Study on Comparison of Biochemistry between *Trogoderma granarium* Everts and *Trogoderma variabile* Ballion

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by

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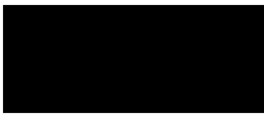
Declaration

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



Signature: T. ALSHUWAILI Date: 09/12/2020

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In the name of God

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Dedication

This thesis is dedicated to My Dad and Mom

My wife and kids Alaa, Dora and Abbas

My brothers and sisters

Without them none of my success would be possible

Thamer Alshuwaili

Abstract

Stored grains are paramount commodities to be preserved and stocked for future supply to the market according to the requirement. However, one of the major problems during storage is insect pests, of which insects from *Trogoderma sp.* especially khapra beetle (*Trogoderma granarium*) is considered the world most dangerous stored grain insect pests. Therefore, it has been listed as quarantine insect pests in many counties. For timely management of quarantine pest, effective and rapid diagnostic methods are required. Until now, diagnostic technology is mainly based on morphology of insects which require trained taxonomists. Recently, diagnostics based on metabolites and hyperspectral imaging coupled with machine learning is gaining importance. However, very little is known about the metabolites in *Trogoderma sp*. and how the host grain, gender, and geographical distribution affect the metabolomic profiling in these species is still unknown.

In this thesis, volatile organic compounds (VOCs) emitted by *Trogoderma variabile* at different life stages were analysed as biomarkers which can help us to understand the biochemistry and metabolomic. Some compounds were identified from *T. variabile* different stages, which could be used as diagnostic tool for this insect. Gas chromatography coupled to mass spectrometry (GC–MS) was used as a technique to study the metabolite profile of *T. variabile* in different host grains. However, there are several factors that affect the volatile organic compounds including extraction time and number of insects. The results indicated that the optimal number of insects required for volatile organic compounds (VOC) extraction at each life stage was 25 and 20 for larvae and adults respectively. Sixteen hours were selected as the optimal extraction time for larvae and adults. Some of the VOCs compounds identified from this insect can be used as biomarkers such as pentanoic acid; diethoxymethyl acetate; 1-decyne; naphthalene, 2-methyl-; n-decanoic acid; dodecane, 1-iodo- and m-camphorene from larvae. While butanoic acid, 2-methyl-; pentanoic acid; heptane, 1,1'-oxybis- 2(3H)-Furanone, 5-ethyldihydro-; pentadecane, 2,6,10-trimethyl-; and 1,14-tetradecanediol VOCs, were found in male, whereas pentadecane; nonanic acid; pentadecane, 2,6,10-trimethyl-; undecanal and hexadecanal were identified from female.

Additionaly, direct immersion-solid phase microextraction (DI-SPME) was employed, followed by gas chromatography mass spectrometry analysis (GC-MS) for the collection,

separation, and identification of the chemical compounds from *T. variabile* adults fed on four different host grains. Results showed that insect host grains have a significant difference on the chemical compounds that were identified from female and male. There were 23 compounds identified from adults reared on canola and wheat. However, there were 26 and 28 compounds detected from adults reared on oats and barley respectively. Results showed that 11-methylpentacosane; 13-methylheptacosane; heptacosane; docosane, 1-iodo- and nonacosane were the most significant compounds that identified form *T. variabile* male reared on different host grains. However, the main compounds identified from female cultured on different host grains include docosane, 1-iodo-; 1-butanamine, N-butyl-; oleic acid; heptacosane; 13-methylheptacosane; hexacosane; nonacosane; 2-methyloctacosane; n-hexadecanoic acid and docosane.

A novel diagnostic tool to identify between *T. granarium* and *T. variabile* were developed using visible near infrared hyperspectral imaging and deep learning models including Convolutional Neural Networks (CNN) and Capsule Network. Ventral orientation showed a better accuracy over dorsal orientation of the insects for both larvae and adult stages. This technology offers a new approach and possibility of an effective identification of *T. granarium* and *T. variabile.* from its body fragments and larvae skins. The results showed high accuracy to identify between *T. granarium and T. variabile.* The accuracy was 93.4 and 96.2% for adults and larvae respectively, and the accuracies of 91.6, 91.7 and 90.3% were achieved for larvae skin, adult fragments, larvae fragment respectively.

