *R. dominica* was to investigate the contribution of hydrocarbons to the phosphine resistance mechanism of resistant strains which avoids the toxic effect of phosphine. The hydrocarbon profiles of *T. castaneum* and *R. dominica* are qualitatively very different and it is possible to differentiate the two species by their chemicals profiles detected and identified from chromatograms (Figures. 6.1 to 6.4). In both species, hydrocarbon profiles mainly consisted of n-alkenes and methyl-branched alkenes (Supplementary materials Tables 6.S1 and 6.S2). The higher amount of hydrocarbons in the resistant insects than in the susceptible ones indicated that their resistance levels affected cuticular lipids metabolism which could contribute to understanding the mechanism of phosphine resistance. Higher levels of hydrocarbons in cuticle of resistant insect strains play an important role to prevent the penetration of phosphine into the insect bodies, and thus further reduce the fumigant toxicity. Nakakita and Kuroda (1986) have suggested that phosphine might

penetrate the insect body through the cuticle layer, although no evidence was provided. This function of hydrocarbons may reduce the amount of phosphine that reaches the mitochondria, which are the target site of phosphine (Price, 1980a; Zuryn et al., 2008). This causes a reduction in the toxicity of the fumigant because no pesticide can perform unless it reaches the target site in the insect body (Pratt, 2003). Our results are consistent with previous studies which indicated that the inhibitory function of phosphine only acts on mitochondria of resistant organism strains in vitro (Dua & Gill, 2004; Pratt, 2003; Price, 1980b), with little or no effect of the fumigant on resistant strains treated in vivo (Price, 1980a; Zuryn et al., 2008). This finding was confirmed when, after treatment with phosphine, the respiration of live insects and nematodes from the phosphine-resistant strains of *R. dominica* and *Caenorhabditis elegans* was not inhibited as much as it was in susceptible strains (Price, 1980a; Zuryn et al., 2008), while the inhibition of respiration of isolated mitochondria of the resistant types treated with phosphine was similar to the response by susceptible types (Price, 1980a; Zuryn et al., 2008). This similar response of both susceptible and resistant strains in vitro proves that there are further mechanisms involved in phosphine resistance (Price, 1980a) and the elimination of phosphine which reduce or avoid phosphine toxicity (Price, 1984). And, despite expectations, the exclusion ratio of phosphine is increased in resistant insects when phosphine concentration is increased (Price, 1981). Therefore, to control resistant insects, extending the treatment time is more effective than increasing the phosphine dosage (Dyte et al., 1983). However, no evidence or plausible explanation has been provided for the mechanism of phosphine elimination. Physical actions like the closure of the spiracles were reported to make a small contribution to preventing phosphine from penetrating insect bodies (Bond & Monro, 1967), but our results demonstrate that cuticular hydrocarbons are also a phosphine barrier in resistant insects. The unique physical properties of these insect hydrocarbons such as their high melting point, mean that they form a barrier on the insect body which varies depending on the carbon chain length and the location of the methyl group in the methyl-branched alkane compounds. The high melting points of these compounds allow them to be a significant factor in protecting insects (Gibbs & Pomonis, 1995). Therefore, the primary function of the cuticular hydrocarbons is to work as a barrier to protect insect bodies from harmful elements (Hadley, 1984). The action of cuticular hydrocarbons prevents the pathogens and chemicals from entering insect bodies (Cohen & Moussian, 2016; El-Sayed et al., 1991), including preventing the penetration of pesticides into the insect bodies (Nelson & Sukkestad, 1970).

For most insects, comparison of the composition of cuticular hydrocarbons from diverse environments shows the importance of these hydrocarbons (Gibbs & Pomonis, 1995). Insects affecting stored grain exhibit a unique hydrocarbon profile which allows them to survive the dusty, dry, and highly entomopathogenic environment inside grain stores (Howard & Lord, 2003). In stored-product insects, cuticular lipids have a stronger role because of the unique cuticular hydrocarbon composition of these insects in comparison with other insects (Howard & Lord, 2003). Moreover, the higher levels of hydrocarbons in the resistant strains of these insects result in elimination of higher levels of phosphine.

6.5 Conclusion

A direct immersion solid-phase microextraction technique followed by GC-MS analysis, was used to extract the hydrocarbons of both *R. dominica* (Fabricius) and *T. castaneum* (Herbst). Both resistant and susceptible strains of the two species studied exhibited a similar pattern regarding cuticular hydrocarbons, although the resistant strains had higher peaks in abundance based on the peak areas than the susceptible strains did. The higher levels of the hydrocarbons on the cuticles of the resistant strains appear to act to eliminate phosphine and protect the insects from its toxic effects.

Supplementary results

Table 6.S1. Extracted and identified hydrocarbon compounds from cuticle layer and homogenised body of *T. castaneum*.

No	ID	Compound name	NIST RI	Calcu-lated RI	GC-MS response (10^5) ± SD, n=4 Cuticular hydrocarbons		Fold change (A/B)	p value (10^{-3})	GC-MS response (10^5) ± SD, n=4 Homogenised body hydrocarbons		Fold change (A/B)	p value (10^{-3})
					Resistant (A)	Susceptible (B)			Resistant (A)	Susceptible (B)		
1	16.28_154	1-Pentadecene	1502	1504	1639±50	1017±161	1.61	0.31	1451±111	1172±113	1.24	7.90
2	31.48_253	Unknown1	-	2505	67±17	8±1	8.24	0.40	25±5	4±1	6.31	12.00
3	33.32_238	Pentacosane	2500	2515	195±8	122.07±24	1.60	1.10	110±15	53±9	2.09	0.59
4	33.89_266	Hexacosane	2600	2612	17±1	18±2	0.93	230.00	13±2	9±1	1.46	6.90
5	34.06_267	Unknown2	-	2618	123±8	72±20	1.72	2.90	78±5	42±5	1.87	0.057
6	34.27_294	2-methylhexacosane	2661	2684	7±1	6±1	1.29	100.00	8.78±1.69	4±0.4	2.18	1.30
7	34.73_296	13-methylheptacosane	2731	2741	509±63	236±43	2.16	0.37	235±67	274±46	0.86	370.00
8	34.82_309	11-methylheptacosane	2734	2750	2399±57	1799±363	1.33	17.00	1609±133	1024±122	1.57	0.64
9	35.02_336	2-methylheptacosane	2762	2766	50±7	29±4	1.71	1.90	31±2	11±2	2.76	0.013
10	35.58_337	3-methylheptacosane	2773	2771	2180±156	1584±352	1.38	21.00	1427±116	839±87	1.70	0.18
11	35.87_323	Octacosane	2800	2815	634±29	393±96	1.62	2.900	323±18	254±37	1.27	1.00
12	36.28_351	3-methyloctacosane	2872	2849	453±12	369±57	1.23	28.00	217±25	128±18	1.69	1.20
13	36.60_365	Nonacosane	2900	2902	134±10	80±17	1.67	1.50	51±6	27±3	1.88	0.58
14	36.85_324	Unknown3	-	2908	162±13	95±21	1.70	1.70	74±8	38±5	1.96	0.28
15	37.08_394	Unknown4	-	2911	91±4	62±14	1.45	8.30	36±7	15±2	2.44	1.20
16	37.49_378	Unknown5	-	2917	427±40	346±50	1.23	45.00	200±34	364±67	0.55	4.40
17	37.58_379	13-methylnonacosane	2930	2927	584±47	501±170	1.17	380.00	233±36	128±18	1.81	2.00
18	37.92_393	11-methylnonacosane	2939	2950	188±31	149±52	1.26	250.00	66±13	35±6	1.87	1.60

19	38.13_421	Nonacosane, 2-methyl-	2962	2961	409±28	282±69	1.45	14.00	156±16	95±13	1.65	0.64
20	38.44_395	3-methylnonacosane	2974	2973	87±6	39±8	2.21	0.068	40±6	18±2	2.17	0.53
21	39.64_239	Triacontane	3000	3003	35±5	2±5	1.44	27.00	16±1	10±1	1.59	0.17
22	42.37_449	Dotriacontane	3200	3203	41±2	37±4	1.12	75.00	12±2	7±1	1.70	4.60

The list consists of only the compounds that were identified; some compounds may present on the GC-MS chromatogram but are not presented due to the lack of the identification; ID includes the retention time and mass to charge ratio that used to identify the chemical; "Unknown" features are compounds that were identified as hydrocarbons by NIST database, but were reported as unknown because of the big difference between the NIST RI and the calculated RI (> 30 difference); RT=retention time; NIST RI=retention indices acquired from National Institute of Standards and Technology database (NIST); Calculated RI=retention indices calculated using n-alkane standard C7-C40; Fold changes obtained from dividing GC-MS response (areas) of resistant insects by the GC-MS response (areas) of susceptible insects; p values were generated by t-tests as ($p \leq 0.05$, n=4); ND=not detected.

Table 6.S2. Extracted and identified hydrocarbon compounds from cuticle layer and homogenised body of *R. dominica*.

NO	ID	Compound name	NIST RI	Calculated RI	GC-MS response (10^5) ±SD, n=4 Cuticular hydrocarbons		Fold change (A/B)	p value (10^{-3})	GC-MS response (10^5) ±SD, n=4 Homogenised body hydrocarbons		Fold change (C/D)	p value (10^{-3})
					Resistant (A)	Susceptible (B)			Resistant (C)	Susceptible (D)		
1	31.46_253	Unknown1	-	2515	35±9	12±3	2.77	6.90	15±4	ND	NA	0.28
2	31.94_281	11-methylpentacosane	2535	2555	330±61	6±1	59.17	0.044	7±1	2±0.3	3.65	0.014
3	32.91_295	Unknown2	-	2628	27±8	17±3	1.64	80.00	11±2	ND	NA	100.00
4	34.41_296	13-methylheptacosane	2731	2741	156±18	260±41	0.60	100.00	236±92	143±35	1.65	0.14
5	34.84_336	2-methylheptacosane	2762	2766	373±87	96±24	3.91	1.40	111±10	33±3	3.38	0.0046
6	35.34_337	3-methylheptacosane	2773	2771	73±15	70±10	1.03	0.16	60±9	35±11	1.70	13.00
7	35.72_323	Octacosane	2800	2815	73±15	57±10	1.28	120.00	33±9	31±6	1.07	700.00
8	37.10_394	Unknown3	-	2912	306±40	306±52	1.00	110.00	147±27	220±31	0.67	12.00

9	37.50_379	13-methylnonacosane	2930	2927	86±12	34±7	2.51	0.82	27±5	3±0.7	8.15	0.14
10	39.64_239	Triacontane	3000	3003	104±10	157±40	0.66	41.00	169±23	101±6	1.67	1.10
11	41.22_435	Hentriacontane	3100	3117	235±63	67±5	3.52	1.80	57±8	27±5	2.15	0.58
12	41.53_436	2-Methylhentriacontane	3162	3152	213±57	68±1	3.13	2.30	36±10	16±2	2.23	7.40
13	42.01_424	3-Methylhentriacontane	3172	3182	69±18	34±10	2.06	13.00	18±1	6±1	3.11	0.0027
14	42.37_449	Dotriacontane	3200	3203	750±130	266±33	2.82	0.36	208±37	72±10	2.89	0.36
15	42.50_477	10-Methyldotriacontane	3235	3218	1001±75	541±36	1.85	0.033	287±51	139±32	2.07	2.70
16	42.60_450	8-Methyldotriacontane	3240	3221	297±37	88±9	3.39	0.0031	47±5	16±3	2.96	0.026
17	42.78_464	Unknown4	-	3231	66±6	19±3	3.53	0.00064	11±1	3±1	3.57	0.0077
18	42.87_466	Unknown5	-	3237	261±8	96±9	2.71	0.00012	57±12	20±3	2.86	0.89
19	42.96_478	Unknown6	-	3249	204±13	61±7	3.34	0.0012	37±7	15±2	2.44	1.10
20	43.22_481	2-Methyldotriacontane	3263	3266	406±49	242±21	1.67	8.90	85±9	37±8	2.21	0.25
21	43.40_416	Unknown7	-	3276	435±86	240±16	1.81	0.67	83±6	38±7	2.17	0.056
22	44.03_463	15-Methyltritriacontane	3333	3323	435±86	242±44	1.80	7.00	84±8	30±5	2.84	0.032
23	44.47_479	Unknown8	-	3351	951±87	443±49	2.15	0.054	159±16	61±14	2.59	0.10
24	44.88_492	Tetratriacontane	3400	3387	134±5	56±4	2.40	0.0003	25±1	9±2	2.82	0.015

The list consists of only the compounds that were identified; some compounds may present on the GC-MS chromatogram but are not presented due to the lack of the identification; ID includes the retention time and mass to charge ratio that used to identify the chemical; "Unknown" features are compounds that were identified as hydrocarbons by NIST database, but were reported as unknown because of the big difference between the NIST RI and the calculated RI (> 30 difference); RT=retention time; NIST RI=retention indices acquired from National Institute of Standards and Technology database (NIST); Calculated RI=retention indices calculated using n-alkane standard C7-C40; Fold changes obtained from dividing GC-MS response (areas) of resistant insects by the GC-MS response (areas) of susceptible insects; p values were generated by t-tests as ($p \leq 0.05$, n=4); ND=not detected.

Statement of Contribution

Title of Paper	Comparison glycerolipids and phospholipids from phosphine susceptible and resistant strains of *Tribolium castaneum* (H.) and *Rhyzopertha dominica* (F.)
Publication Status	○ Published ○ Accepted for Publication ○ Submitted for Publication ◉ Unpublished and Unsubmitted work written in a manuscript style
Publication Details	

Principal Author

Name of Principal Author (Candidate)	Ihab alnajim
Contribution to the Paper	Concept, laboratory works, data analysis and writing manuscript
Overall percentage (%)	65%
Signature	[redacted] Date: 25/ 7/ 2019

Co-Author Contributions

By signing the Statement of Contribution, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate to include the publication in the thesis; and
iii. the sum of all the co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Xin Du
Contribution to the Paper	Laboratory work and editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	Tao Liu
Contribution to the Paper	Concept, editing manuscript
Overall percentage (%)	5%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Supervision and editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Name of Co-Author	YongLin Ren
Contribution to the Paper	Concept, supervision and editing manuscript
Overall percentage (%)	10%
Signature	[redacted] Date: 26/ 7/ 2019

Please cut and paste additional co-author panels here as required.

Chapter 7

Comparison Glycerolipids and Phospholipids from Phosphine Susceptible and Resistant Strains of *Tribolium castaneum* (H.) and *Rhyzopertha dominica* (F.)

Abstract

Insects rely on lipids as their main sources, for the provision of energy needed to perform various bioactivities. Flight, growth, diapause, and metamorphosis are essential processes that cannot be performed without the existence of a sufficient amount of lipids. The lipid composition is affected by ecological, nutritional and genetic factors that relate to insect species. Phosphine resistant and susceptible strains of the two main stored-grain insects, *T. castaneum* and *R. dominica*, were tested to determine their lipid content. The lipid investigation consists of glycerolipids and phospholipids in order to determine their possible roles in phosphine resistance. Liquid chromatography-mass spectroscopy (LCMS) was used to analyse glycerolipids and phospholipids samples. The results showed differences regarding the majority of the lipids that were investigated. The resistant insects of both species have a higher amount of lipids than the susceptible insects.

The lipid content of phosphine resistant and susceptible strains of *T. castaneum* and *R. dominica* was significantly different. Resistant strains showed a higher amount of glycerolipids and phospholipids. The role of these differences as an important factor in the mechanism of phosphine resistance was also discussed.

7.1 Introduction

Phosphine is the most effective fumigant that is being used to control stored product pests (Chaudhry, 1997). However, A strong resistance to phosphine was reported in most of the stored grain insects like the lesser grain borer, *Rhyzopertha dominica* (F.), in the flat grain beetle, *Cryptolestes ferrugineus* (S.), and in the rust-red flour beetle, *Tribolium castaneum* (H.) (Collins, 1998; Jagadeesan et al., 2012; Jagadeesan et al., 2016). A study on the dihydrolipoamide dehydrogenase (DLD) gene that contributes to biochemical processes showed the importance of this enzyme in phosphine resistance (Schlipalius et al., 2012). Hence, it is undoubtedly that biochemical differences, such as lipid composition occurred because of the expected different metabolic processes between the susceptible and resistant insects.

The composition of Lipids occur naturally, and lipids have an essential role in the metabolism of insects and plants (Ad et al., 1985). Lipids are the most abundant content of some insect bodies, reaching 75% based on their dry weight (Pino Moreno & Ganguly, 2016). The obvious advantage of using lipids for reproduction, transformation and flight explains the important role of these components in the continuation of insects on this planet (Gilbert & Chino, 1974).

Additionally, lipids are considered to be the main reserved form of energy that can be used by insects for different bioprocesses like diapause (Hahn & Denlinger, 2007), growth (Ziegler & Van Antwerpen, 2006) and flight (Beenakkers et al., 1984). The composition of lipids is influenced by many factors which include genetic, ecological conditions and nutritional status; therefore, lipids varied across insect species (Ad et al., 1985). Lipids are stored in the form of triglycerides (TGs) inside cells called fat bodies that are the main cells to provide the insects with their energy requirements by storing large quantities of triglycerides (Arrese & Soulages, 2010). TGs can be stored in an anhydrous form thereby allowing the use of lipids as an essential substance for metabolism, which allows the accumulation of a large reservoir of energy that can be used during long periods of energy demand (Downer & Matthews, 1976). Diglycerides (DGs), on the other hand, are the major lipids in the insect hemolymph (Chang, 1974). The conversion pathway of TG to DG was suggested to be through the cleavage of TG to monoglycerides (MG) followed by the conversion to DG, releasing one fatty acid to the haemolymph (Arrese & Wells, 1997). The importance of DG was described in a study about locust flight when the amount of DG was levelled up in the haemolymph to threefold of its normal concentration to supply the energy requirements (Jutsum & Goldsworthy, 1976).

The phospholipids are a large group of lipids that contain a polar and non-polar end, consisting of two layers, hydrophobic layer that contains two fatty acids and hydrophilic layer of phosphate group connected by glycerol or alcohol (Li et al., 2015; Singh et al., 2017). The importance of phospholipids is derived from their main function as a major part of cellular membranes, it acts as a barrier to separate the cells from the surrounding environment and allow each cell to perform its specific function (Bohdanowicz & Grinstein, 2013).

From the phosphine mode of action, three possible factors may contribute to phosphine resistance: respiratory, neural and metabolic (Nath et al., 2011). The difference in the metabolism of the susceptible and the resistant strains may prevent metabolic crises or supplies the energy demand in resistant individuals(Nath et al., 2011). This may be affected by the genetic differences that were previously recognised in many resistant insect species(Nguyen, 2016). The possibility of the existence of metabolic differences between resistant and susceptible strains led us to implement this study and assume that there are differences in the amount of lipids between susceptible and resistant strains of *R. dominica* and *T. castaneum* and that this difference may have a significant role in phosphine resistance.

7.2 Materials and Methods

7.2.1 The insect cultures

Susceptible and resistant Adult insects, *Tribolium castaneum* (MUWTCSS-6000 and MUWTCSR) and *Rhyzopertha dominica* (MUWRDSS-7 and MUWRDSR-675) were obtained from the Department of Primary Industries and Regional Development (DPIRD), Australia. Narrow aged insects (2-3 days) were cultured by incubating 3000 adult insects with 1000 g of food, broken wheat for *R. dominica* and wheat flour/yeast 12:1 ratio for *T. castaneum* in 2-L jars sealed with meshed lids. The Parents' insects were removed after four days and the remaining cultural medium were incubated at 28±1°C and 70±2% RH. As new insects emerge, the adults were transferred to a new food to keep insects with narrow age together. The insects used in the experiments were one month old. The flour was made from freshly harvested wheat (Australian Standard Wheat). Before using, the wheat was sterilised by keeping at -20°C freezer for seven days and were stored at 4°C until use. The grain was milled using a Wonder Mill (Model WM2000, Korea), and the flour was kept at 4°C.

7.2.2 Generation of phosphine gas and determination of resistance factor

Phosphine was generated by dissolving commercial tablets (Quickphos, United Phosphorus Limited (UPL), NSW, Australia) of aluminium phosphide in 10% sulphuric acid solution, and the final purity of phosphine was 86% (FAO, 1975). To determine the resistance factor, bioassays were implemented by using different concentrations of phosphine (control (air), 0.005, 0.01, 0.02, 0.03, 0.04, 0.1, 0.3, 0.6, 1, 2, 3 and 4 mg/L) with three replicates of each concentration. Fifty adult insects in 1000 mL flasks for each replicate were fumigated with phosphine for 20 h for the susceptible and resistant strains followed by a one-week recovery period at 25±1°C and 65±5% RH.

7.2.3 Chemicals and apparatuses

The extraction and analysis of lipids were performed using the following materials: acetonitrile ≥ 99.9% *v/v* (Fisher Chemical, USA), methanol ≥ 99.9% *v/v*, 2-propanol ≥ 99.9% *v/v* and chloroform ≥ 99.9% *v/v* (Sigma- Aldrich, USA). The extraction was performed using a 2 mL micro tube (Benchmark Scientific, USA), 2 mL clear screw HPLC vials (Agilent Technology, USA), different volumes of micropipettes (Dragon lab, China), bead bug micro tube homogenizer (Model DI030-E, Benchmark Scientific, USA), Dynamica Velocity 13µ micro

centrifuge (Dynamica Pty Ltd, United Kingdom)., and an ultrasonic cleaner (Omegasonics, Model PS-20A, USA).

7.2.4 Ultra performance liquid chromatography-quadrupole-mass spectrometry (UPLC-Q-Tof-MS) and analytical conditions

Samples were analysed using Waters Acquity UPLC-Q-Tof. Data acquisition and processing were performed using the Masslynx software (version 4.1).