Table of contents

Declaration .... **ii**
Acknowledgments .... **iii**
Dedication .... **iv**
Abstract .... **v**
Table of contents .... **vii**
List of tables .... **x**
List of figures .... **xi**
Publications .... **xiii**
List of Abbreviations .... **xiv**
Chapter One 1
General Introduction and Literature Review 1
1.1. General Introduction **2**
1.1.1. Grains **4**
1.1.2. Stored grain insects **5**
1.1.3. Management control of stored grain insects **6**
1.1.3.1. Chemical control **7**
1.1.3.2. Biological control **7**
1.1.3.3. Physical control **8**
1.1.3.4. Modified atmosphere **9**
1.1.4. Detection and identification **10**
1.2. Literature review of *Trogoderma spp.* diagnostic and metabolic response to host grains **12**
1.2.1. Damage caused by and economic importance of *Trogoderma spp.* **12**
1.2.2. Host range **14**
1.2.3. Classification of target insects **15**
1.2.4. Life cycle **15**
1.2.4.1. Eggs **16**
1.2.4.2. Larvae **16**
1.2.4.3. Pupa **17**
1.2.4.4. Adult **17**
1.2.5. Diagnostic tools for *Trogoderma* spp. **17**
1.2.5.1. Morphology **18**
1.2.5.2. Molecular methods **19**
1.2.6. Hyperspectral imaging technique coupled with deep learning **20**
1.2.6.1. Hyperspectral imaging technique **20**
1.2.6.2. Deep learning **21**
1.2.6.2.1. Convolution neural network (CNN) **22**

1.2.6.2.1. Capsule networks **23**
1.2.7. X-Ray **23**
1.2.8. *Trogoderma spp.* metabolic response to host grain **24**
1.2.8.1. Solid phase microextraction (SPME) **24**
1.2.8.2. Gas chromatography-mass spectrometry (GC-MS) **27**
1.2.8.3. Flame ionisation detector (FID) **28**
1.2.8.4. Volatile organic compounds (VOCs) **29**
1.2.8.5. Insect Lipids **32**
1.3. Research Gaps and aim of the study **33**
Chapter Two 36
Optimization and Validation for Identification of Volatile Organic Compounds (VOCs) released from *Trogoderma variabile* Ballion using Headspace Solid Phase Microextraction (SPME) Coupled with GC-FID/MS 36
2.1. Abstract **37**
2.2. Introduction **37**
2.3. Materials and Methods 39
2.3.1. Insect culture **39**
2.3.2. Apparatus and equipment **39**
2.3.3. Optimization of number of insects **40**
2.3.4. Optimization of extraction time **40**
2.3.5. Analysis and identification of real samples using optimized method **40**
2.3.6. Data analysis **41**
2.4. Results and Discussion 41
2.4.1. Insect densities **41**
2.4.2. Extraction time **45**
2.4.3. Analysis and identification of real samples using optimized method **48**
2.5. Conclusions **51**
Chapter 3 53
Study on metabolic response of male and female *Trogoderma variabile* (Ballion) on different host grain using Direct Immersion Solid-Phase Microextraction (DI-SPME) coupled with Gas Chromatography Mass Spectrometry (GC-MS) 53
3.1. Abstract **54**
3.2 Introduction **54**
3.3. Materials and Methods 56
3.3.1. Insect culture **56**
3.3.2. Apparatus and equipment **57**
3.3.3. The extraction and analysis method **57**
3.3.4. Data collection and analysis **57**
3.4. Results and Discussion 58

3.4.1. Effect of insect gender of *T. variabile* on the compound production 58

3.4.2. Effect of host grains (canola, oats, wheat and barley) on the compound production 63

3.5. Conclusions 67

Chapter Four 69

Identification and diagnosis of whole body and fragments of *Trogoderma granarium* and *Trogoderma variabile* using visible near infrared hyperspectral imaging technique coupled with deep learning 69

4.1. Abstract 70

4.2. Introduction 70

4.3. Materials and Methods 73

4.3.1. Insects 73

4.3.2. Hyperspectral imaging system 73

4.3.3. Spectral sample preparation 74

4.3.4. Image acquisition 74

4.3.5. Deep learning model for pest classification 76

4.3.5.1. Dataset description 76

4.3.5.2. Convolutional Neural Network (CNN) 76

4.3.5.3. Capsule Network 79

4.4. Result and discussion 80

4.4.1. Training of algorithms 80

4.4.2. Optimisation of larvae and adult orientation 82

4.4.3. Classification using larvae and adults of *T. granarium* and *T. variabile* 83

4.4.4. Classification between larvae skins, fragments of adults and larvae of *T. granarium* and *T. variabile* 84

4.5. Conclusion 85

Chapter Five 86

General Discussion 86

5.1. General discussion 87

5.2. Conclusion 90

References 91

List of tables

Table 1-1. Advantages and disadvantages of different detection techniques for stored-product insects in grain. 10

Table 3-1. Compounds peak areas (10^5) detected from *T. variabile* male and female reared on canola, oats, wheat, and barley 61

Table 3-2. Compounds that were detected and not detected from *T. variabile* female and male reared on canola, oats, wheat and barley......66

Table 4-1. Number of images acquired for each insect species, including specimen type and orientation...... 75

Table 4-2. Binary Classification between *T. granarium* and *T. variabile* for identification accuracy using both CNN and Capsule Network for larvae (ventral and dorsal), adult (ventral and dorsal), larvae skin, fragments of adults and larvae. 82

Table 4-3. Confusion metrics using CNN and Capsule Network for both larvae and adult with ventral and dorsal orientation......83

List of figures

Figure 1-1. Map showing the current known world distribution of the khapra beetle countrywise. 4
Figure 1-2. Solid phase microextraction device. (Aulakh et al., 2005b) 26
Figure 1-3. Gas chromatography-mass spectrometry 28
Figure 1-4. Diagram showing extraction and analysis of compounds using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) (Schmidt and Podmore, 2015) 31
Figure 2-1a and b. Partial Least Squares Discriminant Analysis (PLS-DA) shows volatile profiles from different *T. variabile* stages in different extraction time based on VOCs with three biological replicates. 43
Figure 2-2a and b. Variable Importance in Projection (VIP) shows important features identified by PLS-DA from data of different insect densities based on Volatile organic compounds (VOCs) with three biological replicates. Codes in left side are the metabolic ID (FID indicates GC-FID detector and numbers indicate the retention times (min) while the right side indicate the high relative abundance of the corresponding metabolites in each group under study. 44
Figure 2-3a and b. Partial Least Squares Discriminant Analysis (PLS-DA) shows volatile profiles from different *T. variabile* stages in different extraction time based on VOCs with three biological replicates. 46
Figure 2-4a and b. Variable Importance in Projection (VIP) shows important features identified by PLS-DA from data of different insect densities based on Volatile organic compounds (VOCs) with three biological replicates. Codes in left side are the metabolic ID (FID indicates GC-FID detector and numbers indicate the retention times min) while the right side indicate the high relative abundance of the corresponding metabolites in each group under study. 47
Figure 2-5a, b and c. Volatile organic compounds (VOCs) detected from *T. variabile* different stages using headspace microextraction technique and gas chromatography mass spectrometry. 50
Figure 3-1a and b. a. Score plot of principle components analysis (PCA) for chemical compounds obtained from *T. variabile* female reared on different host grains (canola, oats, wheat and barley), b. loading plot shows the most significant compounds that participated in the differentiation. 64