Lipids: The compounds separation was performed on a Waters Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm). The binary gradient run consisted of eluents A (60% acetonitrile: 40% water (*w:w*)) and B (90% 2-propanol: 10% acetonitrile (*w/w*)) with 10 µM ammonium formate and 0.1% formic acid at room temperature with a flow rate at 0.25 mL/min and a 2 µL injection volume. Optimal separation was accomplished using the following solvent gradient elution: mobile B started with 40%, increased to 92.1% (1−16 min), then ramped back to 40% (17-17.5 min), followed by two and half minutes of re-equilibration with a total run time of 20 min. All features were analysed in a positive ionisation mode and were monitored in a full scan mode. The optimum MS parameters were as follows: capillary voltage 3.1 kV, sample cone 45 V, extraction 5.0 V, ion guide voltage 3.0 V, desolvation gas temperature 350°C with 350 L/min, collision cell 0.6 mL/min of UHP Argon and detector voltage 1820V.

Sugar: Compounds separation was implemented on a shodex asahipak NH2P-40 2D column (2.0 I.D X 150 mm). The binary gradient runs involves eluents A (0.1 % formic acid in acetonitrile (v:v)) and B (chloroform) at room temperature with a flow rate at 0.15 mL/min and a 3 µL injection volume. Optimal separation was accomplished using the following solvent gradient elution: mobile B started with 0.5%, increased to 36.0% (1−10 min), 60.0% (10-11 min) and 99.0% (11-17 min), then ramped back to 0.5% (17-18.5 min), followed by one and half minutes of re-equilibration with a total run time of 20 min. All features were analysed in a negative ionization mode and were monitored in a full scan mode. Similar MS parameters as lipids were used except the capillary voltage which was 2.1 kV.

7.2.5 Extraction procedures

All the insects used in this research were cleaned by allowing them to move on a wet tissue paper for 15 min, and then the insects were transferred into clean dry tissue paper for 10 min. The cleaned insects were frozen to death and stored using liquid nitrogen. Adult insects were collected in 2 mL micro tube using a small clean brush.

7.2.5.1 Total lipids content, glycerolipids and phospholipids

Fifteen adult insects were homogenized (0.01799±0.00081 g and 0.01881±0.00134 g for resistant and susceptible strains of *R. dominica* respectively), and (0.02255±0.00071 g and 0.02459±0.00117 g for resistant and susceptible strains of *T. castaneum* respectively) in 1 mL of chloroform/methanol (2:1, *w/w*) after adding three milling balls using the beadbug micro tube homogenizer at 400 RPM for 1 min. The supernatant was transferred into a 2 mL GC clear vial, which was already weighed as (W_{vial}) after filtering using 3 mL syringe (Terumo Syringe, Australia) coupled with 13 mm 0.2 µm Agilent Captiva Econo Filters (Agilent Technologies, USA). The chloroform/methanol extract was blown to dryness under general nitrogen flow. The same vial was weighed as ($W_{vial+lipids}$) for calculating total lipids weight (W_{lipids}) according to the following equation:

$$(W_{lipids}) = (W_{vial+lipids}) - (W_{vial})$$

After calculating the total lipids, 600 µl of HPLC solvent (Isopropanol/Acetonitrile/Water (2:1:1, *w/w/w*) was added to reconstitute the dried lipid components for UPLC-Q-Tof analysis. The ultrasonic cleaner was also used to assist the dissolution of the lipids as an observation.

7.2.5.2 Extraction of sugars

Fifteen adult insects were homogenised in 600 µl methanol/water (20:2, *w/w*) using the beadbug micro tube homogenizer after adding three milling balls at 400 rpm for 1 min. The extract was centrifuged at 13, 500 rpm for 3 min using the Dynamica Velocity 13µ micro centrifuge. Four hundreds µl of the supernatant were transferred into an insert glass that was installed inside a 2 mL clear screw HPLC vial. The extract was injected directly into the LCMS same day of the preparation.

7.2.6 Data processing and analysis

All the samples were analysed in four biological replicates. The LCMS data samples were analysed as one patch to ensure that the parameters will be applied equally in all the samples. Peak deconvolution, filtering, scaling and integration were extracted and aligned using the MassLynx software (Waters, USA). Chromatographic peaks were extracted from 1 to 20 min with a retention time error window of 0.2 min and the mass spectral peaks detected ranged from 50 to 2000 m/z with a mass error window of 7 ppm. Data matrix consisting of retention time m/z was generated together with peak intensity based on peak area for all features. The mass spectra of the lipids were loaded into LIPID MAPS Lipidomics Gateway

(http://www.lipidmaps.org/tools/ms/lm_mass_form.php). The identification search was only restricted to two main lipids categories, which include glycerolipids and phospholipids. The following parameters were applied to get an appropriate identification: mass tolerance ±0.1 m/z and ion adducts of positive mode ([M+H] +) and ([M+NH4] +). The loaded spectra were compared with the matched spectra, which were obtained from the lipid maps to identify the compounds. The compounds with the highest spectrum match factor were chosen as the lipid compound candidates.

The qualitative analysis of sugar was limited to five types of sugar: glucose, fructose, maltose, trehalose, and sucrose. The identification was implemented by comparing the mass spectra obtained for the unknown samples with the mass spectra of external standard solutions of the above sugars. Individual external standards were purchased from Merck-Sigma Aldrich Co., D-(+)-Glucose ≥ 99.5%, D-(+)-Fructose ≥ 99%, D-(+)-Maltose ≥ 99%, Trehalose ≥ 99% and Sucrose ≥ 99.5%. All the individual external standard chemicals were prepared by dilution with methanol in 20 mL clear glass vial, the final concentration was 2.5 µM. Data were normalized with internal standards before statistical evaluation. Statistical analysis was employed to evaluate and visualise the data through MetaboAnalyst 4.0 using volcano plot analysis (Chong et al, 2018). Samples were uploaded to Metaboanalyst as columns (unpaired); data filtering were characterized by using mean intensity value. Sample normalization, data transformation and data scaling were specified as a "NONE" mode. Volcano plot was analysed at P-value threshold of 0.05 and fold change threshold ≥ 2.

7.3 Results

7.3.1 Lipids and sugar importance for survival

The lipid experiments were started with the evaluation of the total lipids content of both *T. castaneum* and *R. dominica* according to the Floch method (Folch et al., 1957). The results showed a significant difference in the quantity of total lipids (Figure 7.1). The resistant insects of both *T. castaneum* and *R. dominica* were shown to have more lipids than the susceptible insects. These results have led us to suggest that lipids have an important survival role with regard to phosphine resistance. Therefore, we evaluated the importance of lipids in respect to the use of these components for survival in comparison with sugar, and that was also to confirm the importance of lipids for resistant insects as a source of energy required to treat the toxic effect of phosphine. We determined the short and long term effect of starving on the amount of sugar and lipids in the resistant insect bodies. Quick consumption pattern of sugar was

observed from both *R. dominica* and *T. castaneum*. The sugar amount was virtually consumed after 1 day of starving in comparison with the 0 day, specifically with *T. castaneum*, whereas the lipids content of both species of insect was comparatively high until after 7 days of starving (Figures. 7.2 and 7.3).

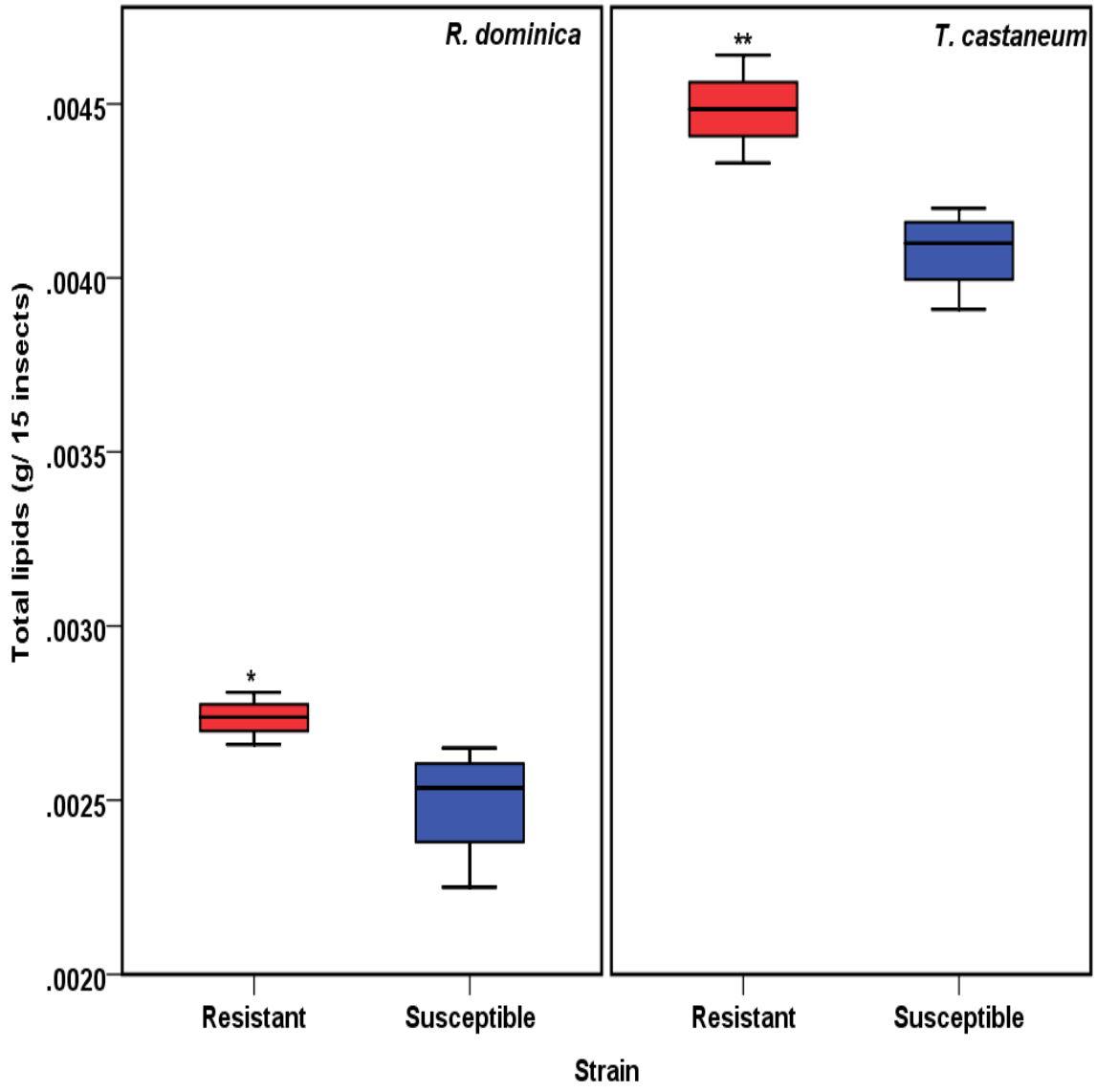

Figure 7.1. Total lipid content obtained from phosphine susceptible and resistant strains of *T. castaneum* and *R. dominica*. Each value in the figure represents four different biological insect sets. The values were statistically analysed by T-test. * = P≤0.01, ** = P≤ 0.001.

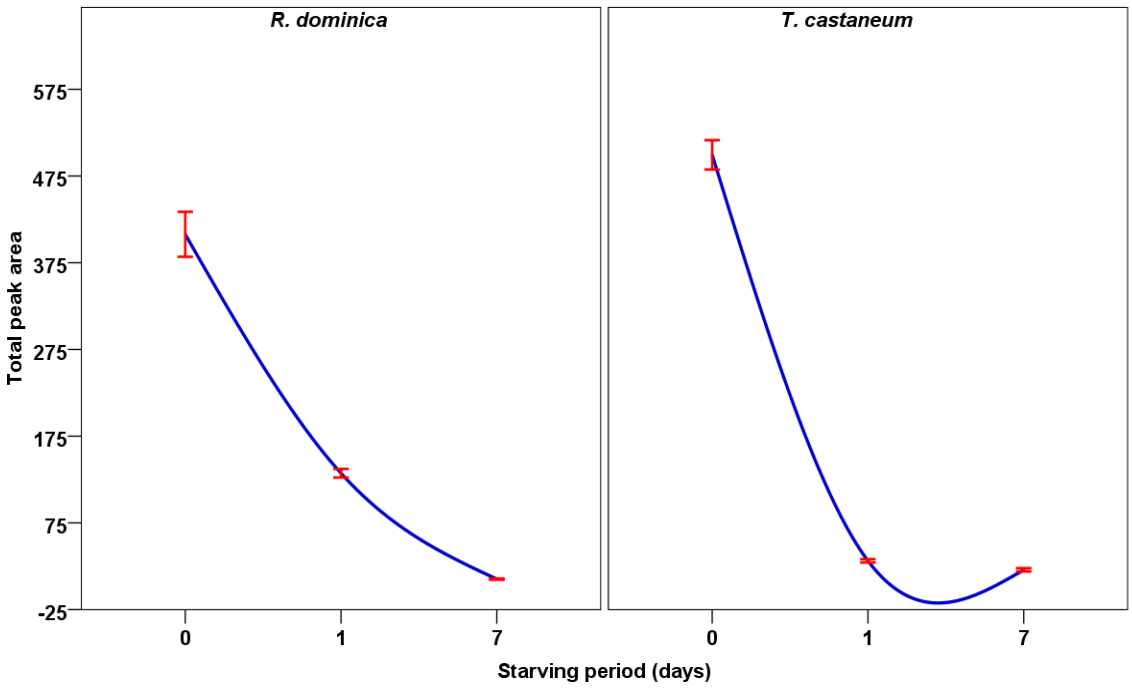

Figure 7.2. Effect of starving on the sugar content of phosphine resistant insects of *R. dominica* and *T. castaneum*.

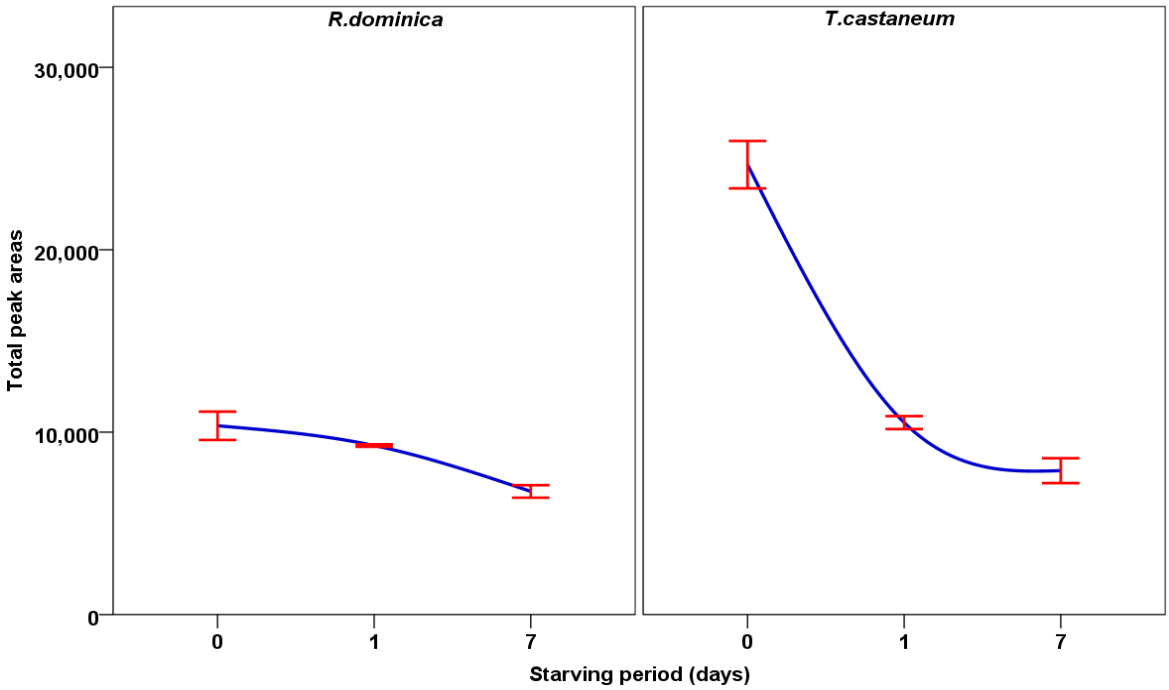

Figure 7.3. Effect of the starving on the lipids content of phosphine resistant insects of *R. dominica* and *T. castaneum*.

7.3.2 Glycerolipids and phospholipids from susceptible and resistant strains

Insect samples of susceptible and resistant strains of both the studied insects were tested to determine the differences with regard to the phospholipids and glycerolipids for the predictable role of these lipids in phosphine resistance. Significant variance was observed with regard to the major peaks of LCMS base peak intensities chromatograms when comparing susceptible and resistant strains of both insect species that were tested in this study. This might be an important observation that should be considered when studying phosphine resistance. A comparison of the lipids data obtained from the profiles of susceptible and resistant insects of the two studied species is shown in Figures. 7.4 and 7.5. The main difference between the susceptible and resistant strains of *T. castaneum* in the chromatograms was in the area between RT= 6.67 to 10.64 min (Figure 7.4). Whereas, the differences between the two strains of *R. dominica* includes majority of the base peak intensity chromatogram (Figure 7.5).

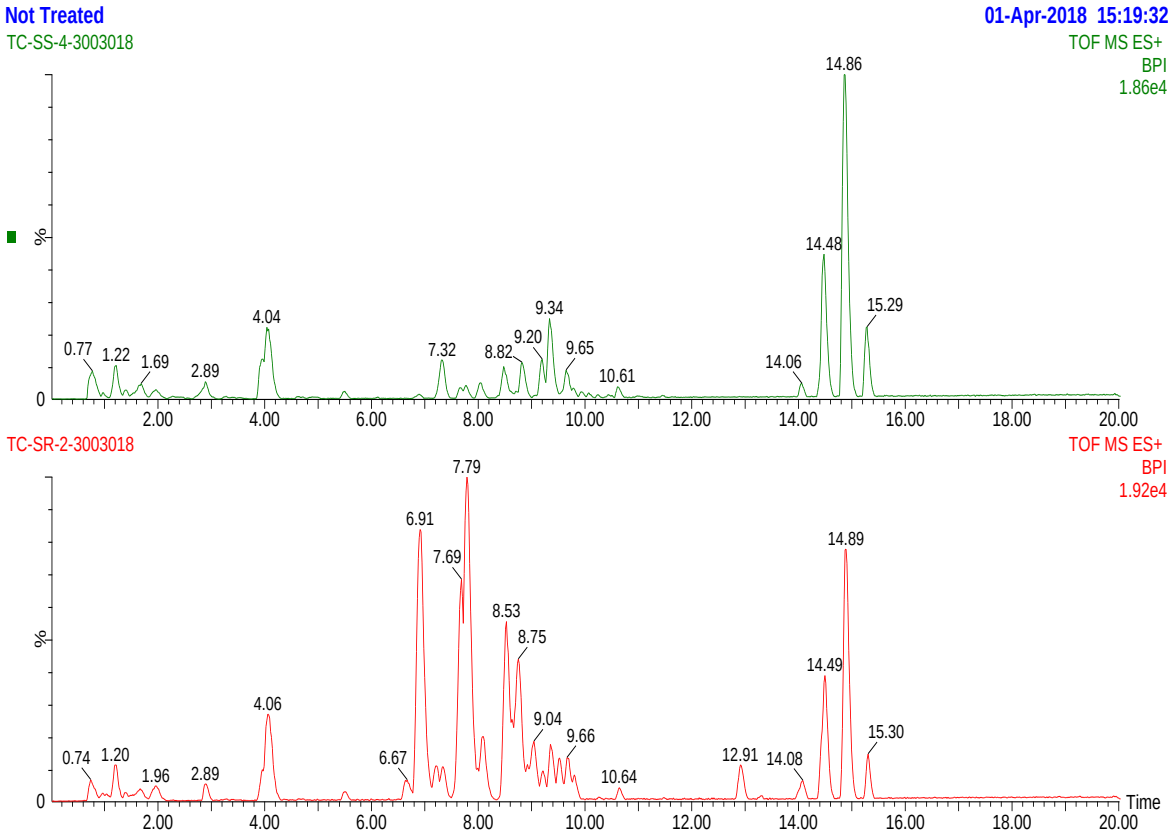

Figure 7.4. Base peak intensity (BPI) chromatograms are showing the differences of the lipid content obtained from susceptible (green) and resistant (red) strains of *T. castaneum*.

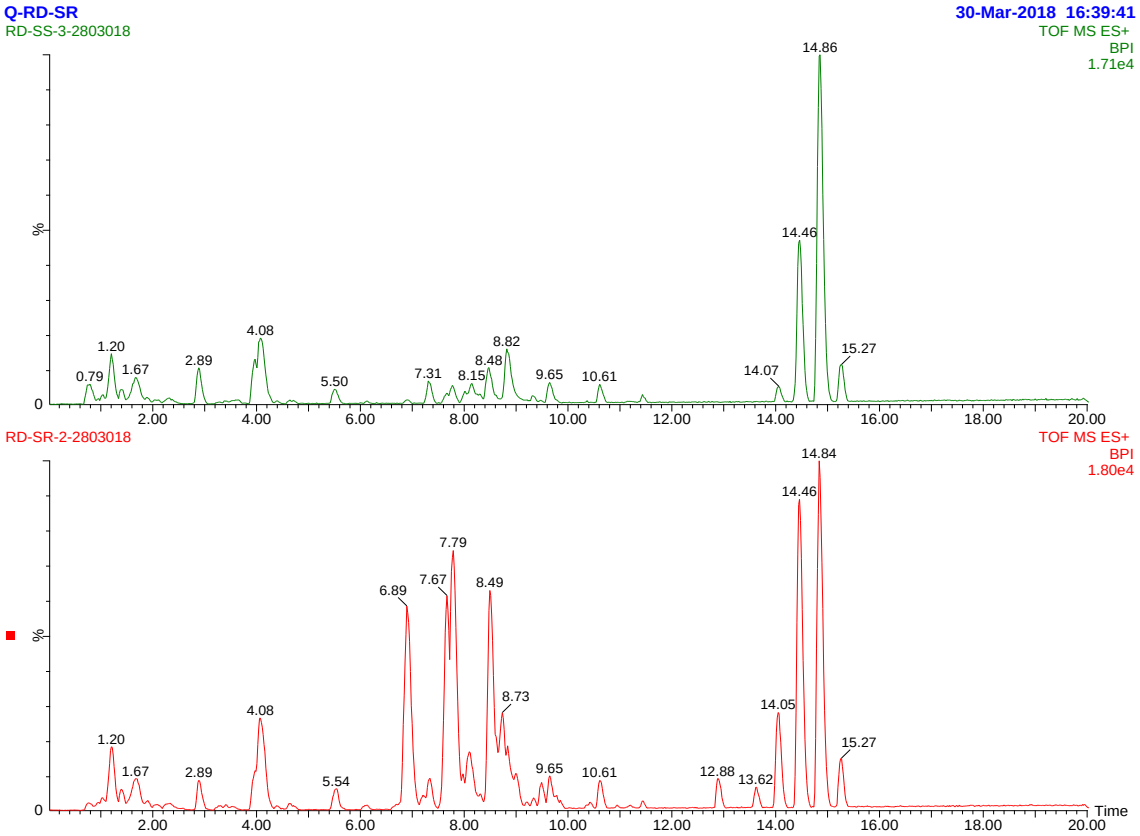

Figure 7.5. Base peak intensity (BPI) chromatograms are showing the differences in the lipid content obtained from susceptible (green) and resistant (red) strains of *R. dominica*.