Figure 3-2a and b. a. Score plot of principle components analysis (PCA) for chemical compounds obtained from *T. variabile* male reared on different host grains (canola, oats, wheat and barley), b. loading plot shows the most significant compounds that participated in the differentiation. 65

Figure 4-1. The CNN architecture used for the pest image classification. CNN contains 5 layers and the last layer consists a dense (fully connected) network. 77

Figure 4-2. Schematic diagram showing Capsule Network, which has 2 convolutional layers and a fully connected layer to perform the pest classification. 78

Figure 4-3. Capsule Networks loss plot for the training and testing datasets. The plot demonstrates the Capsule Networks adaptability to the dataset. The graph shows that the Capsule Network trains and achieve the optimal loss values within a few epochs of training. 79

Figure 4-4. Loss graph for CNN for binary classification. The CNN's training and testing aligns with each other which indicates that the CNN has not over trained. 81

Publications

Thamer Alshuwaili, Xin Du, Yonglin Ren and Manjree Agarwal (2020). Optimization and Validation for Identification of Volatile Organic Compounds (VOCs) released from *Trogoderma variabile* Ballion using Headspace Solid Phase Microextraction (SPME) Coupled with GC-FID/MS (Chapter 2: Current Analytical Chemistry will be submitted)

Thamer Al-Shuwaili, Manjree Agarwal, Ihab Alnajim, Xin Du and Yonglin Ren (2020). Study on metabolic response of male and female *Trogoderma variabile* (Ballion) on different host grain using Direct Immersion Solid-Phase Microextraction (DI-SPME) Coupled with Gas Chromatography Mass Spectrometry (GC-MS) (Molecules, will be submitted)

Manjree Agarwal, **Thamer Al-Shuwaili**, Anupiya Nugaliyadde, Panghao Wang, Kok Wai Wong and Yonglin Ren (2020). Identification and diagnosis of whole body and fragments of *Trogoderma granarium* and *Trogoderma variabile* using visible near infrared hyperspectral imaging technique coupled with deep learning (Published in Computers and Electronics in Agriculture)

List of Abbreviations

ANOVA	Analysis of Variance
℃	Degree Celsius
C7-C40	N-alkanes Standard
CNN	Convolutional Neural Network
DISPME	Direct immersion solid phase microextraction
DPIRD	Department of Primary Industries and Regional Development
DVB/CAR/PDMS	Divinylbenzene/ Carboxin /Polydimethylsiloxane
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography- mass spectrometry
He	Helium
HS-SPME	Headspace Solid phase microextraction
LED	Light Emitting Diode
mL	Millilitre
Mm	Millimetre
MS	Mass Spectrometric
Ms	Millisecond
N	Number of Replicates
n.d.	Not Detected
Nm	Nanometre
NIST	National Institute of Standards of Technology
PCA	Principal Components Analysis
PLS-DA	Partial Least Squares- Discriminant Analysis
RH	Relative Humidity
RI	Retention Index
RT	Retention Time
SABC	State Agricultural Biotechnology Centre
S.D.	Standered Deviation
SPSS	Statistical Package for the Social Sciences
T.G.	*Trogoderma granarium*
T.V.	*Trogoderma variabile*
VIP Score	Variable importance in Projection of Partial Least Squares

VNIH	Visible Near Infrared Hyperspectroscopy
VOCs	Volatile Organic Compounds
$\sum$	Sum