As further investigated, the lipids obtained from the insect samples using LCMS were characterized. The identification was only specified to glycerolipids and phospholipids. Forty-five features of *T. castaneum* and sixty-seven from *R. dominica* were identified as lipids belong either to glycerolipids or phospholipids (supplementary materials Tables 7.S1 and 7.S2). The statistical analysis revealed significant differences regarding the two categories of lipids. The resistant insects showed higher compounds in abundance than the susceptible of both lipid categories (Figures. 7.6 and 7.7). For *T. castaneum*, seventeen features belong to glycerolipids, while eight from phospholipids were identified as significant features using Volcano plot statistical analysis (at P-value ≤ 0.05 and fold change ≥ 2). While eighteen features belong glycerolipids and eight under phospholipids were selected to be significant features between resistant and susceptible strains of *R. dominica* using Volcano plot statistical analysis. The fold changes results revealed a higher quantity of lipids obtained from resistant strain in comparison with susceptible insects in both species. High difference ratios were obtained for the majority of lipids obtained from *T. castaneum* ranged for glycerolipids from 1.13 to 53.10 fold and for

phospholipids from 1.05 to 20.00 fold (supplementary materials Table 7.S1). Whereas, the fold changes of glycerolipids for *R. dominica is* between 1.04 to 31.50 fold and for phospholipids from 1.04 to 10.10 fold (supplementary materials Table 7.S2).

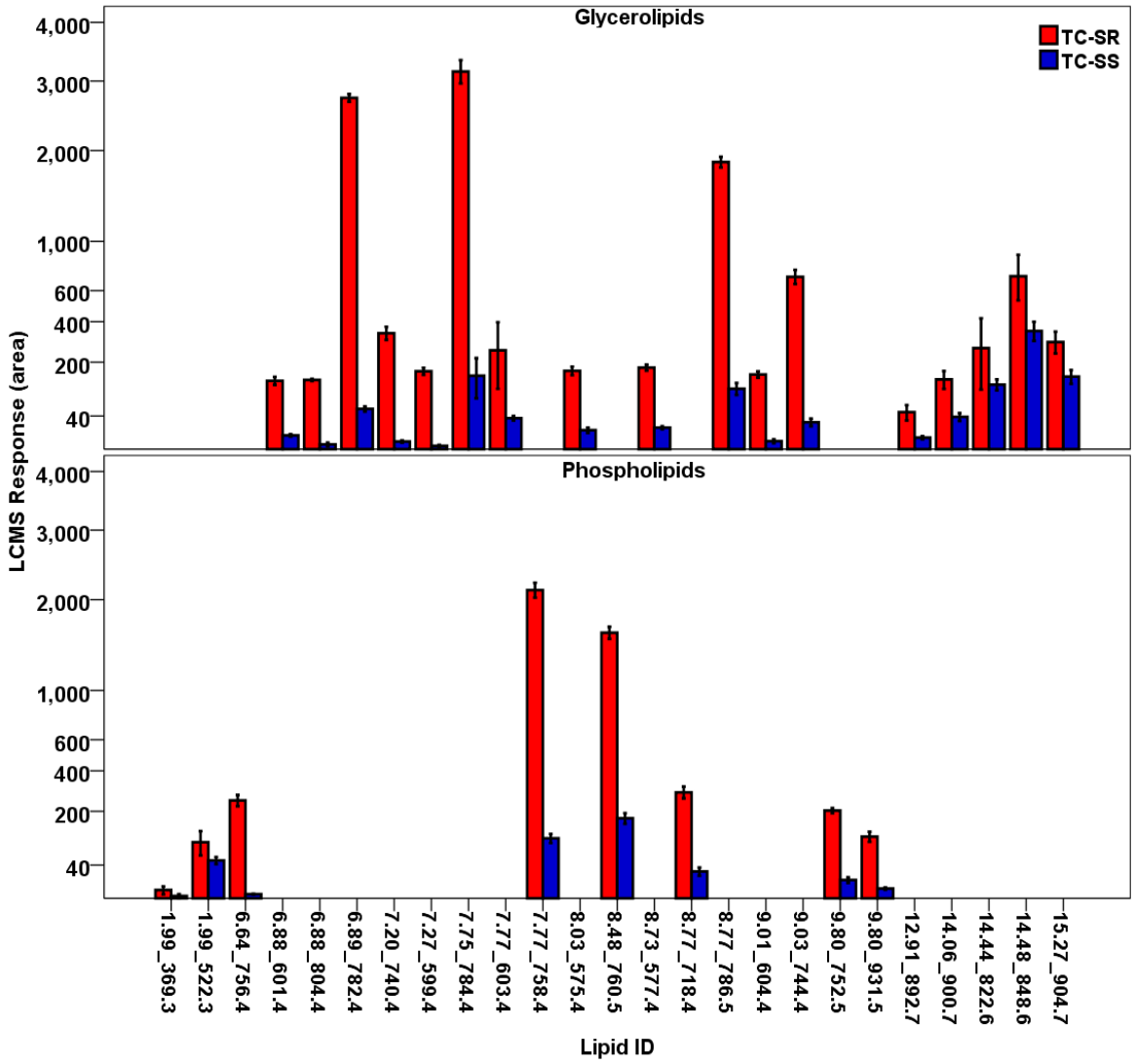

Figure 7.6. Lipids identified as significant features by Volcano plot ($p \leq 0.05$, $FC \geq 2$) obtained from phosphine susceptible (TC-SS) and resistant (TC-SR) strains of *T. castaneum*.

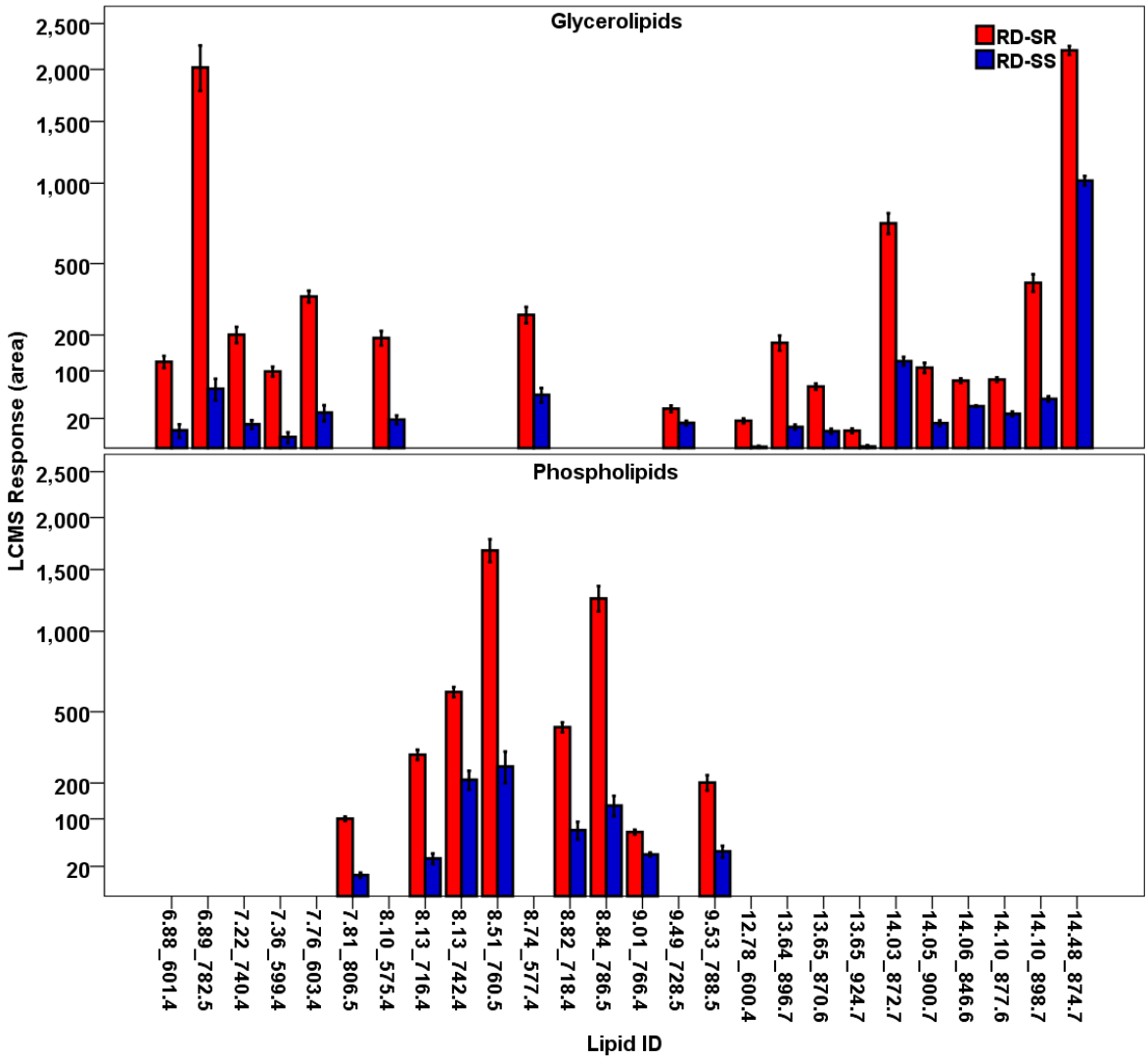

Figure 7.7. Lipids identified as significant features by Volcano plot ($p \leq 0.05$, $FC \geq 2$) obtained from phosphine susceptible (TC-SS) and resistant (TC-SR) strains of *R. dominica*.

7.4 Discussion

7.4.1 Lipids and sugar importance for survival

Our examination aimed to provide an overview of the variances of the lipid amounts presented in insect bodies of phosphine resistant and susceptible insects. The more lipids in resistant insects of both *T. castaneum* and *R. dominica* may show that lipids have significant role to protect resistant insects from phosphine. The result indicated that insects are rely more on lipids than sugar, since sugar was found to be consumed in short term starving while lipids continued to be at a high level even after long term starving, This because lipids are reserved to recover

the lack of energy, specifically during the starving periods (Arrese & Soulages, 2010). These results are consistent with previous studies findings that indicated that insects usually use sugar in the short term(Ziegler, 1991). On the other hand, lipids are suitable for long term use, by releasing diglycerides to the haemolymph to be used as an energy source (Ad et al., 1985; Ziegler, 1991). This was also approved by another study when the injection of trehalose caused a reduction in the lipid concentration of the haemolymph, thereby indicating that lipids are only used when trehalose concentration is low (Ad et al., 1985). The apparent proof that lipids are used over the long term is the constant using of lipids from the larval stage into pupae and early adult stage (Arrese & Soulages, 2010). The importance of that fact is that more lipids in resistant insects can provide more energy to survive the effect of phosphine that causes a decrease of energy, thereby affecting mitochondria.

7.4.2 Glycerolipids and phospholipids from susceptible and resistant strains

The finding that lipids are in abundance in the resistant strain with respect to the susceptible strain indicates the importance of these compounds to reduce the harm toxic effect of phosphine. That because lipids can play an important role by offering a consistent energy source to face up the energy requested. In addition, to protect the mitochondria from phosphine, which was reported to be a respiratory inhibitor in the mitochondria of insects and rats (Dua et al., 2010; Price & Dance, 1983), by disturbing the energy production of mitochondria (Nath et al., 2011). which shows why lack of energy is one of the plausible reasons for mortality due to exposure to phosphine (Nath et al., 2011; Price, 1980b). Therefore, a conventional explanation for the mechanism of phosphine toxicity is its effect on the metabolic rate (Nath et al., 2011). This was supported by different results, showing that artificially raising of energy demand increased the sensitivity of the *Caenorhabditis elegance* toward phosphine (Zuryn et al., 2008). In our study, lipids were acquired from whole insects and this shows that the amount of the lipid is higher in the resistant strain in comparison to susceptible insects. This outcome supports the thought that this high amount of lipids provides additional energy sources for survival from the toxic effect of phosphine. The results of this study include glycerolipids, which are considered as one of the main sources of energy. Glycerolipids includes triglycerides, which is served along with glycogen as the main energy stored in the insect bodies (Steele, 1982). However, glycogen can be consumed quickly in short-term usage (Ziegler, 1991). On the other hand, triglycerides have a higher caloric content than glycogen and they are the main sources of releasing fatty acids, which can be used for energy production (Athenstaedt & Daum, 2006). Stored fatty acids are utilised in a different form for many purposes such as the provision of

energy to perform the metabolic activities (Arrese & Soulages, 2010). That is why higher concentrations of triglycerides that were found in this study may explain a possible mechanism for resistant insects to avoid the effect of phosphine on the mitochondria, which causes a reduction in the energy that causes death.

Another advantage of lipids that may support the demand of energy caused by phosphine is that, fatty acids play a role in the synthesis of energy components like trehalose (McDougall & Steele, 1988) and proline (Gäde & Auerswald, 2002). Therefore, higher lipid content of the resistant strains regarding the glycerolipids like triglycerides and diglycerides may have a significant role to supply high amounts of fatty acids and support understanding of phosphine resistance. Higher content of diglycerides that can be obtained from different metabolic pathways in the resistant insects is very important when compared with susceptible strains which also play an essential role to these components as the main source for triglycerides synthesis (Arrese & Soulages, 2010). Diglycerides are also important because they are the core lipids that exist in insect hemolymph after triglycerides degradation (Tsuchida et al., 1998). According to their importance as explained above, the significant adifferences, regarding triglycerides and diglycerides between the resistant and susceptible strains obtained in this study is that the resistant insects are using these compounds to survive from phosphine especially after long-term exposure.

On the other hand, phospholipids were also found to be significantly higher in resistant insects in comparison with susceptible insects. This finding may also support the assumption that resistant insects are relying on lipids to survive from phosphine effect. This comes from the fact that phospholipids exist in the organisms as essential compounds to maintain life activity and are also vital components of cellular and semi cellular membranes (Singh et al., 2017). This function may reduce or prevent phosphine penetration to the cells, thereby causing more exclusion of phosphine, this is consistent with one of the accepted explanations of the phosphine resistance mechanism (Price, 1984). Additionally, phospholipids are essential materials for improving nerve cells function (Pepeu et al., 1996). And because of the effect on the neural system, is one of the toxicity mechanisms of phosphine (Al-Azzawi et al., 1990). Therefore, higher concentration of phospholipids in the resistant insects may improve the functions of the neural system in these insects. Furthermore, phospholipid of the mitochondrial membrane that is rich in unsaturated fatty acids play an essential role in mitochondrial energy by affecting the activity of proteins of the mitochondrial inner membrane (Hoch, 1992). Phospholipids also contribute a large amount to the mitochondrial membrane lipid environment

and have a significant role in the mitochondrial respiratory chain by affecting the physical properties of the mitochondrial membrane (Horvath & Daum, 2013). Hence, respiration was found to be affected by the reduction of the phospholipids amount due to the effect on mitochondria. Therefore, higher concentration of phospholipids in resistant strains may enhance mitochondria function and reduce phosphine toxicity.

In addition to the effect of the mitochondrial respiratory chain, the reduction of phospholipids was also observed to be synchronous with a significant reduction of adenosine triphosphate (ATP) (Baker et al., 2016). A study by Price and Walter (1987) on lesser grain borer *R. dominica*, demonstrated that ATPs were reduced from 2.75 to 1.64 nmoles/insect after treating the insect with phosphine. Phosphine causes a severe reduction of cytochrome oxidase activities and affects nicotinamide adenine dinucleotide (NAD) and succinic dehydrogenase activities, thereby leading to a reduction in respiration and causing a decline in the synthesis and ATP level (Dua et al., 2010). Therefore, improving the mitochondrial energy and raising the mitochondria function by higher content of phospholipids will certainly affect phosphine toxicity and energy production. Another benefit of the high amount of phospholipid in the resistant strain in comparison with susceptible strain is that the phosphatidic acids produced from the glycerophosphate pathway are also considered as one of the main sources of triglycerides composition. this allows the presence of more energy sources (Arrese & Soulages, 2010), which can provide more energy to the resistant insects to resist phosphine.

7.5 Conclusion

In conclusion, lipid content was investigated in phosphine resistant and susceptible strains of *R. dominica* and *T. castaneum*. The total lipid content was found to be higher in resistant strains in comparison with susceptible strains. Results obtained from using LCMS showed that the majority of glycerolipids and phospholipids in the resistant insects were higher than in the susceptible insects. The importance of the amount of lipids was discussed as a possible reason for the resistant insects' resistance to the fumigant, which comes from the importance of the lipids as the main sources of energy and their contribution to the cell walls.

Supplementary results

Table 7.S1. Glycerolipids and phospholipids candidates obtained from *T. castaneum*

No	Lipids ID	Input Mass	Matched Mass	Lipid category	Candidate lipid	Formula	Ion	LCMS response		Fold Change (A/B)
								Resistant (A)	Susceptible (B)	
1	1.99_369.3	369.3532	369.3727	phospholipids	LPA(13:0)	C16H34O7P	$[M+H]^+$	5.02±0.83	2.31±1.44	2.17
2	1.99_522.3	522.2812	522.2826	phospholipids	LPC(18:1)	C26H53NO7P	$[M+NH4]^+$	99.91±16.34	49.16±7.18	2.03
3	1.99_544.2	544.3822	544.3609	phospholipids	LPC(20:4)	C28H51NO7P	$[M+NH4]^+$	31.13±4.84	43.99±6.80	0.70
4	4.04_621.2	621.3114	621.3034	phospholipids	LPI(20:4)	C29H50O12P	$[M+H]^+$	832.66±52.05	790.97±101.41	1.05
5	4.04_666.2	666.3628	666.3613	phospholipids	LPI(22:4)	C31H57NO12P	$[M+NH4]^+$	68.50±8.76	61.30±13.01	1.11
6	5.60_457.2	457.2211	457.2561	phospholipids	LPG(14:0)	C20H42O9P	$[M+H]^+$	2.00±0.56	1.09±0.13	1.84
7	5.60_437.3	437.3476	437.3625	glycerolipids	MG(24:3),	C27H49O4	$[M+H]^+$	7.89±0.73	4.95±0.90	1.59
8	6.88_782.4	782.4642	782.4896	glycerolipids	DGDG(23:1)	C38H72NO15	$[M+NH4]^+$	2717.16±44.73	54.27±5.57	50.10
9	6.88_804.4	804.4523	804.474	glycerolipids	DGDG(25:4)	C40H70NO15	$[M+NH4]^+$	133.56±4.30	4.31±1.27	31.00
10	6.88_701.4	701.4454	701.4259, 701.5715	glycerolipids	MGDG(31:8), TG(41:4)	C40H61O10, C44H77O6	$[M+H]^+$	74.04±4.77	45.81±9.18	1.62
11	6.88_601.4	601.4694	601.4826, 601.4979	glycerolipids	MGDG(23:2), DG(35:5)	C32H57O10, C38H65O5	$[M+H]^+$	125.56±9.54	11.98±1.26	10.50
12	7.20_740.4	740.5026	740.5307	glycerolipids	MGDG(32:4)	C41H74NO10	$[M+NH4]^+$	338.85±42.19	6.39±0.85	53.10
13	7.27_599.4	599.4126	599.379, 599.467	glycerolipids	MGDG(23:3), DG(35:6)	C32H55O10, C38H63O5	$[M+H]^+$	159.43±10.07	3.36±0.63	47.50
14	7.22_703.4	703.4723	703.4416, 703.5871	glycerolipids	MGDG(31:7), TG(41:3)	C40H63O10, C44H79O6	$[M+H]^+$	273.52±50.17	354.10±23.76	0.77
15	7.75_784.4	784.4761	784.5053	glycerolipids	DGDG(23:0)	C38H74NO15	$[M+NH4]^+$	3137.94±229.11	117.26±8.82	26.80
16	7.75_758.4	758.4732	758.4967, 758.4814	phospholipids	PG(34:5), PI(27:0)	C40H73NO10P, C36H73NO13P	$[M+NH4]^+$	2087.66±76.10	104.59±13.47	20.00

17	7.77_806.4	806.4587	806.4814	phospholipids	PS(38:7), PC(37:7(OH))	C44H73NO10P, C45H77NO9P	$[M+NH4]^+$	110.65±13.14	8.09±0.64	13.70
18	7.77_603.4	603.4434	603.4103, 603.4983	glycerolipids	MGDG(23:1), DG(35:4)	C32H59O10, C38H67O5	$[M+H]^+$	313.60±17.51	36.05±4.00	8.70
19	8.04_729.4	729.4751	729.4701, 729.6028	phospholipids	PE(38:8), PS(34:2)	C43H71NO8P, C40H75NO10P	$[M+H]^+$	306.08±6.74	176.42±29.97	1.74
20	8.03_575.4	575.453	575.467	glycerolipids	DG(33:4)	C36H63O5	$[M+H]^+$	158.93±7.77	18.01±3.30	8.82
21	8.48_760.5	760.4868	760.5123,	phospholipids	PG(34:4)	C40H75NO10P	$[M+NH4]^+$	1561.83±22.12	172.36±20.44	9.06
22	8.52_731.4	731.4872	731.5668, 731.6184	glycerolipids	MGDG(32:0), TG(43:3)	C41H79O10, C46H83O6	$[M+H]^+$	362.89±10.73	319.09±50.36	1.13
23	8.77_786.5	786.4706	786.427, 786.5151	glycerolipids	DGDG(24:6), MGDG(36:9)	C39H64NO15, C45H72NO10	$[M+NH4]^+$	1827.19±44.90	106.02±17.82	17.14
24	8.83_663.3	663.3986	663.4103, 663.4983	glycerolipids	MGDG(28:6),DG(40:9)	C37H59O10, C43H67O5	$[M+H]^+$	359.03±23.18	296.30±23.71	1.21
25	8.77_718.4	718.4232	718.4525, 718.5017	phospholipids	PC(30:2(OH)), PE(33:2(OH))	C38H73NO9P, C38H73NO9P	$[M+NH4]^+$	287.33±35.33	29.15±6.48	9.86
26	8.83_664.3	664.3537	664.3456	phospholipids	LPI(22:5), PS(27:1)	C31H55NO12P, C33H63NO10P	$[M+NH4]^+$	154.02±1.65	132.65±10.12	1.16
27	8.73_577.4	577.4341	577.3946, 577.4826	glycerolipids	MGDG(21:0), DG(33:3),	C30H57O10, C36H65O5	$[M+H]^+$	174.22±10.17	21.30±2.10	8.18
28	9.03_744.4	757.4978	757.5824, 757.6341	glycerolipids	MGDG(34:1), TG(45:4)	C43H81O10, C48H85O6	$[M+H]^+$	704.50±65.61	29.31±6.03	24.00
29	9.04_604.4	604.4458	604.4055, 604.4935	glycerolipids	MGDG(22:2), DG(34:5)	C31H58NO10, C37H66NO5	$[M+NH4]^+$	147.95±7.35	6.72±1.56	22.00
30	9.37_772.4	772.4944	772.5933, 772.64	glycerolipids	MGDG(34:2), TG(45:5)	C43H82NO10, C48H86NO6	$[M+NH4]^+$	470.23±76.31	749.62±135.62	0.63
31	9.37_795.4	795.4863	795.5042, 795.51	glycerolipids	MGDG(38:10), DGDG(25:0)	C47H71O10, C40H75O15	$[M+H]^+$	14.07±2.71	24.61±2.25	0.57

32	9.37_762.4	762.4745	762.5151, 762.427	glycerolipids	MGDG(34:7), DGDG(22:4)	C43H72NO10, C37H64NO15	$[M+NH4]^+$	25.54±1.07	12.98±0.96	1.97
33	9.80_931.5	931.5	931.5331	phospholipids	PI(42:10)	C51H80O13P	$[M+H]^+$	103.54±11.13	8.10±0.99	12.8
34	9.80_752.5	752.50	752.5072, 752.5225	phospholipids	PE(36:6(OH)), PC(34:5)	C41H71NO9P, C42H75NO8P	$[M+H]^+$	201.86±18.26	17.15±4.22	11.80
35	12.93_892.6	892.6547	892.7389	glycerolipids	TG(54:8)	C57H98NO6	$[M+NH4]^+$	48.15±9.55	9.74±1.50	4.95
36	13.30_895.6	895.6443	895.681	glycerolipids	TG(56:12)	C59H91O6	$[M+H]^+$	41.92±7.31	3.86±3.73	10.90
37	14.06_900.6	900.6378	900.7076	glycerolipids	TG(55:11)	C58H94NO6	$[M+NH4]^+$	101.13±2.30	15.72±2.58	6.43
38	14.49_874.6	874.6416	874.6919	glycerolipids	TG(53:10)	C56H92NO6	$[M+NH4]^+$	1566.44±180.26	868.03±114.93	1.80
39	14.49_848.6	848.6331	848.6763	glycerolipids	TG(51:9)	C54H90NO6	$[M+NH4]^+$	909.28±89.13	347.89±51.29	2.61
40	14.49_822.6	822.6191	822.6606	glycerolipids	TG(49:8)	C52H88NO6	$[M+NH4]^+$	293.52±16.92	118.59±17.31	2.48
41	14.90_876.6	876.6555	876.7076	glycerolipids	TG(53:9)	C56H94NO6	$[M+NH4]^+$	3310.62±139.19	2189.69±296.72	1.51
42	14.90_850.6	850.644	850.6919	glycerolipids	TG(51:8)	C54H92NO6	$[M+NH4]^+$	2272.31±153.12	1155.83±164.14	1.97
43	14.90_902.6	902.6696	902.7232	glycerolipids	TG(55:10)	C58H96NO6	$[M+NH4]^+$	474.79±64.08	212.74±22.57	2.23
44	15.32_878.6	878.6759	878.7232	glycerolipids	TG(53:8)	C56H96NO6	$[M+NH4]^+$	838.12±60.35	391.75±57.23	2.14
45	15.32_904.6	904.6899	904.7389	glycerolipids	TG(55:9)	C58H98NO6	$[M+NH4]^+$	370.22±57.50	145.12±24.04	2.55

Lipids ID= contains retention time and mass spectrum of the lipid, Input Mass= mass spectra obtained from LCMS, Matched mass= mass spectra obtained from LIPID MAPS database, Ion= LCMS run mode, MG=Monoglycerol, DG=Diglycerol, TG=triglycerol, MGDG=Monogalactosyldiacylgylcerol, DGDG=Digalactosyldiacylgylcerol, PG=Phosphtatidylglycerol, PI=Phosphtatidylinositol, PS=Phosphtatidylserine, PC=Phosphtatidylcholine, PE=Phosphtatidylethanolamine,LPG= Lysyl-phosphatidylglycerol, LPI= Lysophosphatidylinositol, LPA= Lysophosphatidic acid.

Table 7.S2. Glycerolipids and phospholipids candidates obtained from *R. dominica*

No	Feature ID	Input Mass	Matched Mass	Lipid category	Name	Formula	Ion	LCMS response		Fold change (A/B)
								Resistant (A)	Susceptible (B)	
1	1.20_333.2	331.2029	331.2843	glycerolipids	MG(16:0)	C19H39O4	$[M+H]^+$	494.20±39.19	373.22±30.01	1.32
2	1.42_372.2	372.2362	372.3108	glycerolipids	MG(18:2)	C21H42NO4	$[M+NH4]^+$	165.21±8.96	123.47±11.27	1.34
3	1.42_504.3	504.3135	504.4047	glycerolipids	MG(28:6)	C31H54NO4	$[M+NH4]^+$	194.33±5.74	150.90±5.74	1.29
4	1.42_522.3	522.3278	522.4153	glycerolipids	DG(28:4)	C31H56NO5	$[M+NH4]^+$	133.57±9.96	79.67±15.17	1.68
5	1.77_497.3	497.3189	497.2874	phospholipids	LPG(17:1)	C23H46O9P	$[M+H]^+$	55.96±3.28	79.22±5.13	0.71
6	2.85_440.3	440.3844	440.4462	phospholipids	LPA (P-18:0)	C21H47NO6P	$[M+NH4]^+$	131.61±38.07	228.69±74.20	0.58
7	2.85_387.2	387.2717	387.3469	glycerolipids	MG(20:0)	C23H47O4	$[M+H]^+$	22.74±2.45	26.03±0.93	0.87
8	2.85_397.3	397.3575	397.404	phospholipids	LPA (O-16:0)	C19H42O6P	$[M+H]^+$	8.03±2.17	7.60±2.14	1.06
9	2.85_560.3	560.3673	560.283	phospholipids	LPI(14:1)	C23H47NO12P	$[M+NH4]^+$	22.75±1.48	26.00±0.18	0.88
10	4.006_621.2	621.2667	621.3034	phospholipids	LPI(20:4)	C29H50O12P	$[M+H]^+$	875.73±15.65	764.82±134.20	1.15
11	4.006_468.4	468.4175	468.4775	phospholipids	LPA (P-20:0)	C23H51NO6P	$[M+NH4]^+$	147.30±42.49	142.10±46.77	1.04
12	6.91_782.5	782.504	782.4896	glycerolipids	DGDG(23:1),	C38H72NO15	$[M+NH4]^+$	2019.82±235.61	68.25±17.86	29.60
13	6.91_701.5	701.5061	701.5715, 701.4259	glycerolipids	TG(41:4), MGDG(31:8)	C44H77O6, C40H61O10	$[M+H]^+$	48.53±7.57	27.33±4.27	1.78
14	6.91_805.4	805.4935	805.4944	glycerolipids	DGDG(26:2)	C41H73O15	$[M+H]^+$	48.53±7.57	27.33±4.27	1.72
15	6.91_601.4	601.4752	601.4826, 601.3946	glycerolipids	DG(35:5), MGDG(23:2)	C38H65O5, C32H57O10	$[M+H]^+$	122.02±15.25	10.84±3.47	11.30
16	7.32_703.5	703.5203	703.5355, 703.5871	glycerolipids	MGDG(30:0), TG(41:3)	C39H75O10, C44H79O6	$[M+H]^+$	274.50±37.66	197.51±29.57	1.39
17	7.32_740.4	740.4688	740.5307	glycerolipids	MGDG(32:4)	C41H74NO10	$[M+NH4]^+$	200.90±26.49	14.97±3.73	13.40
18	7.32_599.4	599.4777	599.4823, 599.379	glycerolipids	MGDG(23:3), DG(35:6)	C32H55O10, C38H63O5	$[M+H]^+$	98.52±11.18	6.03±0.87	16.30
19	7.32_725.4	725.4998	725.5198	glycerolipids	MGDG(32:3)	C41H73O10	$[M+H]^+$	21.10±2.32	16.90±2.12	1.25

20	7.73_603.4	603.495	603.4983, 603.4103	glycerolipids	DG(35:4), MGDG(23:1)	C38H67O5, C32H59O10	$[M+H]^+$	345.30±25.45	29.48±8.50	11.70
21	7.73_806.5	806.5099	806.4814	phospholipids	PS(38:7), PC(37:7(OH))	C44H73NO10P, C45H77NO9P	$[M+NH4]^+$	100.43±4.92	11.88±1.87	8.45
22	8.04_742.4	742.4846	742.4583, 742.5464	glycerolipids	DGDG(20:0), MGDG(32:3)	C35H68NO15, C41H76NO10	$[M+NH4]^+$	606.81±28.74	224.17±32.49	2.71
23	8.04_729.5	729.5348	729.4701, 729.6028	phospholipids	PE(38:8), PS(34:2)	C43H71NO8P, C40H75NO10P	$[M+H]^+$	244.84±19.95	136.24±18.63	1.80
24	8.04_716.4	716.4632	716.4861, 716.4345	phospholipids	LPG(32:5), PI(24:0)	C38H71NO9P, C33H67NO13P	$[M+NH4]^+$	303.01±20.56	30.08±6.45	10.10
25	8.04_575.4	575.4651	575.467	glycerolipids	DG(33:4)	C36H63O5	$[M+H]^+$	190.19±23.05	19.17±4.16	9.92
26	8.51_760.5	760.5245	760.5123	phospholipids	PG(34:4)	C40H75NO10P	$[M+NH4]^+$	1675.19±107.08	278.83±55.74	6.01
27	8.51_731.5	731.5506	731.5668, 731.6184	glycerolipids	MGDG(32:0), TG(43:3)	C41H79O10, C46H83O6	$[M+H]^+$	436.03±22.29	340.56±46.97	1.28
28	8.51_753.5	753.5414	753.5511, 753.6028	glycerolipids	MGDG(34:3), TG(45:6),	C43H77O10, C48H81O6	$[M+H]^+$	40.47±1.43	42.42±6.82	0.95
29	8.51_744.5	744.5378	744.481, 744.4658,	phospholipids	PG(33:5), PI(26:0)	C39H71NO10P, C35H71NO13P	$[M+NH4]^+$	206.92±21.10	124.11±17.26	1.67
30	8.78_718.4	718.4864	718.4525, 718.5017	phospholipids	PC(30:2(OH), PE(33:2(OH))	C38H73NO9P, C38H73NO9P	$[M+NH4]^+$	423.19±23.43	80.76±16.35	5.24
31	8.78_786.5	786.5374	786.427, 786.5151	glycerolipids	DGDG(24:6), MGDG(36:9)	C39H64NO15, C45H72NO10	$[M+NH4]^+$	1252.82±103.59	142.53±25.80	8.79
32	8.78_577.4	577.4322	577.3946, 577.4826	glycerolipids	MGDG(21:0), DG(33:3)	C30H57O10, C36H65O5	$[M+H]^+$	270.85±30.97	55.24±11.17	4.90
33	8.78_663.4	663.4093	663.4103, 663.4983	glycerolipids	MGDG(28:6), DG(40:9)	C37H59O10, C43H67O5	$[M+H]^+$	609.72±74.05	507.66±27.32	1.20
34	9.01_766.4	766.4847	766.4583, 766.5464	glycerolipids	DGDG(22:2),MGDG(34:5)	C37H68NO15, C43H76NO10	$[M+NH4]^+$	71.82±4.59	31.68±3.10	2.27

35	9.01_757.5	757.5558	757.5824, 757.6341	glycerolipids	MGDG(34:1), TG(45:4)	C43H81O10, C48H85O6	$[M+H]^+$	51.64±2.38	55.87±6.82	0.92
36	9.45_788.5	788.5247	788.5307, 788.6187	glycerolipids	MGDG(36:8), DG(48:11)	C45H74NO10, C51H82NO5	$[M+NH4]^+$	201.30±24.97	43.00±8.75	4.68
37	9.45_772.5	772.5918	772.5933, 772.64	glycerolipids	MGDG(34:2), TG(45:5)	C43H82NO10, C48H86NO6	$[M+NH4]^+$	99.85±5.59	84.51±9.65	1.18
38	9.45_759.5	759.5811	759.5981, 759.6497	glycerolipids	MGDG(34:0), TG(45:3)	C43H83O10, C48H87O6	$[M+H]^+$	58.75±1.45	45.27±5.51	1.30
39	9.45_728.5	728.5168	728.5307, 728.5824	glycerolipids	MGDG(31:3), TG(42:6),	C40H74NO10, C45H78NO6	$[M+NH4]^+$	31.43±4.19	14.37±1.82	2.19
40	9.45_702.4	702.4594	702.4341, 702.4188,	phospholipids	PG(30:5), PI(23:0)	C36H65NO10P, C32H65NO13P,	$[M+NH4]^+$	84.14±7.79	41.91±2.24	2.00
41	9.68_730.5	730.5211	730.4654	phospholipids	PG(32:5)	C38H69NO10P	$[M+NH4]^+$	243.07±13.67	182.51±19.88	1.33
42	9.68_752.5	752.5024	752.5072, 752.5225	phospholipids	PE(36:6(OH), PC(34:5)	C41H71NO9P, C42H75NO8P	$[M+H]^+$	39.41±3.15	33.47±3.80	1.18
43	9.68_641.4	641.4714	641.5139, 641.4259	glycerolipids	DG(38:6), MGDG(26:3)	C41H69O5, C35H61O10	$[M+H]^+$	14.66±1.26	7.88±1.39	1.86
44	10.59_566.5	566.5204	566.4779	glycerolipids	DG(31:3)	C34H64NO5	$[M+NH4]^+$	15.23±1.20	18.96±3.34	0.80
45	10.59_588.4	588.4946	588.4986	glycerolipids	MG(34:6)	C37H66NO4	$[M+NH4]^+$	1.74±0.39	2.61±0.74	0.67
46	12.75_600.4	600.48	600.5198	glycerolipids	TG(32:0)	C35H70NO6	$[M+NH4]^+$	17.54±2.35	0.56±0.07	31.5
47	13.63_896.7	896.7049	896.7702	glycerolipids	TG(54:6)	C57H102NO6	$[M+NH4]^+$	175.31±23.13	12.12±2.46	14.5
48	13.63_870.6	870.6881	870.7545	glycerolipids	TG(52:5)	C55H100NO6	$[M+NH4]^+$	67.03±5.73	9.21±2.25	7.28
49	13.63_924.7	924.7244	924.8015	glycerolipids	TG(56:6)	C59H106NO6	$[M+NH4]^+$	8.99±1.70	0.88±0.21	10.2
50	14.03_872.7	872.702	872.7702	glycerolipids	TG(52:4)	C55H102NO6	$[M+NH4]^+$	729.15±64.44	131.25±17.75	5.56
51	14.03_898.7	898.7174	898.7858	glycerolipids	TG(54:5)	C57H104NO6	$[M+NH4]^+$	406.46±40.88	51.77±9.89	7.85
52	14.03_877.6	877.6541	877.728	glycerolipids	TG(54:7)	C57H97O6	$[M+H]^+$	80.83±4.60	25.55±2.90	3.16
53	14.03_846.6	846.6855	846.7545	glycerolipids	TG(50:3)	C53H100NO6	$[M+NH4]^+$	78.61±4.63	36.13±0.66	2.18
54	14.03_900.7	900.7314	900.7076	glycerolipids	TG(55:11)	C58H94NO6	$[M+NH4]^+$	107.36±12.25	16.55±3.90	6.49

55	14.46_874.7	874.7136	874.6919	glycerolipids	TG(53:10)	C56H92NO6	$[M+NH4]^+$	2201.20±47.19	1105.18±102.89	2.00
56	14.46_848.7	848.704	848.6763	glycerolipids	TG(51:9)	C54H90NO6	$[M+NH4]^+$	364.11±10.02	335.58±11.22	3.15
57	14.46_880.6	880.6754	880.7389	glycerolipids	TG(53:7)	C56H98NO6	$[M+NH4]^+$	90.99±4.68	59.83±3.47	1.52
58	14.85_876.7	876.7307	876.7076	glycerolipids	TG(53:9)	C56H94NO6	$[M+NH4]^+$	1030.29±120.17	1355.54±62.69	0.76
59	14.85_850.7	850.7169	850.6919	glycerolipids	TG(51:8)	C54H92NO6	$[M+NH4]^+$	66.44±2.03	63.60±3.82	1.04
60	14.85_902.7	902.7433	902.7232	glycerolipids	TG(55:10)	C58H96NO6	$[M+NH4]^+$	327.72±35.09	207.45±23.05	1.58
61	14.85_881.6	881.699	881.7593	glycerolipids	TG(54:5)	C57H101O6	$[M+H]^+$	110.66±3.85	117.84±10.57	0.94
62	14.85_855.6	855.6716	855.7436	glycerolipids	TG(52:4)	C55H99O6	$[M+H]^+$	56.90±4.62	62.22±2.34	0.91
63	15.26_878.7	878.7495	878.7232	glycerolipids	TG(53:8)	C56H96NO6	$[M+NH4]^+$	297.63±42.81	239.60±19.02	1.24
64	15.26_905.7	905.7624	905.7593	glycerolipids	TG(56:7)	C59H101O6	$[M+H]^+$	83.15±9.77	53.37±4.27	1.56
65	15.26_883.7	883.7335	883.681	glycerolipids	TG(55:11)	C58H91O6	$[M+H]^+$	20.88±1.10	16.90±0.51	1.24
66	15.26_909.6	909.6959	909.7906	glycerolipids	TG(56:5)	C59H105O6	$[M+H]^+$	8.24±2.20	5.48±0.14	1.5
67	15.26_852.7	852.7589	852.7076	glycerolipids	TG(51:7)	C54H94NO6	$[M+NH4]^+$	14.22±1.03	9.74±0.58	1.46

Lipids ID= contains retention time and mass spectrum of the lipid, Input Mass= mass spectra obtained from LCMS, Matched mass= mass spectra obtained from LIPID MAPS database, Ion=LCMS run mode, MG=Monoglycerol, DG=Diglycerol, TG=triglycerol, MGDG=Monogalactosyldiacylgylcerol, DGDG=Digalactosyldiacylgylcerol, PG=Phosphtatidylglycerol, PI=Phosphtatidylinositol, PS=Phosphtatidylserine, PC=Phosphtatidylcholine, PE=Phosphtatidylethanolamine,LPG=Lysyl-phosphatidylglycerol, LPI=Lysophosphatidylinositol, LPA=Lysophosphatidic acid.

Chapter 8

General Discussion

8. General discussion

Despite there are many studies that have focused on resistance to phosphine; however, the resistant mechanism remains largely unknown. In our study, we determined some of the potential reasons that may contribute to phosphine resistance by studying the metabolic and physiological differences of different resistance level insect strains. The study consisted of two economic important stored insects, including *T. castaneum* and *R. dominica*. The study was started by determining the respiration rate of phosphine resistant and susceptible insect strains. A significant pattern of respiration of phosphine susceptible was observed in comparison with resistant strains. The high respiration by the susceptible insects correlated with high production of volatile organic compounds (VOCs), proving that susceptible insects might have higher metabolism rate which causes a rapid loss of the energy which might be one reason of the death when they are treated with phosphine. The respiration of both susceptible and resistant strains was not affected by different oxygen levels, although the toxic effect of phosphine required aerobic respiration. Phosphine had a negative effect on the respiration of both susceptible and resistant strains at a range of concentrations (0 to 20 ppm); however, we recorded for the first time different response by a resistant strain of *Tribolium castaneum*. The respiration had raised after treating the insect with a high concentration of phosphine. This associated with high emission of (VOCs), suggesting that the metabolism of the insects might be elevated to face up the lack of the energy caused by phosphine. To study the VOCs, we developed a novel method based on headspace solid-phase microextraction. The method included optimization the ideal conditions to analyse the VOCs from the insect strains. The method then applied to determine the differences between susceptible and resistant strains of both *T. castaneum* and *R. dominica*.

To analyse cuticular lipids, DI-SPME method coupled with GC-MS without derivatization was explored for the first time, the lipids included hydrocarbons and fatty acids. The method was used to analyse the hydrocarbons from susceptible and resistant strains of both studied insects. We recorded that resistant insects had abundant cuticular hydrocarbons in comparison with susceptible insects. This might be critical to protecting the insects from phosphine by preventing the penetration of the fumigant. Other important factors that might increase the ability of the resistant insects is the higher content of the lipids, such as glycerolipids and phospholipids that were found to be higher in the resistant insects in this study. The major findings that were described in different chapters in this thesis are represented in a conceptual diagram (Figure 8.1).

8.1 Phosphine resistance

In our study, we focused on the insect resistance toward hydrogen phosphide. The phenomenon threats the use of this fumigant, which is considered the most important pesticide used in the grain industry. The fumigant has many characteristics that qualified it to be widely used to protect stored grain all around the world, including the absence of residue (Bruce et al., 1962; Scudamore & Goodship, 1986), and it's having no negative effect on seeds (Ahmad, 1976; Krishnasamy & Seshu, 1990; SITTISUANG, 1985). This study provides new information about the metabolic differences between phosphine-resistant and -susceptible strains. The information may strongly contribute to the understanding of the mechanism of phosphine resistance.

The importance of this study comes from the spread of phosphine resistance in many countries across different insect species (Benhalima et al., 2004; Cao et al., 2003; Champ & Dyte, 1976; Collins, 2006; Daglish et al., 2014; Herron, 1990; Jagadeesan et al., 2012; Mills, 1983; Nayak et al., 2003; Opit et al., 2012; Pacheco et al., 1990; Pimentel et al., 2009; Pimentel et al., 2010; Song et al., 2011; Zettler et al., 1989; Zettler, 1991).

Therefore, phosphine resistance as a threat to the grain industry has become a global issue (Benhalima et al., 2004; Champ & Dyte, 1976; Nayak et al., 2003; Opit et al., 2012). Hence, studying resistance to phosphine is an important issue that should be taken into consideration. In our study, we studied the metabolic side of different resistant level insect strains for its possible effect to resist phosphine. Because of resistance has reached such a high level that it impacts the entire grain industry (Collins, 2009). That because the grain industry strongly relies on phosphine and resistance to phosphine has led to applying higher concentrations to control resistant populations. In some cases, it may not be possible to complete control (Lilford et al., 2009).

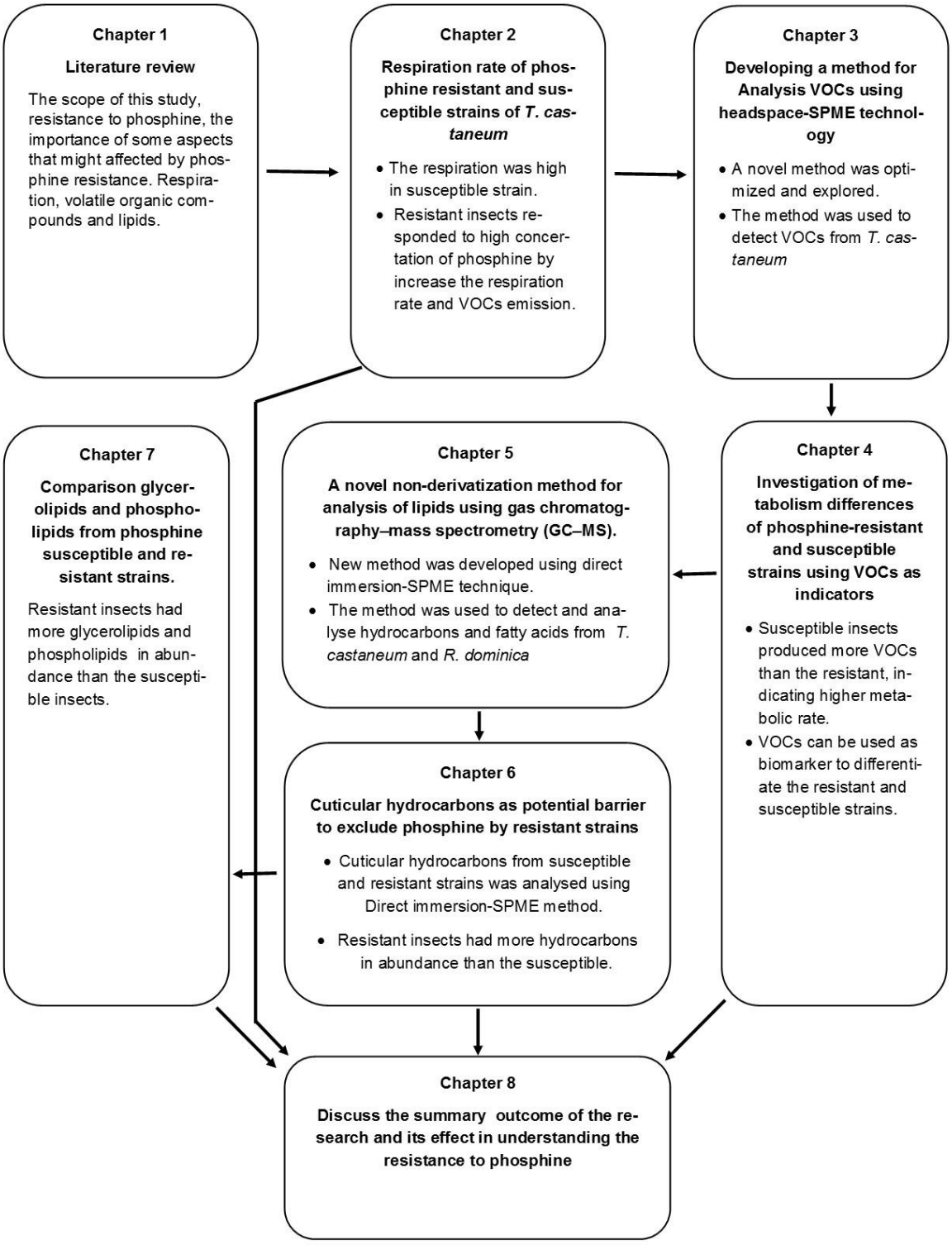

Figure 8.1. Thesis structure and some outcome of the study.

8.2 Mechanism of phosphine resistance

From the results in our study, resistance to phosphine may include different mechanisms, including the difference in the metabolism of resistant and susceptible strains. In previous studies, phosphine resistance was found to be associated a negative fitness cost, reducing the growth and development of the population (Bajracharya et al., 2016; Sousa et al., 2009). Flight, ambulation and the ability to find food sources were significantly slower than with the susceptible strains (Malekpour et al., 2016), and egg production was fewer in number (Saxena & Bhatia, 1980). The fitness cost was suggested to be a mechanism used by resistant insects to resist pesticides by reducing the rate of the metabolism and energy demand (Araújo et al., 2008; Guedes et al., 2006). However, one study indicated that a resistant population of *T. castaneum* showed higher growth and developmental rates compared to their contrary susceptible population (Bajracharya et al., 2016). In our study, we found that susceptible insects had higher respiration rate than the resistant strains. This was found to be associated with higher emission of volatile organic compounds, which might prove that susceptible insects have a higher metabolic rate, which might cause a rapid loss of the energy that causes death. Although a study indicated that there is no evidence that resistant insects have more biochemical defences than susceptible insects (Chaudhry & Price, 1992). However, genetic studies have previously confirmed that the resistant populations are genetically different from their contrary susceptible populations (Chen et al., 2015; Collins et al., 2002; Jagadeesan et al., 2016; Kaur et al., 2013; Kocak et al., 2015; Nguyen, 2016; Nguyen et al., 2015; Oppert et al., 2015). Analysis revealed that both rph1 and rph2 are responsible for weak resistance; however, when the two alleles synergise, strong resistance was observed (Jagadeesan et al., 2012; Schlipalius et al., 2008). Phosphine resistance is controlled by the DLD gene, which controls four main metabolic steps; a mutation in the DLD gene resulted in the rph2 allele that is responsible for phosphine resistance (Schlipalius et al., 2012). Therefore, resistance to phosphine may include physiological and metabolic changes that affect the bioactivities of insects (Daglish et al., 2014; Malekpour et al., 2016). Phosphine resistance was found to be correlated with the metabolic rate. Immature stages of insects that have lower metabolic activities such as respiration were more resistant to phosphine than the adult insects (Bell, 1976; Hole et al., 1976; Howe, 1973). Therefore, lower metabolism by resistant strains found in this study using VOCs and

respiration as indicators may indicate that resistant insects using the mechanism to resist phosphine.

Since phosphine is a fumigant, there was a belief that entry to the animal body occurs through the respiratory system, and therefore, the uptake of phosphine is relational to the rate of the respiration of the exposed animal (Chaudhry, 1997). The amount of phosphine that is taken up by the resistant strain and the susceptible strains of several insect species such as *T. castaneum* (H.), *R. dominica* (F.) and *Sitophilus granarius* (L.) was investigated as a mechanism for resisting the fumigant. This proves that in resistant insects the uptake of phosphine is a lower amount than with the susceptible insects (Bond et al., 1969; Bond & Upitis, 1973; Chaudhry & Price, 1989; Nakakita & Kuroda, 1986; Pratt, 2003; Price, 1984; Price, 1981; Price & Dance, 1983; Reichmuth, 1994). The higher uptake of phosphine by susceptible insects was even observed in the larval, pupal and adult stages (Nakakita & Kuroda, 1986). Moreover, the lower uptake continues to be less in resistant insects, even when live resistant strains were compared with freshly dead susceptible insects (Pratt, 2003; Price, 1980a). Consistent with these results, respiration of resistant *T. castaneum, R. dominica* and *S. granarius* were found to be lower than with the susceptible insects (Pimentel et al., 2007; Pimentel et al., 2009; Pimentel et al., 2008). In this study, we found that susceptible insect respiration is higher than the resistant strains, and this may support the thought that resistant insects are using this mechanism to reduce the amount of phosphine that enters to the insect body.

However, the less respiration by resistant insects as a mechanism to resist phosphine might not be appropriate for all cases of resistance. Since a study confirmed that the uptake and toxicity of phosphine were found to be not affected by the opening or closing of the spiracles in roaches (Bond et al., 1969). The lower uptake of phosphine was found not to be related to the effect of phosphine on the respiration rate, as the consumption of oxygen in the resistant strain was not affected by phosphine as much as susceptible insects (Price, 1980a). The overall results suggested that "active exclusion" is a mechanism involved in phosphine resistance (Price, 1984). Even a parallel phosphine uptake had little effect on resistant insects compared to the susceptible insects (Chaudhry & Price, 1990). This indicates that there are more detoxification processes involved (Chaudhry, 2000), including the inability of the fumigant to reach the target site that is sensitive to phosphine, the absence of the susceptible site, or a membrane system that excludes phosphine (Chaudhry, 2000). In this study, phospholipids that were found to be higher in resistant insects in comparison with susceptible insects might also provide evidence that exclusion is a possible mechanism to resist phosphine. That because phospholipids are the

main components in cell membranes and the surrounding environment, which may provide more protection to the mitochondria that consider the main site that is affected by phosphine. This may support the assumption that the mitochondrial membrane works as a barrier to reduce phosphine penetration. Since mitochondrial membrane consists of double layers, a permeable outer membrane and an impervious mitochondrial inner membrane (MIM), suggesting that MIM provides a physical barrier against phosphine uptake or by activation of the electron transportation (Nath et al., 2011). Despite this, phosphine causes severe damage to the function of the mitochondria of *Caenorhabditis elegans*. However, phosphine causes an increase in the basal mitochondrial membrane potential of the resistant strain, resulting in more protection against the fumigant (Zuryn et al., 2008).

Additionally, inhibition of respiration in mitochondria isolated from resistant and susceptible strain was similar when the mitochondria were treated in vitro, suggesting that this inhibitory function is not related to resistance to phosphine (Price, 1980b). This indicating that physiological status is an important factor for avoiding the effect of phosphine (Nath et al., 2011). The lower uptake of phosphine by the resistant strain in comparison with susceptible (Bond et al., 1969; Bond & Upitis, 1973; Chaudhry & Price, 1989; Nakakita & Kuroda, 1986; Pratt, 2003; Price, 1984; Price, 1981; Price & Dance, 1983; Reichmuth, 1994) suggests that there is a barrier to prevent phosphine from entering the insect body (Chaudhry, 1997). Nakakita and Kuroda (1986) suggested that phosphine may penetrate the insect body through the cuticle layer, although there was no evidence were supplied to prove that. The result of cuticular hydrocarbons in this study might provide evidence that resistant insects are using these lipid compounds to prevent phosphine from penetration to inside the insect bodies, resulting in reduce or prevent the toxic effect of phosphine. This may support the conclusion that the exclusion of phosphine through the cuticle layer is one of the possible mechanisms that reduce the toxicity of phosphine. This comes from the functions of the cuticular lipids as a protector shield, preventing pathogens, chemicals and other harmful elements like pesticides from entering the insects' bodies (Cohen & Moussian, 2016; El-Sayed et al., 1991; Gibbs, 1998; Gołębiowski et al., 2012; Gołębiowski et al., 2008a; Nelson & Sukkestad, 1970; Wang & Leger, 2005).

The energy production is strongly affected by a mitochondrial membrane (Nath et al., 2011). The result of our study proved that in resistant strains higher content of the phospholipids, which are considered vital components of cellular and semi cellular membranes (Li et al., 2015; Singh et al., 2017),. Phospholipids have big roles that affect the physical properties of the

mitochondrial membrane (Horvath & Daum, 2013; Ren et al., 2014). The higher amount of phospholipids may lead to the enhancement of the mitochondrial function, thus increasing the energy production that raises the resistance to phosphine. This may be supported by the result obtained from lesser grain borer *R. dominica*, which demonstrated that adenosine triphosphate (ATP) was reduced from 2.75 to 1.64 nmoles/insect after treating the insect with phosphine (Price & Walter, 1987).

Energy supply is a vital factor that may contribute in more survival from phosphine effect. The inhibitory effect of phosphine on mitochondrial respiration may result in a reduction of the energy metabolism (Nath et al., 2011), since the effect of phosphine on cytochrome oxidase lead to a decrease in the synthesis of ATP (Dua et al., 2010). A study on *R. dominica* proved that ATP was reduced after treating the insect with phosphine (Price & Walter, 1987). Nath et al. (2011) indicated that there are two possible explanations for the relationship between the toxicity of phosphine and metabolism: the increase in the metabolic rate or insufficient energy production that results in death. That was proved by evidence that increasing the energy demand artificially raised the sensitivity toward phosphine (Valmas et al., 2008). In our study, we found that glycerolipids like triglycerides and diglycerides were significantly higher in the resistant strains of both species. Since triglycerides are considered as the main reserve of energy (Hahn & Denlinger, 2007), differences in triglycerides and diglycerides obtained from the resistant and susceptible strains in this study may support the point that resistant insects are using lipids to survive from phosphine in short- and long- term exposure.

References

Ad, M.T., Van der Horst, D.J., Van Marrewijk, W.J. 1985. Insect lipids and lipoproteins, and their role in physiological processes. *Progress in lipid research*, **24**(1), 19-67.

Adam, B.D., Phillips, T., Flinn, P. 2006. The economics of IPM in stored grain: Why don't more grain handlers use IPM? *Proceedings of the 9th International Working Conference on Stored Product Protection. Edited by I. Lorini et al.. Campinas, Sao Paulo, BRAZIL: ABRAPOS*. pp. 3-12.

Ahmad, M. 1976. Effect of phosphine fumigation on the germination of edible legume seeds. *Journal of Stored Products Research*.

Al-Hakkak, Z., Al-Azzawi, M., Al-Adhamy, B., Khalil, S. 1989. Inhibitory action of phosphine on acetylcholinesterase of *Ephestia cautella* (Lepidoptera: Pyralidae). *Journal of Stored Products Research*, **25**(3), 171-174.

Al-Azzawi, M.J., Al-Hakkak, Z.S., Al-Adhami, B.W. 1990. In vitro inhibitory effects of phosphine on human and mouse serum cholinesterase. *Toxicological & Environmental Chemistry*, **29**(1), 53-56.

Aldai, N., Murray, B.E., Nájera, A.I., Troy, D.J., Osoro, K. 2005. Derivatization of fatty acids and its application for conjugated linoleic acid studies in ruminant meat lipids. *Journal of the Science of Food and Agriculture*, **85**(7), 1073-1083.

Alnajim, I., Agarwala, M., Liu, T., Ren, Y. 2019. A Novel Method for the Analysis of Volatile Organic Compounds (VOCs) from Red Flour Beetle *Tribolium castaneum* (H.) Using Headspace-SPME Technology. *Current Analytical Chemistry* **15**, 1-9.

Andersen, S.O. 1979. Biochemistry of insect cuticle. *Annual review of entomology*, **24**(1), 29-59.

Ansell, M., Dyte, C., Smith, R. 1990. The inheritance of phosphine resistance in *Rhyzopertha dominica* and *Tribolium castaneum*. *Proc 5th Int Working Conf on Stored Product Protection, Bordeaux, France. Institut National de la Recherche Agronomique*. pp. 961-969.

Araújo, R., Guedes, R., Oliveira, M., Ferreira, G. 2008. Enhanced proteolytic and cellulolytic activity in insecticide-resistant strains of the maize weevil, *Sitophilus zeamais*. *Journal of Stored Products Research*, **44**(4), 354-359.

Arbogast, R. 1991. Beetles: coleoptera. *Ecology and management of food-industry pests. FDA Technical Bulletin*, **4**, 131-176.

Arnaud, L., Lognay, G., Verscheure, M., Leenaers, L., Gaspar, C., Haubruge, E. 2002. Is dimethyldecanal a common aggregation pheromone of *Tribolium* flour beetles? *Journal of chemical ecology*, **28**(3), 523-532.

Arrese, E.L., Soulages, J.L. 2010. Insect fat body: energy, metabolism, and regulation. *Annual review of entomology*, **55**, 207-225.

Arrese, E.L., Wells, M.A. 1997. Adipokinetic hormone-induced lipolysis in the fat body of an insect, *Manduca sexta*: synthesis of sn-1, 2-diacylglycerols. *Journal of lipid research*, **38**(1), 68-76.

Arroyo, F.T., Moreno, J., Daza, P., Boianova, L., Romero, F. 2007. Antifungal activity of strawberry fruit volatile compounds against *Colletotrichum acutatum*. *Journal of agricultural and food chemistry*, **55**(14), 5701-5707.

Arthur, C.L., Pawliszyn, J. 1990. Solid phase microextraction with thermal desorption using fused silica optical fibers. *Analytical chemistry*, **62**(19), 2145-2148.

Athenstaedt, K., Daum, G. 2006. The life cycle of neutral lipids: synthesis, storage and degradation. *Cellular and Molecular Life Sciences CMLS*, **63**(12), 1355-1369.

Auger, J., Rousset, S., Thibout, E., Jaillais, B. 1998. Solid-phase microextraction–gas chromatography–direct deposition infrared spectrometry as a convenient method for the determination of volatile compounds from living organisms. *Journal of Chromatography A*, **819**(1-2), 45-50.

Bajracharya, N., Opit, G., Talley, J., Gautam, S., Payton, M. 2016. Assessment of fitness effects associated with phosphine resistance in *Rhyzopertha dominica* (F.)(Coleoptera: Bostrichidae) and *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae). *African Entomology*, **24**(1), 39-49.

Baker, C.D., Basu Ball, W., Pryce, E.N., Gohil, V.M. 2016. Specific requirements of nonbilayer phospholipids in mitochondrial respiratory chain function and formation. *Molecular biology of the cell*, **27**(14), 2161-2171.

Balasubramanian, S., Panigrahi, S. 2011. Solid-phase microextraction (SPME) techniques for quality characterization of food products: a review. *Food and Bioprocess Technology*, **4**(1), 1-26.

Baldwin, R., Fasulo, T.R. 2003. Confused Flour Beetle, *Tribolium Confusum* Jacquelin du Val (Insecta: Coleoptera: Tenebrionidae) and Red Flour Beetle, *Tribolium Castaneum* (Herbst)(Insecta: Coleoptera: Tenebrionidae). *USA: University of Florida IFAS Extension*.

Banks, H. 1990. Behaviour of gases in grain storages. *Fumigation and Controlled Atmosphere Storage of Grain, Proceedings of International Conference, Singapore*. Australian Centre for International Agricultural Research Canberra, Australia. pp. 96-107.

Barbosa, R.R., Braga, M.V., Blomquist, G.J., Queiroz, M.M.d.C. 2017. Cuticular hydrocarbon profiles as a chemotaxonomic tool for three *blowfly* species (Diptera: Calliphoridae) of forensic interest. *Journal of natural history*, **51**(25-26), 1491-1498.

Beenakkers, A.T. 1973. Influence of flight on lipid metabolism in *Locusta migratoria*. *Insect Biochemistry*, **3**(11), 303-308.

Beenakkers, A.T., Van der Horst, D., Van Marrewijk, W. 1984. Insect flight muscle metabolism. *Insect Biochemistry*, **14**(3), 243-260.

Belda-Iniesta, C., de Castro Carpeno, J., Carrasco, J., Moreno, V., Saenz, E.C., Feliu, J., Sereno, M., Rio, F.G., Barriuso, J., Baron, M.G. 2007. New screening method for lung cancer by detecting volatile organic compounds in breath. *Clinical and Translational Oncology*, **9**(6), 364-368.

Bell, C. 1976. The tolerance of developmental stages of four stored product moths to phosphine. *Journal of Stored Products Research*, **12**(2), 77-86.

Bell, C., Wilson, S. 1995. Phosphine tolerance and resistance in *Trogoderma granarium* Everts (Coleoptera: Dermestidae). *Journal of Stored Products Research*, **31**(3), 199-205.

Belliardo, F., Bicchi, C., Cordero, C., Liberto, E., Rubiolo, P., Sgorbini, B. 2006. Headspace-solid-phase microextraction in the analysis of the volatile fraction of aromatic and medicinal plants. *Journal of chromatographic science*, **44**(7), 416-429.

Benhalima, H., Chaudhry, M., Mills, K., Price, N. 2004. Phosphine resistance in stored-product insects collected from various grain storage facilities in Morocco. *Journal of Stored Products Research*, **40**(3), 241-249.

Bennett, J.W., Inamdar, A.A. 2015. Are some fungal volatile organic compounds (VOCs) mycotoxins? *Toxins*, **7**(9), 3785-3804.

Berson, J.D., Simmons, L.W. 2019. Female cuticular hydrocarbons can signal indirect fecundity benefits in an insect. *Evolution*, **73**(5), 982-989.

Bland, J.M., Osbrink, W.L., Cornelius, M.L., Lax, A.R., Vigo, C.B. 2001. Solid-phase microextraction for the detection of termite cuticular hydrocarbons. *Journal of Chromatography A*, **932**(1-2), 119-127.

Blomquist, G.J., Chu, A.J., Remaley, S. 1980. Biosynthesis of wax in the honeybee, *Apis mellifera* L. *Insect Biochemistry*, **10**(3), 313-321.

Blomquist, G.J., Jackson, L.L. 1979. Chemistry and biochemistry of insect waxes. *Progress in lipid research*, **17**(4), 319-345.

Bocchini, P., Andalò, C., Bonfiglioli, D., Galletti, G.C. 1999. Solid-phase microextraction gas chromatography/mass spectrometric analysis of volatile organic compounds in water. *Rapid communications in mass spectrometry*, **13**(21), 2133-2139.

Bohdanowicz, M., Grinstein, S. 2013. Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis. *Physiological reviews*, **93**(1), 69-106.

Bond, E. 2007. Manual of fumigation for insect control. *FAO plant production and protection paper*, 54.

Bond, E., Monro, H. 1967. The role of oxygen in the toxicity of fumigants to insects. *Journal of Stored Products Research*, **3**(4), 295-310.

Bond, E., Robinson, J., Buckland, C. 1969. The toxic action of phosphine absorption and symptoms of poisoning in insects. *Journal of Stored Products Research*, **5**(4), 289-298.

Bond, E., Upitis, E. 1973. Response of three insects to sublethal doses of phosphine. *Journal of Stored Products Research*, **8**(4), 307-313.

Bond, E.J. 1984. *Manual of fumigation for insect control*.

Boserup, E. 2017. *The conditions of agricultural growth: The economics of agrarian change under population pressure*. Routledge.

Bousquet, Y. 1990. Beetles associated with stored products in Canada: An identification guide Agriculture and Agri-Food Canada, Canadian Governement Publishing Centre, Otawa.

Boyer, S., Zhang, H., Lempérière, G. 2012. A review of control methods and resistance mechanisms in stored-product insects. *Bulletin of entomological research*, **102**(2), 213-229.

Braga, M.V., Pinto, Z.T., de Carvalho Queiroz, M.M., Matsumoto, N., Blomquist, G.J. 2013. Cuticular hydrocarbons as a tool for the identification of insect species: Puparial cases from Sarcophagidae. *Acta tropica*, **128**(3), 479-485.

Brondz, I., Ekeberg, D., Høiland, K., Bell, D.S., Annino, A.R. 2007. The real nature of the indole alkaloids in Cortinarius infractus: Evaluation of artifact formation through solvent extraction method development. *Journal of Chromatography A*, **1148**(1), 1-7.

Bruce, R., Robbins, A., Tuft, T. 1962. Fumigant Residues, Phosphine Residues from Phostoxin-Treated Grain. *Journal of Agricultural and Food Chemistry*, **10**(1), 18-21.

Buckner, J.S., Hagen, M.M., Nelson, D.R. 1999. The composition of the cuticular lipids from nymphs and exuviae of the silverleaf whitefly, *Bemisia argentifolii*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **124**(2), 201-207.

Buckner, J.S., Mardaus, M.C., Nelson, D.R. 1996. Cuticular lipid composition of *Heliothis virescens* and *Helicoverpa zea* pupae. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **114**(2), 207-216.

Buckner, J.S., Pitts-Singer, T.L., Guédot, C., Hagen, M.M., Fatland, C.L., Kemp, W.P. 2009. Cuticular lipids of female solitary bees, *Osmia lignaria* Say and *Megachile rotundata* (F.)(Hymenoptera: Megachilidae). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **153**(2), 200-205.

Bumbrah, G.S., Krishan, K., Kanchan, T., Sharma, M., Sodhi, G.S. 2012. Phosphide poisoning: a review of literature. *Forensic science international*, **214**(1-3), 1-6.

Bursell, E. 1970. An introduction to insect physiology. *An introduction to insect physiology*.

Buszewski, B., Ulanowska, A., Ligor, T., Denderz, N., Amann, A. 2009. Analysis of exhaled breath from smokers, passive smokers and non-smokers by solid-phase microextraction gas chromatography/mass spectrometry. *Biomedical Chromatography*, **23**(5), 551-556.

Cai, L., Koziel, J.A., O'Neal, M.E. 2007. Determination of characteristic odorants from *Harmonia axyridis* beetles using in vivo solid-phase microextraction and multidimensional gas chromatography–mass spectrometry–olfactometry. *Journal of Chromatography A*, **1147**(1), 66-78.

Camarasu, C.C. 2000. Headspace SPME method development for the analysis of volatile polar residual solvents by GC-MS. *Journal of pharmaceutical and biomedical analysis*, **23**(1), 197-210.

Campbell, J.F., Arthur, F.H., Mullen, M.A. 2004. Insect management in food processing facilities. *Advances in food and nutrition research*, **48**(2), 239-295.

Campbell, J.F., Toews, M.D., Arthur, F.H., Arbogast, R.T. 2010. Long-term monitoring of Tribolium castaneum in two flour mills: seasonal patterns and impact of fumigation. *Journal of Economic Entomology*, **103**(3), 991-1001.

Cao, Y., Song, Y., Sun, G. 2003. A survey of psocid species infesting stored grain in China and resistance to phosphine in field populations of *Liposcelis entomophila* (Enderlein)(Psocoptera: Liposcelididae). *Credland, PF, Armitage, DM, Bell, CH, Cogan, PM, Highley, E.(Eds.)*, 662-667.

Caputo, B., Dani, F., Horne, G., N'fale, S., Diabate, A., Turillazzi, S., Coluzzi, M., Costantini, C., Priestman, A., Petrarca, V. 2007. Comparative analysis of epicuticular lipid profiles of sympatric and allopatric field populations of *Anopheles gambiae* ss molecular forms and *An. arabiensis* from Burkina Faso (West Africa). *Insect biochemistry and molecular biology*, **37**(4), 389-398.

Carballeira, N. 2008. New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. *Progress in lipid research*, **47**(1), 50-61.

Carlson, D., Mayer, M., Silhacek, D., James, J., Beroza, M., Bierl, B. 1971. Sex attractant pheromone of the house fly: isolation, identification and synthesis. *Science*, **174**(4004), 76-78.

Casanova, J. 2002. An overview of the scientific aspects of ozone depletion and their impact on the environment. *Proc. Int. Conf. on alternatives to Methyl bromide Sevilla, Spain*. pp. 5-8.

Cerkowniak, M., Puckowski, A., Stepnowski, P., Gołębiowski, M. 2013. The use of chromatographic techniques for the separation and the identification of insect lipids. *Journal of Chromatography B*, **937**, 67-78.

Champ, B.R., Dyte, C.E. 1976. *Report of the FAO global survey of pesticide susceptibility of stored grain pests*. FAO.

Chang, F. 1974. Effects of vertebrate adipokinetic hormones on the rate of in vitro lipid release in insects. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **49**(4), 567-578.

Chaudhry, M. 2000. Phosphine resistance. *Pesticide Outlook*, **11**(3), 88-91.

Chaudhry, M. 1997. Review A Review of the Mechanisms Involved in the Action of Phosphine as an Insecticide and Phosphine Resistance in Stored-Product Insects. *Pesticide Science*, **49**(3), 213-228.

Chaudhry, M., Bell, H., Savvidou, N., MacNicoll, A. 2004. Effect of low temperatures on the rate of respiration and uptake of phosphine in different life stages of the cigarette beetle *Lasioderma serricorne* (F.). *Journal of Stored Products Research*, **40**(2), 125-134.

Chaudhry, M.Q., Price, N.R. 1989. Biochemistry of phosphine uptake in susceptible and resistant strains of two species of stored product beetles. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology*, **94**(2), 425-429.

Chaudhry, M.Q., Price, N.R. 1992. Comparison of the oxidant damage induced by phosphine and the uptake and tracheal exchange of 32P-radiolabelled phosphine in the susceptible and resistant strains of *Rhyzopertha dominica* (F.)(Coleoptera: Bostrychidae). *Pesticide Biochemistry and Physiology*, **42**(2), 167-179.

Chaudhry, M.Q., Price, N.R. 1990. Insect mortality at doses of phosphine which produce equal uptake in susceptible and resistant strains of *Rhyzopertha dominica* (F.)(Coleoptera: Bostrychidae). *Journal of Stored Products Research*, **26**(2), 101-107.

Chefurka, W., Kashi, K., Bond, E. 1976. The effect of phosphine on electron transport in mitochondria. *Pesticide Biochemistry and Physiology*, **6**(1), 65-84.

Chen, Z., Schlipalius, D., Opit, G., Subramanyam, B., Phillips, T.W. 2015. Diagnostic molecular markers for phosphine resistance in US populations of Tribolium castaneum and *Rhyzopertha dominica*. *PloS one*, **10**(3), e0121343.

Chin, K., Mai, X., Meaklim, J., Scollary, G., Leaver, D. 1992. The interaction of phosphine with haemoglobin and erythrocytes. *Xenobiotica*, **22**(5), 599-607.

Chino, H., Downer, R. 1982. Insect hemolymph lipophorin: a mechanism of lipid transport in insects. *Advances in biophysics*, **15**, 67-92.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S., Xia, J. 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic acids research*, **46**(W1), W486-W494.

Cogburn, R.R., Burkholder, W.E., Williams, H.J. 1984. Field tests with the aggregation pheromone of the lesser grain borer (Coleoptera: Bostrichidae). *Environmental Entomology*, **13**(1), 162-166.

Cohen, E. 1972. Incorporation of [1-14C] Palmitate into lipids and the pattern of haemolymph proteins in diapause and non-diapause larvae of the beetle *Trogoderma granarium*. *Insect Biochemistry*, **2**(6), 161-166.

Cohen, E., Moussian, B. 2016. *Extracellular Composite Matrices in Arthropods*. Springer, Switzerland.

Collins, P. 2006. Resistance to chemical treatments in insect pests of stored grain and its management. *Proceedings of the 9th International Working Conference on Stored Product Protection*.

Collins, P. 2009. Strategy to manage resistance to phosphine in the Australian grain industry. *CRC Plant Biosecurity*, 1-13.

Collins, P.J. 1998. Resistance to grain protectants and fumigants in insect pests of stored products in Australia. *Proceedings of the Australian Postharvest Technical Conference*. CSIRO, Canberra, Australia. pp. 55-57.

Collins, P.J., Daglish, G.J., Bengston, M., Lambkin, T.M., Pavic, H. 2002. Genetics of resistance to phosphine in *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Journal of economic entomology*, **95**(4), 862-869.

Crespo, R., Pedrini, N., Juarez, M., Dal Bello, G. 2008. Volatile organic compounds released by the entomopathogenic fungus *Beauveria bassiana*. *Microbiological research*, **163**(2), 148-151.

Daglish, G.J., Collins, P.J., Pavic, H., Kopittke, R.A. 2002. Effects of time and concentration on mortality of phosphine-resistant *Sitophilus oryzae* (L) fumigated with phosphine. *Pest Management Science: formerly Pesticide Science*, **58**(10), 1015-1021.

Daglish, G.J., Nayak, M.K., Pavic, H. 2014. Phosphine resistance in *Sitophilus oryzae* (L.) from eastern Australia: Inheritance, fitness and prevalence. *Journal of Stored Products Research*, **59**, 237-244.

Daglish, G.J., Nayak, M.K., Pavic, H., Smith, L.W. 2015. Prevalence and potential fitness cost of weak phosphine resistance in *Tribolium castaneum* (Herbst) in eastern Australia. *Journal of stored products research*, **61**, 54-58.

Dallinga, J., Robroeks, C., Van Berkel, J., Moonen, E., Godschalk, R., Jöbsis, Q., Dompeling, E., Wouters, E., Van Schooten, F. 2010. Volatile organic compounds in exhaled breath as a diagnostic tool for asthma in children. *Clinical & Experimental Allergy*, **40**(1), 68-76.

Davoli, E., Gangai, M., Morselli, L., Tonelli, D. 2003. Characterisation of odorants emissions from landfills by SPME and GC/MS. *Chemosphere*, **51**(5), 357-368.

De Lacy Costello, B., Evans, P., Ewen, R., Gunson, H., Jones, P.R., Ratcliffe, N.M., Spencer-Phillips, P.T. 2001. Gas chromatography–mass spectrometry analyses of volatile organic compounds from potato tubers inoculated with *Phytophthora* infestans or *Fusarium coeruleum*. *Plant Pathology*, **50**(4), 489-496.

De Pasquale, C., Guarino, S., Peri, E., Alonzo, G., Colazza, S. 2007. Investigation of cuticular hydrocarbons from *Bagrada hilaris* genders by SPME/GC-MS. *Analytical and bioanalytical chemistry*, **389**(4), 1259-1265.

Desbois, A.P., Smith, V.J. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied microbiology and biotechnology*, **85**(6), 1629-1642.

Diehl, P.A. 1973. Paraffin synthesis in the oenocytes of the desert locust. *Nature*, **243**(5408), 468.

Diehl, P.A. 1975. Synthesis and release of hydrocarbons by the oenocytes of the desert locust, *Schistocerca gregaria*. *Journal of Insect Physiology*, **21**(6), 1237-1246.

Donahaye, E.J. 2000. Current status of non-residual control methods against stored product pests. *Crop Protection*, **19**(8-10), 571-576.

Donahaye, E.J., Bell C., Jayes D., Noyas R., Phillips T.W. 2007. Integrated pest management strategies used in stored grains in Brazil to manage phosphine resistance. . *Proceeding International conference controlled atmosphere and fumigation in stored product, Gold coast Australia.*, **8-13th August,** , pp. 293-300.

Donahaye EJ, N.S., Bell C, Jayes D, Noyas R, Phillips TW 2007. Integrated pest management strategies used in stored grains in Brazil to manage phosphine resistance. *Proceeding*

International conference controlled atmosphere and fumigation in stored product, Gold coast Australia. 8-13th August, pp. 293-300.

Downer, R., Matthews, J. 1976. Patterns of lipid distribution and utilisation in insects. *American Zoologist*, **16**(4), 733-745.

Du, J. 2001. Plant-insect chemical communication and its behavior control. *Acta Phytophysiologica Sinica*, **27**(3), 193-200.

Dua, R., Gill, K.D. 2004. Effect of aluminium phosphide exposure on kinetic properties of cytochrome oxidase and mitochondrial energy metabolism in rat brain. *Biochimica et Biophysica Acta (BBA)-General Subjects*, **1674**(1), 4-11.

Dua, R., Sunkaria, A., Kumar, V., Gill, K.D. 2010. Impaired mitochondrial energy metabolism and kinetic properties of cytochrome oxidase following acute aluminium phosphide exposure in rat liver. *Food and chemical toxicology*, **48**(1), 53-60.

Dyte, C., Mills, K., Price, N. 1983. Recent work on fumigant-resistant insect strains. *Proceedings of the 6th British Pest Control Conf*. pp. 7-10.

Edde, P.A. 2012. A review of the biology and control of *Rhyzopertha dominica* (F.) the lesser grain borer. *Journal of Stored Products Research*, **48**, 1-18.

El-Sayed, G.N., Ignoffo, C.M., Leathers, T.D. 1991. Effects of cuticle source and concentration on germination of conidia of two isolates of *Nomuraea rileyi*. *Mycopathologia*, **113**(2), 95-102.

Emekci, M., Navarro, S., Donahaye, E., Rindner, M., Azrieli, A. 2002. Respiration of *Tribolium castaneum* (Herbst) at reduced oxygen concentrations. *Journal of Stored Products Research*, **38**(5), 413-425.

Emekci, M., Navarro, S., Donahaye, J., Rindner, M., Azrieli, A. 2001. Respiration of stored product pests in hermetic conditions. *Proceedings of the International Conference on Controlled Atmosphere and Fumigation in Stored Products*. Executive Printing Services Clovis, CA. pp. 26-35.

Evers, T., Millar, S. 2002. Cereal grain structure and development: some implications for quality. *Journal of Cereal Science*, **36**(3), 261-284.

Ezquerro, Ó., Pons, B., Tena, M.a.T. 2003. Multiple headspace solid-phase microextraction for the quantitative determination of volatile organic compounds in multilayer packagings. *Journal of Chromatography A*, **999**(1-2), 155-164.

FAO. 1975. Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides. Tentative method for adults of some major pest species of stored cereals, with methyl bromide and phosphine. FAO Method No. 16. *Plant Protection Bulletin*, **23**(1), 12–25.

Farag, M.A., Ryu, C.-M., Sumner, L.W., Paré, P.W. 2006. GC–MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. *Phytochemistry*, **67**(20), 2262-2268.

Ferveur, J.-F., Cortot, J., Rihani, K., Cobb, M., Everaerts, C. 2018. Desiccation resistance: effect of cuticular hydrocarbons and water content in *Drosophila melanogaster* adults. *PeerJ*, **6**, e4318.

Fluck, E. 1973. The chemistry of phosphine. in: *Inorganic Chemistry*, Springer, pp. 1-64.

Folch, J., Lees, M., Sloane Stanley, G. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J biol Chem*, **226**(1), 497-509.

Gäde, G., Auerswald, L. 2002. Beetles' choice—proline for energy output: control by AKHs. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **132**(1), 117-129.

Garcia-Esteban, M., Ansorena, D., Astiasarán, I., Ruiz, J. 2004. Study of the effect of different fiber coatings and extraction conditions on dry cured ham volatile compounds extracted by solid-phase microextraction (SPME). *Talanta*, **64**(2), 458-466.

Georghiou, G.P. 1986. The magnitude of the resistance problem. *Pesticide resistance: Strategies and tactics for management*.

Georghiou, G.P., Mellon, R.B. 1983. Pesticide resistance in time and space. in: *Pest resistance to pesticides*, Springer, pp. 1-46.

Gianelli, M.P., Flores, M., Toldra, F. 2002. Optimisation of solid phase microextraction (SPME) for the analysis of volatile compounds in dry-cured ham. *Journal of the Science of Food and Agriculture*, **82**(14), 1703-1709.

Gibbs, A., Pomonis, J.G. 1995. Physical properties of insect cuticular hydrocarbons: the effects of chain length, methyl-branching and unsaturation. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **112**(2), 243-249.

Gibbs, A.G. 1998. Water-proofing properties of cuticular lipids. *American Zoologist*, **38**(3), 471-482.

Gilbert, L.I., Chino, H. 1974. Transport of lipids in insects. *Journal of lipid research*, **15**(5), 439-456.

Ginzel, M.D., Millar, J.G., Hanks, L.M. 2003. (Z)-9-Pentacosene− contact sex pheromone of the locust borer, *Megacyllene robiniae*. *Chemoecology*, **13**(3), 135-141.

Ginzel, M.D., Moreira, J.A., Ray, A.M., Millar, J.G., Hanks, L.M. 2006. (Z)-9-Nonacosene—major component of the contact sex pheromone of the beetle *Megacyllene caryae*. *Journal of chemical ecology*, **32**(2), 435-451.

Goh, H.H., Khairudin, K., Sukiran, N.A., Normah, M., Baharum, S.N. 2016. Metabolite profiling reveals temperature effects on the VOC s and flavonoids of different plant populations. *Plant Biology*, **18**, 130-139.

Gołębiowski, M., Boguś, M.I., Paszkiewicz, M., Stepnowski, P. 2010. The composition of the free fatty acids from *Dendrolimus pini exuviae*. *Journal of insect physiology*, **56**(4), 391-397.

Gołębiowski, M., Boguś, M.I., Paszkiewicz, M., Stepnowski, P. 2011. Cuticular lipids of insects as potential biofungicides: methods of lipid composition analysis. *Analytical and bioanalytical chemistry*, **399**(9), 3177-3191.

Gołębiowski, M., Cerkowniak, M., Dawgul, M., Kamysz, W., BOGUŚ, M.I., Stepnowski, P. 2013. The antifungal activity of the cuticular and internal fatty acid methyl esters and alcohols in *Calliphora vomitoria*. *Parasitology*, **140**(8), 972-985.

Gołębiowski, M., Dawgul, M., Kamysz, W., Boguś, M.I., Wieloch, W., Włóka, E., Paszkiewicz, M., Przybysz, E., Stepnowski, P. 2012. The antimicrobial activity of the alcohols from *Musca domestica*. *Journal of Experimental Biology*, jeb. 073155.

Gołębiowski, M., Maliński, E., Boguś, M.I., Kumirska, J., Stepnowski, P. 2008a. The cuticular fatty acids of *Calliphora vicina*, *Dendrolimus pini* and *Galleria mellonella* larvae and their role in resistance to fungal infection. *Insect biochemistry and molecular biology*, **38**(6), 619-627.

Gołębiowski, M., Maliński, E., Nawrot, J., Stepnowski, P. 2008b. Identification and characterization of surface lipid components of the dried-bean beetle *Acanthoscelides obtectus* (Say)(Coleoptera: Bruchidae). *Journal of stored products research*, **44**(4), 386-388.

Gołębiowski, M., Maliński, E., Nawrot, J., Szafranek, J., Stepnowski, P. 2007. Identification of the cuticular lipid composition of the Western Flower Thrips *Frankliniella occidentalis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **147**(2), 288-292.

Guedes, R.N.C., Oliveira, E., Guedes, N., Ribeiro, B., Serrão, J. 2006. Cost and mitigation of insecticide resistance in the maize weevil, *Sitophilus zeamais*. *Physiological Entomology*, **31**(1), 30-38.

Guerenstein, P.G., Hildebrand, J.G. 2008. Roles and effects of environmental carbon dioxide in insect life. *Annu. Rev. Entomol.*, **53**, 161-178.

Hadley, N.F. 1984. Cuticle: ecological significance. in: *Biology of the Integument*, Springer, pp. 685-693.

Hagstrum, D.W., Flinn, P.W. 1994. Survival of *Rhyzopertha dominica* (Coleoptera: Bostrichidae) in stored wheat under fall and winter temperature conditions. *Environmental Entomology*, **23**(2), 390-395.

Hagstrum, D.W., Phillips, T.W., Cuperus, G. 2012. Stored product protection. *Kansas State University, KSRE Publ. S–156*.

Hahn, D.A., Denlinger, D.L. 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. *Journal of Insect Physiology*, **53**(8), 760-773.

Hameed, A., Freed, S., Hussain, A., Iqbal, M., Hussain, M., Naeem, M., Sajjad, A., Hussnain, H., Sadiq, M.A., Tipu, A.L. 2012. Toxicological effects of neem (*Azadirachta indica*), Kanair (*Nerium oleander*) and spinosad (Tracer 240 SC) on the red flour beetle (Tribolium castaneum)(Herbst.). *African Journal of Agricultural Research*, **7**(4), 555-560.

Harein, P., Davis, R. 1992. Control of stored-grain insects. *Storage of cereal grains and their products*, **4**, 491-534.

Haruhito, K., Haruo, C. 1982. Transport of hydrocarbons by the lipophorin of insect hemolymph. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, **710**(3), 341-348.

Herron, G.A. 1990. Resistance to grain protectants and phosphine in coleopterous pests of grain stored on farms in New South Wales. *Australian Journal of Entomology*, **29**(3), 183-189.

Hobbs, S., Bond, E. 1989. Response of *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae) to sublethal treatments with phosphine. *Journal of Stored Products Research*, **25**(3), 137-146.

Hoch, F.L. 1992. Cardiolipins and biomembrane function. *Biochimica et Biophysica Acta (BBA)-Reviews on Biomembranes*, **1113**(1), 71-133.

Hodges, R., Robinson, R., Hall, D. 1996. Quinone contamination of dehusked rice by *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae). *Journal of Stored Products Research*, **32**(1), 31-37.

Hoffman, A.G., Downer, R. 1979. End product specificity of triacylglycerol lipases from intestine, fat body, muscle and haemolymph of the American cockroach, *Periplaneta americana* L. *Lipids*, **14**(11), 893-899.

Hole, B.D., Bell, C., Mills, K., Goodship, G. 1976. The toxicity of phosphine to all developmental stages of thirteen species of stored product beetles. *Journal of Stored Products Research*, **12**(4), 235-244.

Horvath, S.E., Daum, G. 2013. Lipids of mitochondria. *Progress in lipid research*, **52**(4), 590-614.

Howard, R.W., Lord, J.C. 2003. Cuticular lipids of the booklouse, Liposcelis bostrychophila: hydrocarbons, aldehydes, fatty acids, and fatty acid amides. *Journal of chemical ecology*, **29**(3), 615-627.

Howard, R.W., Mueller, D.D. 1987. Defensive chemistry of the flour beetle*Tribolium brevicornis* (LeC). *Journal of chemical ecology*, **13**(7), 1707-1723.

Howe, R. 1973. The susceptibility of the immature and adult stages of *Sitophilus granarius* to phosphine. *Journal of Stored Products Research*, **8**(4), 241-262.

Hsu, C.-H., Han, B.-C., Liu, M.-Y., Yeh, C.-Y., Casida, J.E. 2000. Phosphine-induced oxidative damage in rats: attenuation by melatonin. *Free Radical Biology and Medicine*, **28**(4), 636-642.

Hsu, C.-H., Quistad, G.B., Casida, J.E. 1998. Phosphine-induced oxidative stress in Hepa 1c1c7 cells. *Toxicological sciences*, **46**(1), 204-210.

Inui, Y., Itioka, T. 2007. Species-specific leaf volatile compounds of obligate *Macaranga myrmecophytes* and host-specific aggressiveness of symbiotic Crematogaster ants. *Journal of chemical ecology*, **33**(11), 2054-2063.

Jagadeesan, R., Collins, P.J., Daglish, G.J., Ebert, P.R., Schlipalius, D.I. 2012. Phosphine resistance in the rust red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae): inheritance, gene interactions and fitness costs. *PLos one*, **7**(2), e31582.

Jagadeesan, R., Collins, P.J., Nayak, M.K., Schlipalius, D.I., Ebert, P.R. 2016. Genetic characterization of field-evolved resistance to phosphine in the rusty grain beetle, *Cryptolestes ferrugineus* (Laemophloeidae: Coleoptera). *Pesticide biochemistry and physiology*, **127**, 67-75.

Jayas, D.S., White, N.D. 2003. Storage and drying of grain in Canada: low cost approaches. *Food control*, **14**(4), 255-261.

Jeleń, H.H., Obuchowska, M., Zawirska-Wojtasiak, R., Wasowicz, E. 2000. Headspace solid-phase microextraction use for the characterization of volatile compounds in vegetable oils of different sensory quality. *Journal of Agricultural and Food Chemistry*, **48**(6), 2360-2367.

Jhumur, U.S., Dötterl, S., Jürgens, A. 2007. Electrophysiological and behavioural responses of mosquitoes to volatiles of *Silene otites* (Caryophyllaceae). *Arthropod-Plant Interactions*, **1**(4), 245.

Jian, F., Jayas, D.S., White, N.D. 2000. Toxic action of phosphine on the adults of copra mite *Tyrophagus putrescentiae* [Astigmata: Acaridae]. *Phytoprotection*, **81**(1), 23-28.

Jutsum, A., Goldsworthy, G. 1976. Fuels for flight in Locusta. *Journal of Insect Physiology*, **22**(2), 243-249.

Kashi, K.P. 1981. Response of five species of stored-product insects to phosphine in oxygen-deficient atmospheres. *Pesticide Science*, **12**(2), 111-115.

Kataoka, H., Lord, H.L., Pawliszyn, J. 2000. Applications of solid-phase microextraction in food analysis. *Journal of chromatography A*, **880**(1-2), 35-62.

Katase, H., Chino, H. 1984. Transport of hydrocarbons by haemolymph lipophorin in *Locusta migratoria*. *Insect biochemistry*, **14**(1), 1-6.

Kaur, R., Daniels, E.V., Nayak, M.K., Ebert, P.R., Schlipalius, D.I. 2013. Determining changes in the distribution and abundance of a *Rhyzopertha dominica* phosphine resistance allele in farm grain storages using a DNA marker. *Pest management science*, **69**(6), 685-688.

Kaur, R., Subbarayalu, M., Jagadeesan, R., Daglish, G., Nayak, M., Naik, H., Ramasamy, S., Subramanian, C., Ebert, P., Schlipalius, D. 2015. Phosphine resistance in India is characterised by a dihydrolipoamide dehydrogenase variant that is otherwise unobserved in eukaryotes. *Heredity*, **115**(3), 188.

Kesselmeier, J., Staudt, M. 1999. Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. *Journal of atmospheric chemistry*, **33**(1), 23-88.

Keville, R., Kannowski, P.B. 1975. Sexual excitation by pheromones of the confused flour beetle. *Journal of insect physiology*, **21**(1), 81-84.

Khorramshahi, A., Burkholder, W. 1981. Behavior of the lesser grain borer *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Journal of Chemical Ecology*, **7**(1), 33-38.

Kiaya, V. 2014. Post-harvest losses and strategies to reduce them. *Technical Paper on Postharvest Losses, Action Contre la Faim (ACF)*.

Kleeberg, K., Liu, Y., Jans, M., Schlegelmilch, M., Streese, J., Stegmann, R. 2005. Development of a simple and sensitive method for the characterization of odorous waste gas emissions by means of solid-phase microextraction (SPME) and GC–MS/olfactometry. *Waste Management*, **25**(9), 872-879.

Kocak, E., Schlipalius, D., Kaur, R., Tuck, A., Ebert, P., Collins, P., Yılmaz, A. 2015. Determining phosphine resistance in rust red flour beetle, *Tribolium castaneum* (Herbst.)(Coleoptera: Tenebrionidae) populations from Turkey. *Turk J Entomol*, **39**, 129-136.

Kofroňová, E., Cvačka, J., Vrkoslav, V., Hanus, R., Jiroš, P., Kindl, J., Hovorka, O., Valterová, I. 2009. A comparison of HPLC/APCI-MS and MALDI-MS for characterising

triacylglycerols in insects: Species-specific composition of lipids in the fat bodies of bumblebee males. *Journal of Chromatography B*, **877**(30), 3878-3884.

Koidsumi, K. 1957. Antifungal action of cuticular lipids in insects. *Journal of Insect Physiology*, **1**(1), 40-51.

Kolattukudy, P. 1976. Introduction to natural waxes. *Chemistry and biochemistry of natural waxes*.

Krishnasamy, V., Seshu, D. 1990. Phosphine fumigation influence on rice seed germination and vigor. *Crop science*, **30**(1), 82-85.

Landolt, P.J., Phillips, T.W. 1997. Host plant influences on sex pheromone behavior of phytophagous insects. *Annual review of entomology*, **42**(1), 371-391.

Laopongsit, W., Srzednicki, G., Craske, J. 2014. Preliminary study of solid phase micro-extraction (SPME) as a method for detecting insect infestation in wheat grain. *Journal of stored products research*, **59**, 88-95.

Laothawornkitkul, J., Jansen, R., Smid, H., Bouwmeester, H., Muller, J., Van Bruggen, A. 2010. Volatile organic compounds as a diagnostic marker of late blight infected potato plants: A pilot study. *Crop protection*, **29**(8), 872-878.

Leesch, J.G. 1992. Carbon dioxide on the penetration and distribution of phosphine through wheat. *Journal of economic entomology*, **85**(1), 157-161.

Li, J., Wang, X., Zhang, T., Wang, C., Huang, Z., Luo, X., Deng, Y. 2015. A review on phospholipids and their main applications in drug delivery systems. *Asian journal of pharmaceutical sciences*, **10**(2), 81-98.

Lilford, K., Fulford, G., Schlipalius, D., Ridley, A. 2009. Fumigation of stored grain insects a two locus model of phosphine resistance. *The 18th World IMACS Congress and MODSIM09, International Congress on Modelling and Simulation, Cairns, Australia*.

Liu, Y., Ma, T., Zhang, W., Wu, J., Cheng, Z. 2004. The antimicrobial effect of plant volatiles. *Journal of Jishou University*, **25**, 39-42.

Locke, M. 1974. The structure and formation of the integument in insects. in: *The Physiology of Insecta (Second Edition)*, Elsevier, pp. 123-213.

Lockey, K.H. 1978. Hydrocarbons of adult *Tribolium castaneum* Hbst. and *Tribolium confusum* Duv.(Coleoptera: Tenebrionidae). *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **61**(3), 401-407.

Lorini, I., Ferreira Filho, A. 2004. Integrated pest management strategies used in stored grain in Brazil to manage phosphine resistance. *Proceedings of the International Conference on Controlled Atmosphere and Fumigation in Stored Products, Gold-Coast Australia*. pp. 293-300.

Malekpour, R., Rafter, M.A., Daglish, G.J., Walter, G.H. 2016. Influence of phosphine resistance genes on flight propensity and resource location in *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae): the landscape for selection. *Biological journal of the Linnean Society*, **119**(2), 348-358.

Marco, A., Navarro, J., Flores, M. 2004. Volatile compounds of dry-fermented sausages as affected by solid-phase microextraction (SPME). *Food Chemistry*, **84**(4), 633-641.

Matisová, E., Sedláková, J., Slezáčková, M., Welsch, T. 1999. Solid-phase Microextraction of Volatile Polar Compounds in Water. *Journal of High Resolution Chromatography*, **22**(2), 109-115.

Mau, Y.S., Collins, P.J., Daglish, G.J., Nayak, M.K., Ebert, P.R. 2012. The rph2 gene is responsible for high level resistance to phosphine in independent field strains of *Rhyzopertha dominica*. *PloS one*, **7**(3), e34027.

McDougall, G., Steele, J. 1988. Free fatty acids as a source of energy for trehalose synthesis in the fat body of the American cockroach (*Periplaneta americana*). *Insect biochemistry*, **18**(6), 591-597.

McMahon, H.T., Gallop, J.L. 2005. Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature*, **438**(7068), 590.

Melander, A.L. 1914. Can insects become resistant to sprays. *J. Econ. Entomol*, **7**(1), 167.

Miekisch, W., Schubert, J.K., Noeldge-Schomburg, G.F. 2004. Diagnostic potential of breath analysis—focus on volatile organic compounds. *Clinica chimica acta*, **347**(1-2), 25-39.

Mills, K. 1983. Resistance to the fumigant hydrogen phosphide in some stored-product species associated with repeated inadequate treatments. *Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie*, **4**(1/3), 98-101.

Misra, U., Bhargava, S., Nag, D., Kidwai, M., Lal, M. 1988. Occupational phosphine exposure in Indian workers. *Toxicology letters*, **42**(3), 257-263.

Monnin, T., Malosse, C., Peeters, C. 1998. Solid-phase microextraction and cuticular hydrocarbon differences related to reproductive activity in queenless ant *Dinoponera quadriceps*. *Journal of Chemical Ecology*, **24**(3), 473-490.

Mugisha-Kamatenesi, M., Deng, A., Ogendo, J., Omolo, E., Mihale, M., Otim, M., Buyungo, J., Bett, P. 2008. Indigenous knowledge of field insect pests and their management around Lake Victoria basin in Uganda. *African Journal of Environmental Science and Technology*, **2**(10), 342-348.

Munro, J.W. 1966. Pests of stored products. *Pests of stored products.*

Naeem, A. 2016. Biochemical response in phosphine resistant and susceptible adult beetles of *Trogoderma granarium* to the sublethal dose. *Journal of Entomology and Zoology Studies*, **4**(6), 582-587.

Nakakita, H., KATSUMATA, Y., OZAWA, T. 1971. The effect of phosphine on respiration of rat liver mitochondria. *The Journal of Biochemistry*, **69**(3), 589-593.

Nakakita, H., Kuroda, J. 1986. Differences in phosphine uptake between susceptible and resistant strains of insects. *Journal of Pesticide Science*, **11**(1), 21-26.

Nakamura, S., Daishima, S. 2005. Simultaneous determination of 22 volatile organic compounds, methyl-tert-butyl ether, 1, 4-dioxane, 2-methylisoborneol and geosmin in water by headspace solid phase microextraction-gas chromatography–mass spectrometry. *Analytica Chimica Acta*, **548**(1-2), 79-85.

Nansen, C., Meikle, W.G., Campbell, J., Phillips, T.W., Subramanyam, B. 2008. A binomial and species-independent approach to trap capture analysis of flying insects. *Journal of economic entomology*, **101**(6), 1719-1728.

Nath, N.S., Bhattacharya, I., Tuck, A.G., Schlipalius, D.I., Ebert, P.R. 2011. Mechanisms of phosphine toxicity. *Journal of toxicology*, **2011**.

Nayak, M., Collins, P., Pavic, H., Cao, Y. 2003. Developments in phosphine resistance in China and possible implications for Australia. *Stored Grain in Australia 2003. Proceedings of the Australian Postharvest Technical Conference. CSIRO Stored Grain Research Laboratory: Canberra, Australia*. pp. 156-159.

Nayak, M.K., Collins, P.J. 2008. Influence of concentration, temperature and humidity on the toxicity of phosphine to the strongly phosphine-resistant psocid Liposcelis bostrychophila Badonnel (Psocoptera: Liposcelididae). *Pest management science*, **64**(9), 971-976.

Nayak, M.K., Holloway, J.C., Emery, R.N., Pavic, H., Bartlet, J., Collins, P.J. 2013. Strong resistance to phosphine in the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens)(Coleoptera: Laemophloeidae): its characterisation, a rapid assay for diagnosis and its distribution in Australia. *Pest management science*, **69**(1), 48-53.

Neethirajan, S., Karunakaran, C., Jayas, D., White, N. 2007. Detection techniques for stored-product insects in grain. *Food Control*, **18**(2), 157-162.

Nelson, D.R. 1978. Long-chain methyl-branched hydrocarbons: occurrence, biosynthesis, and function. in: *Advances in insect physiology*, Vol. 13, Elsevier, pp. 1-33.

Nelson, D.R., Charlet, L.D. 2003. Cuticular hydrocarbons of the sunflower beetle, *Zygogramma exclamationis*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **135**(2), 273-284.

Nelson, D.R., Fatland, C.L., Buckner, J.S., Freeman, T.P. 1999. External lipids of adults of the giant whitefly, *Aleurodicus dugesii*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **123**(2), 137-145.

Nelson, D.R., Freeman, T.P., Buckner, J.S. 2000. Waxes and lipids associated with the external waxy structures of nymphs and pupae of the giant whitefly, *Aleurodicus dugesii*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **125**(2), 265-278.

Nelson, D.R., Sukkestad, D.R. 1970. Normal and branched aliphatic hydrocarbons from the eggs of the tobacco hornworm. *Biochemistry*, **9**(23), 4601-4611.

Nelson, D.R., Tissot, M., Nelson, L.J., Fatland, C.L., Gordon, D.M. 2001. Novel wax esters and hydrocarbons in the cuticular surface lipids of the red harvester ant, *Pogonomyrmex barbatus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **128**(3), 575-595.

Nguyen, D.T. 2006. Analysis of the behaviour of Rhyzopertha dominica (Coleoptera: Bostrichidae) towards host volatiles, M. Sc. Thesis, University of Greenwich, UK.

Nguyen, T.T. 2016. Genetics of phosphine resistance in the rice weevil *Sitophilus oryzae* (L.)(Coleoptera: Curculionidae).

Nguyen, T.T., Collins, P.J., Ebert, P.R. 2015. Inheritance and characterization of strong resistance to phosphine in *Sitophilus oryzae* (L.). *PloS one*, **10**(4), e0124335.

Nicolas, G., Sillans, D. 1989. Immediate and latent effects of carbon dioxide on insects. *Annual review of entomology*, **34**(1), 97-116.

Nilsson, T., Larsen, T.O., Montanarella, L., Madsen, J.Ø. 1996. Application of head-space solid-phase microextraction for the analysis of volatile metabolites emitted by *Penicillium* species. *Journal of Microbiological Methods*, **25**(3), 245-255.

Niu, Y., Hardy, G., Hua, L., Trengove, R., Agarwal, M., Cheng, H., Ren, Y. 2012. Optimization of HS-SPME-GC method for detection of stored grain insects.

Niu, Y., Hua, L., Hardy, G., Agarwal, M., Ren, Y. 2015. Analysis of volatiles from stored wheat and *Rhyzopertha dominica* (F.) with solid phase microextraction–gas chromatography mass spectrometry. *Journal of the Science of Food and Agriculture*, **96**(5), 1697-1703.

Opit, G., Phillips, T.W., Aikins, M.J., Hasan, M. 2012. Phosphine resistance in *Tribolium castaneum* and *Rhyzopertha dominica* from stored wheat in Oklahoma. *Journal of Economic Entomology*, **105**(4), 1107-1114.

Oppert, B., Guedes, R.N., Aikins, M.J., Perkin, L., Chen, Z., Phillips, T.W., Zhu, K.Y., Opit, G.P., Hoon, K., Sun, Y. 2015. Genes related to mitochondrial functions are differentially expressed in phosphine-resistant and-susceptible *Tribolium castaneum*. *BMC genomics*, **16**(1), 968.

Oppert, B., Morgan, T. 2013. Improved high-throughput bioassay for Rhyzopertha dominica (F.)(Coleoptera: Bostrichidae). *Journal of stored products research*, **52**, 68-73.

Pacheco, I., Santori, M., Taylor, R. 1990. Survey of phosphine resistance in stored grain insect pests in the state of Sao Paulo. *Coletanea do Instituto de Tecnologia de Alimentos*, **20**(2), 144-154.

Padın, S., Dal Bello, G., Fabrizio, M. 2002. Grain loss caused by *Tribolium castaneum*, *Sitophilus oryzae* and *Acanthoscelides obtectus* in stored durum wheat and beans treated with Beauveria bassiana. *Journal of Stored Products Research*, **38**(1), 69-74.

Park, T. 1932. Studies in population physiology: the relation of numbers to initial population growth in the flour beetle *Tribolium confusum* Duval. *Ecology*, **13**(2), 172-181.

Pawliszyn, J. 1995. New directions in sample preparation for analysis of organic compounds. *TrAC Trends in Analytical Chemistry*, **14**(3), 113-122.

Pecoraino, G., Scalici, L., Avellone, G., Ceraulo, L., Favara, R., Candela, E.G., Provenzano, M.C., Scaletta, C. 2008. Distribution of volatile organic compounds in Sicilian groundwaters analysed by head space-solid phase micro extraction coupled with gas chromatography mass spectrometry (SPME/GC/MS). *Water research*, **42**(14), 3563-3577.

Pepeu, G., Pepeu, I.M., Amaducci, L. 1996. A review of phosphatidylserine pharmacological and clinical effects. Is phosphatidylserine a drug for the ageing brain? *Pharmacological Research*, **33**(2), 73-80.

Perera, R.M., Marriott, P.J., Galbally, I.E. 2002. Headspace solid-phase microextraction—Comprehensive two-dimensional gas chromatography of wound induced plant volatile organic compound emissions. *Analyst*, **127**(12), 1601-1607.

Perez-Mendoza, J. 1999. Survey of insecticide resistance in Mexican populations of maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae). *Journal of Stored Products Research*, **35**(1), 107-115.

Pimentel, M., Faroni, L., Guedes, R., Neto, A., Garcia, F. 2007. Phosphine resistance, respiration rate and fitness consequences in *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae). *Proceedings of the 9th International Working Conference on Stored Product Protection*. pp. 344-351.

Pimentel, M., LRD'A, F., Guedes, R., Sousa, A., Tótola, M. 2009. Phosphine resistance in Brazilian populations of *Sitophilus zeamais* motschulsky (Coleoptera: Curculionidae). *Journal of Stored Products Research*, **45**(1), 71-74.

Pimentel, M.A., Faroni, L.R.D.A., da Silva, F.H., Batista, M.D., Guedes, R.N. 2010. Spread of phosphine resistance among Brazilian populations of three species of stored product insects. *Neotropical Entomology*, **39**(1), 101-107.

Pimentel, M.A.G., Faroni, L.R.A., Corrêa, A.S., Guedes, R.N.C. 2012. Phosphine-induced walking response of the lesser grain borer (*Rhyzopertha dominica*). *Pest management science*, **68**(10), 1368-1373.

Pimentel, M.A.G., Faroni, L.R.D.A., Tótola, M.R., Guedes, R.N.C. 2008. Phosphine resistance, respiration rate and fitness consequences in stored-product insects. *Pest Management Science: formerly Pesticide Science*, **63**(9), 876-881.

Pino Moreno, J., Ganguly, A. 2016. Determination of fatty acid content in some edible insects of Mexico. *Journal of Insects as Food and Feed*, **2**(1), 37-42.

Potter, W., Garry, V., Kelly, J., Tarone, R., Griffith, J., Nelson, R. 1993. Radiometric assay of red cell and plasma cholinesterase in pesticide appliers from Minnesota. *Toxicology and applied pharmacology*, **119**(1), 150-155.

Pratt, S.J. 2003. A new measure of uptake: desorption of unreacted phosphine from susceptible and resistant strains of *Tribolium castaneum* (Herbst)(Coleoptera: Tenebrionidae). *Journal of Stored Products Research*, **39**(5), 507-520.

Price, L., Mills, K. 1988. The toxicity of phosphine to the immature stages of resistant and susceptible strains of some common stored product beetles, and implications for their control. *Journal of Stored Products Research*, **24**(1), 51-59.

Price, N. 1984. Active exclusion of phosphine as a mechanism of resistance in *Rhyzopertha dominica* (F.)(Coleoptera: Bostrychidae). *Journal of Stored Products Research*, **20**(3), 163-168.

Price, N. 1981. A Comparison Of The Uptake And Metabolism Of 32 P-Radiolabelled Phosphine In Susceptible And Resistant Strains Of The Lesser Grain Borer (*Rhyzopertha Dominica*) Vol-69, MPKV; Maharastra.

Price, N. 1980a. The effect of phosphine on respiration and mitochondrial oxidation in susceptible and resistant strains of *Rhyzopertha dominica*. *Insect Biochemistry*, **10**(1), 65-71.

Price, N. 1980b. Some aspects of the inhibition of cytochrome c oxidase by phosphine in susceptible and resistant strains of *Rhyzopertha dominicia*. *Insect Biochemistry*, **10**(2), 147-150.

Price, N.R., Dance, S.J. 1983. Some biochemical aspects of phosphine action and resistance in three species of stored product beetles. *Comparative biochemistry and physiology. C, Comparative pharmacology and toxicology*, **76**(2), 277-281.

Price, N.R., Walter, C.M. 1987. A comparison of some effects of phosphine, hydrogen cyanide and anoxia in the lesser grain borer, *Rhyzopertha dominica* (F.)(Coleoptera:

Bostrychidae). *Comparative biochemistry and physiology. C, Comparative pharmacology and toxicology*, **86**(1), 33-36.

Qiu, R., Qu, D., Hardy, G.E.S.J., Trengove, R., Agarwal, M., Ren, Y. 2014a. Optimization of headspace solid-phase microextraction conditions for the identification of *phytophthora cinnamomi* rands. *Plant disease*, **98**(8), 1088-1098.

Qiu, R., Qu, D., Trengove, R., Agarwal, M., Hardy, G.E.S.J., Ren, Y. 2014b. Headspace solid-phase microextraction and gas chromatography-mass spectrometry for analysis of VOCs produced by Phytophthora cinnamomi. *Plant Disease*, **98**(8), 1099-1105.

Qui, R., Hardy, G., Dong, Q., Trengove, R., Agarwal, M., Ren, Y. 2012. Optimization of the Headspace Solid-Phase Microextraction Gas Chromatography for Volatile Compounds Determination in Phytophthora Cinnamomi Rands. *World Academy of Science, Engineering and Technology. Proceedings*, **72**, 324-328.

Rees, D.P. 2004. *Insects of stored products*. Csiro Publishing.

Reichmuth, C. 1994. Uptake of phosphine by stored-product pest insects during fumigation. *Proceedings of the 6th International Working Conference on Stored-product Protection*. pp. 157-162.

Ren, M., Phoon, C.K., Schlame, M. 2014. Metabolism and function of mitochondrial cardiolipin. *Progress in lipid research*, **55**, 1-16.

Risticevic, S., Lord, H., Górecki, T., Arthur, C.L., Pawliszyn, J. 2010. Protocol for solid-phase microextraction method development. *Nature protocols*, **5**(1), 122.

Rochat, D., Ramirez-Lucas, P., Malosse, C., Aldana, R., Kakul, T., Morin, J.-P. 2000. Role of solid-phase microextraction in the identification of highly volatile pheromones of two Rhinoceros beetles *Scapanes australis* and *Strategus aloeus* (Coleoptera, Scarabaeidae, Dynastinae). *Journal of Chromatography A*, **885**(1-2), 433-444.

Rodrigues, F.d.M., Mesquita, P.R., De Oliveira, L.S., De Oliveira, F.S., Menezes Filho, A., Pereira, P.A.d.P., de Andrade, J.B. 2011. Development of a headspace solid-phase microextraction/gas chromatography–mass spectrometry method for determination of organophosphorus pesticide residues in cow milk. *Microchemical Journal*, **98**(1), 56-61.

Romer, F. 1980. Histochemical and biochemical investigations concerning the function of larval oenocytes of *Tenebrio molitor* L.(Coleoptera, Insecta). *Histochemistry*, **69**(1), 69-84.

Roux, E., Sreng, L., Provost, E., Roux, M., Clement, J.-L. 2002. Cuticular hydrocarbon profiles of dominant versus subordinate male *Nauphoeta cinerea* cockroaches. *Journal of chemical ecology*, **28**(6), 1221-1235.

Rumpold, B.A., Schlüter, O.K. 2013. Nutritional composition and safety aspects of edible insects. *Molecular nutrition & food research*, **57**(5), 802-823.

Saïd, I., Costagliola, G., Leoncini, I., Rivault, C. 2005. Cuticular hydrocarbon profiles and aggregation in four *Periplaneta species* (Insecta: Dictyoptera). *Journal of insect physiology*, **51**(9), 995-1003.

Saxena, J., Bhatia, S. 1980. Reduction in fecundity of *Tribolium castaneum* (Herbst) due to fumigation and phosphine resistance. *Indian Journal of Entomology*, **42**(4), 796-798.

Saxena, R., Jilani, G., Kareem, A.A. 1988. Effects of neem on stored grain insects. *Focus on phytochemical pesticides*, **1**, 97-111.

Schlipalius, D., Chen, W., Collins, P., Nguyen, T., Reilly, P., Ebert, P. 2008. Gene interactions constrain the course of evolution of phosphine resistance in the lesser grain borer, *Rhyzopertha dominica*. *Heredity*, **100**(5), 506.

Schlipalius, D.I., Cheng, Q., Reilly, P.E., Collins, P.J., Ebert, P.R. 2002. Genetic linkage analysis of the lesser grain borer *Rhyzopertha dominica* identifies two loci that confer high-level resistance to the fumigant phosphine. *Genetics*, **161**(2), 773-782.

Schlipalius, D.I., Valmas, N., Tuck, A.G., Jagadeesan, R., Ma, L., Kaur, R., Goldinger, A., Anderson, C., Kuang, J., Zuryn, S. 2012. A core metabolic enzyme mediates resistance to phosphine gas. *Science*, **338**(6108), 807-810.

Scudamore, K.A., Goodship, G. 1986. Determination of phosphine residues in fumigated cereals and other foodstuffs. *Pesticide science*, **17**(4), 385-395.

Seitz, L., Sauer, D. 1996. Volatile compounds and odors in grain sorghum infested with common storage insects. *Cereal chemistry*.

Seitz, L.M., Ram, M. 2004. Metabolites of lesser grain borer in grains. *Journal of agricultural and food chemistry*, **52**(4), 898-908.

Sekine, T., Sugano, M., Majid, A., Fujii, Y. 2007. Antifungal effects of volatile compounds from black zira (*Bunium persicum*) and other spices and herbs. *Journal of chemical ecology*, **33**(11), 2123-2132.

Sher, F., Ali, S., Shakoori, A. 2004. Phosphine induced changes in various esterase levels in 4th instar larvae of Trogoderma granarium. *Pakistan J. Zool*, **36**(4), 257-260.

Shrivastava, A., Gupta, V.B. 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. *Chronicles of young scientists*, **2**(1), 21.

Singh, R.P., Gangadharappa, H., Mruthunjaya, K. 2017. Phospholipids: unique carriers for drug delivery systems. *Journal of Drug Delivery Science and Technology*, **39**, 166-179.

Singh, S., Bhalla, A., Verma, S.K., Kaur, A., Gill, K. 2006. Cytochrome-c oxidase inhibition in 26 aluminum phosphide poisoned patients. *Clinical Toxicology*, **44**(2), 155-158.

SITTISUANG, P. 1985. The effect of phosphine and methyl bromide on germination of rice and corn seeds. *Journal of Pesticide Science*, **10**(3), 461-468.

Sledge, M.F., Moneti, G., Pieraccini, G., Turillazzi, S. 2000. Use of solid-phase microextraction in the investigation of chemical communication in social wasps. *Journal of Chromatography A*, **873**(1), 73-77.

Snellings, Y., Herrera, B., Wildemann, B., Beelen, M., Zwarts, L., Wenseleers, T., Callaerts, P. 2018. The role of cuticular hydrocarbons in mate recognition in *Drosophila suzukii*. *Scientific reports*, **8**(1), 4996.

Snelson, J.T. 1987. *Grain protectants*. Australian Centre for International Agricultural Research.

Song, G., Qin, T., Liu, H., Xu, G.-B., Pan, Y.-Y., Xiong, F.-X., Gu, K.-S., Sun, G.-P., Chen, Z.-D. 2010. Quantitative breath analysis of volatile organic compounds of lung cancer patients. *Lung Cancer*, **67**(2), 227-231.

Song, X., Wang, P., Zhang, H. 2011. Phosphine resistance in *Rhyzopertha dominica* (Fabricius)(Coleoptera: Bostrichidae) from different geographical populations in China. *African Journal of Biotechnology*, **10**(72), 16367-16373.

Sostaric, T., Boyce, M.C., Spickett, E.E. 2000. Analysis of the volatile components in vanilla extracts and flavorings by solid-phase microextraction and gas chromatography. *Journal of agricultural and food chemistry*, **48**(12), 5802-5807.

Sousa, A., LRD'A, F., Pimentel, M., Guedes, R. 2009. Developmental and population growth rates of phosphine-resistant and-susceptible populations of stored-product insect pests. *Journal of Stored Products Research*, **45**(4), 241-246.

Spencer, I.M., Candy, D.J. 1976. Hormonal control of diacyl glycerol mobilization from fat body of the desert locust, *Schistocerca gregaria*. *Insect Biochemistry*, **6**(3), 289-296.

Steele, J. 1982. Glycogen phosphorylase in insects. *Insect Biochemistry*, **12**(2), 131-147.

Stevenson, E. 1972. Haemolymph lipids and fat body lipases of the southern armyworm moth. *Journal of Insect Physiology*, **18**(9), 1751-1756.

Storey, K.B., Bailey, E. 1978. The intracellular distribution of enzymes of carbohydrate degradation in the fat body of the adult male cockroach. *Insect Biochemistry*, **8**(1), 73-79.

Subramanian S, C.R., Dwivedi V, Srivastava C. 2016. DNA markers for phosphine resistance in populations of *Tribolium castaneum* from Punjab, India. Pp. 414–417. In: Navarro S, Jayas DS, Alagusundaram K, (Eds.). *Proceedings of the 10th International Conference on Controlled Atmosphere and Fumigation in Stored Products (CAF2016), CAF Permanent Committee Secretariat, Winninpeg, Canada.*

SUZUKI, T., NAKAKITA, H., KUWAHARA, Y. 1988. Defensive Secretions and Hydrocarbons of Two *Tribolium* Species and Their Hybrids: Coleoptera: Tenebrionidae. *Applied Entomology and Zoology*, **23**(3), 329-337.

Suzuki, T., Suzuki, T., Manh Huynh, Y., Muto, T. 1975. Hydrocarbon repellents isolated from *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Agricultural and Biological Chemistry*, **39**(11), 2207-2211.

Talukder, F. 2009. Pesticide Resistance in Stored-Product Insects and Alternative Biorational Management: A Brief Review. *Journal of Agricultural and Marine Sciences [JAMS]*, **14**, 9-15.

Talukder, F., Howse, P. 1994. Laboratory evaluation of toxic and repellent properties of the pithraj tree, Aphanamixis polystachya Wall & Parker, against Sitophilus oryzae (L.). *International Journal of Pest Management*, **40**(3), 274-279.

Talukder, F.A. 1995. Isolation and characterization of active secondary pithraj (*Aphanamixis polystachya*) compounds in controlling stored-product insect-pests, University of Southampton.

Tentschert, J., Bestmann, H., Heinze, J. 2002. Cuticular compounds of workers and queens in two Leptothorax ant species—a comparison of results obtained by solvent extraction, solid sampling, and SPME. *Chemoecology*, **12**(1), 15-21.

Thomas, K.K. 1974. Lipid composition of the fat body and haemolymph and its relation to lipid release in *Oncopeltus fasciatus*. *Journal of insect physiology*, **20**(5), 845-858.

Tietz, A. 1969. Studies on the biosynthesis of diglycerides and triglycerides in cell free preparations of the fat body of the locust *Locusta migratoria*. *Israel journal of medical sciences*, **5**(5), 1007.

Tietz, A., Weintraub, H. 1978. Hydrolysis of glycerides by lipases of the fat body of the locust, *Locusta migratoria*. *Insect Biochemistry*, **8**(1), 11-16.

Tietz, A., Weintraub, H. 1980. The stereospecific structure of haemolymph and fat-body 1, 2-diacylglycerol from *Locusta migratoria*. *Insect Biochemistry*, **10**(1), 61-63.

Toolson, E.C. 1982. Effects of rearing temperature on cuticle permeability and epicuticular lipid composition in *Drosophila pseudoobscura*. *Journal of Experimental Zoology*, **222**(3), 249-253.

Toolson, E.C., Kuper-Simbrón, R. 1989. Laboratory evolution of epicuticular hydrocarbon composition and cuticular permeability in *Drosophila pseudoobscura:* effects on sexual dimorphism and thermal-acclimation ability. *Evolution*, **43**(2), 468-473.

Trematerra, P., Sciarreta, A., Tamasi, E. 2000. Behavioural responses of Oryzaephilus surinamensis, Tribolium castaneum and Tribolium confusum to naturally and artificially damaged durum wheat kernels. *Entomologia Experimentalis et Applicata*, **94**(2), 195-200.

Tsuchida, K., Arai, M., Tanaka, Y., Ishihara, R., Ryan, R.O., Maekawa, H. 1998. Lipid transfer particle catalyzes transfer of carotenoids between lipophorins of *Bombyx mori*. *Insect biochemistry and molecular biology*, **28**(12), 927-934.

Tubiello, F.N., Soussana, J.-F., Howden, S.M. 2007. Crop and pasture response to climate change. *Proceedings of the National Academy of Sciences*, **104**(50), 19686-19690.

Tuduri, L., Desauziers, V., Fanlo, J.L. 2001. Potential of solid-phase microextraction fibers for the analysis of volatile organic compounds in air. *Journal of Chromatographic Science*, **39**(12), 521-529.

Tyler, P., Taylor, R., Rees, D. 1983. Insect resistance to phosphine fumigation in food warehouses in Bangladesh. *International Pest Control*.

Unruh, L.M., Xu, R., Kramer, K.J. 1998. Benzoquinone levels as a function of age and gender of the red flour beetle, *Tribolium castaneum*. *Insect Biochemistry and Molecular Biology*, **28**(12), 969-977.

Upadhyay, R.K., Ahmad, S. 2011. Management strategies for control of stored grain insect pests in farmer stores and public ware houses. *World J Agric Sci*, **7**(5), 527-549.

USDA. 1986. Stored-Grain insects. *United states department of agriculture*, **Agricultural handbook**.

Valmas, N., Zuryn, S., Ebert, P.R. 2008. Mitochondrial uncouplers act synergistically with the fumigant phosphine to disrupt mitochondrial membrane potential and cause cell death. *Toxicology*, **252**(1-3), 33-39.

Vance, J.E., Steenbergen, R. 2005. Metabolism and functions of phosphatidylserine. *Progress in lipid research*, **44**(4), 207-234.

Verheggen, F.J., Arnaud, L., Bartram, S., Gohy, M., Haubruge, E. 2008. Aphid and plant volatiles induce oviposition in an aphidophagous hoverfly. *Journal of chemical ecology*, **34**(3), 301-307.

Villaverde, M.L., Girotti, J.R., Mijailovsky, S.J., Pedrini, N., Juárez, M.P. 2009. Volatile secretions and epicuticular hydrocarbons of the beetle *Ulomoides dermestoides*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, **154**(4), 381-386.

Villaverde, M.L., Juárez, M.P., Mijailovsky, S. 2007. Detection of Tribolium castaneum (Herbst) volatile defensive secretions by solid phase microextraction–capillary gas chromatography (SPME-CGC). *Journal of Stored Products Research*, **43**(4), 540-545.

Walker, P., Bailey, E. 1970. Metabolism of glucose, trehalose, citrate, acetate, and palmitate by the male desert locust during adult development. *Journal of insect physiology*, **16**(3), 499-509.

Walter, V. 1990. Stored product pests. In Handbook of Pest Control (Story K, Moreland D. (editors.)). *Franzak & Foster Co., Cleveland, OH.*, 526-529.

Wang, C., Leger, R.J.S. 2005. Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. acridum. *Eukaryotic Cell*, **4**(5), 937-947.

Wei, D.J. 2001. Plant-insect Chemical Communication and Its Behavior Control [J]. *Acta Photophysiologica Sinica*, **3**, 001.

Weintraub, H., Tietz, A. 1973. Triglyceride digestion and absorption in the locust, locust a migratoria. *Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, **306**(1), 31-41.

Wilson, R., Lovejoy, F.H., Jaeger, R.J., Landrigan, P.L. 1980. Acute phosphine poisoning aboard a grain freighter: epidemiologic, clinical, and pathological findings. *Jama*, **244**(2), 148-150.

Winks, R.G. 1975. Fumigants and their applications. In International Training Course in Preservation of Stored Cereals Selected Reference Papers, Part two,. *Australian Development Assistance Bureau, Canberra*, 589 - 591. .

Xia, J., Wishart, D.S. 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Current protocols in bioinformatics*, **55**(1), 14.10. 1-14.10. 91.

Ye, G., Li, K., Zhu, J., Zhu, G., Hu, C. 2007. Cuticular hydrocarbon composition in pupal exuviae for taxonomic differentiation of six necrophagous flies. *Journal of medical entomology*, **44**(3), 450-456.

Yezerski, A., Gilmor, T.P., Stevens, L. 2004. Genetic analysis of benzoquinone production in *Tribolium confusum*. *Journal of chemical ecology*, **30**(5), 1035-1044.

Zeng, L. 1999. Development and countermeasures of phosphine resistance in stored grain insects in Guangdong, China, 642–647. *Proceedings of the Seventh International Working Conference on Stored-product Protection (Eds.: J. Zuxun, L. Quan, L. Yongsheng, T. Xianchang & G. Lianghua),(14–19 October 1998, Beijing, China). Sichuan Publishing House of Science & Technology, Chengdu, China*.

Zettler, J., Halliday, W., Arthur, F. 1989. Phosphine resistance in insects infesting stored peanuts in the southeastern United States. *Journal of Economic Entomology*, **82**(6), 1508-1511.

Zettler, L.J. 1991. Pesticide resistance in *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae) from flour mills in the United States. *Journal of Economic Entomology*, **84**(3), 763-767.

Zhang, Z., Li, G. 2010. A review of advances and new developments in the analysis of biological volatile organic compounds. *Microchemical journal*, **95**(2), 127-139.

Zhou, J., Zhou, L., Hou, D., Tang, J., Sun, J., Bondy, S.C. 2011. Paeonol increases levels of cortical cytochrome oxidase and vascular actin and improves behavior in a rat model of Alzheimer's disease. *Brain research*, **1388**, 141-147.

Ziegler, R. 1991. Changes in lipid and carbohydrate metabolism during starvation in adult *Manduca sexta*. *Journal of Comparative Physiology B*, **161**(2), 125-131.

Ziegler, R., Van Antwerpen, R. 2006. Lipid uptake by insect oocytes. *Insect biochemistry and molecular biology*, **36**(4), 264-272.

Zini, C.A., Augusto, F., Christensen, E., Smith, B.P., Caramão, E.B., Pawliszyn, J. 2001. Monitoring biogenic volatile compounds emitted by Eucalyptus citriodora using SPME. *Analytical chemistry*, **73**(19), 4729-4735.

Zuryn, S., Kuang, J., Ebert, P. 2008. Mitochondrial modulation of phosphine toxicity and resistance in Caenorhabditis elegans. *Toxicological Sciences*, **102**(1), 179-186.