Chapter One

General Introduction and Literature Review

1.1. General Introduction

Grains are the most significant source of food energy worldwide. They are an essential source for many countries because they are high in various nutrients, such as vitamins, magnesium, iron, phosphorus, manganese and selenium. Food grain storage began approximately 4,500 years ago to maintain a stock of food for humans (Saxena et al., 1988). However, there are many problems with the storage process, such as the presence of pests, which is a very critical issue for stored products. Pests enter grain storage areas because they provide an ideal environment for the development and growth of insects compared to external environments (Rees, 2004; Nansen, 2008). One such insect pest is the genus *Trogoderma*, which contains more than 134 species (Háva, 2011). This genus is one of the 100 worst invasive species worldwide. *Trogoderma* spp. is distributed in many countries, including Asia, Africa, North and South America, Europe, Australia and New Zealand (Figure 1-1). The warehouse beetle, *T. variabile* Ballion, 1878 (Coleoptera: Dermestidae), is a significant pest of packaged and processed stored products. The common name "warehouse beetle" was given to it by Okumura (1972), who regarded it as the next most serious dermestid pest after the khapra beetle, *T. granarium* Everts (Cross et al., 1977). Originating in central Asia, this species was first described in the United States by Beal in 1954 (Loschiavo, 1960; Partida and Strong, 1975). This species was found to be most prominent in Western areas, infesting a wide variety of seeds and stored products of both animal and vegetable origin (Vincent and Lindgren, 1975). *Trogoderma* spp. infect a wide range of economically important crops worldwide. According to Rees (2004), *T. variabile and T. granarium* are the most important species causing significant damage to grain stores. Australia is free of *T. granarium* currently; however, the closely related species, *T. variabile*, has successfully invaded and established in this country in 1977. *T. variabile* was first discovered in Griffith, Australia, and has since spread throughout the remainder of Australia (Hartley and Greening, 1983). *T. variabile* first appeared in southern New South Wales in 1977, spread to Queensland and Victoria in 1981 and was later recorded in South Australia in the early 1990s. In 1989, it was recorded in Western Australia. *T. variabile* has become established in Australia despite numerous attempts at eradication (Wright, 1993). It is now a frequent pest of storage structures and is becoming a pest of bulk-stored canola in Australia. It is now widely distributed from northern New South Wales to South Australia, east of Port Augusta, with limited distribution in Queensland and Western Australia (Rees et al., 2003). *T. variabile* shows great similarity to other *Trogoderma* species, especially *T. granarium* that is not currently present in Australia. Many techniques have been developed to

identify between species, including detection probes, kernel staining, Berlese funnel method, acoustic techniques, X-ray imaging, nuclear magnetic resonance imaging, thermal imaging and the solid phase microextraction (SPME) method (Neethirajan et al., 2007). Some of these techniques are expensive and time-consuming, have potential health hazard, and are less efficient. Manual sampling traps and probes are the most common methods used on farms, whereas manual inspection, sieving, and the Berlese funnel method are used in grain storage and handling facilities (Neethirajan et al., 2007). Early monitoring and detection of insects in stored food grains are required to apply corrective actions. The capability of early detection, monitoring, cost, reliability, and labour requirements are the major factors considered when selecting the most appropriate method. Detection of hidden infestation is an important concern for mitigating losses in bulk storage warehouses, which enables the early fumigation actions or disposal of the grain (Banga et al., 2018).

There are no studies regarding the use of headspace solid phase microextraction (HS-SPME) for the diagnosis of *T. variabile*. The SPME method has been widely used for the analysis of volatile compounds and successfully employed to monitor and diagnose grain insect species. The HS-SPME technique is a new, simple, fast, highly sensitive and solvent-free sample preparation technique for the extraction of volatile compounds (Bicchi et al., 2000; Wardencki et al., 2004). The direct immersion solid-phase microextraction (DI-SPME) method coupled with gas chromatography-mass spectrometry (GC-MS) has been used to extract and analyse fatty acids and hydrocarbons because it is a fast, reliable and affordable technique (Braga et al., 2013). It can be used as an alternative method when the taxonomical identification of an insect is not possible due to its damaged condition or if its DNA is too degraded (Braga et al., 2013). Given these drawbacks, an alternative technique is to use a hyperspectral imaging system. Hyperspectral images are high-resolution images that are used for geolocation identification, plant species identification, and identifying pest damage on plants (Camps-Valls, 2013; Cao et al., 2015). Hyperspectral imaging combines the properties of imaging and spectroscopy; therefore, it can attain both spatial and spectral information from an object, making this technique more sensitive and reliable.

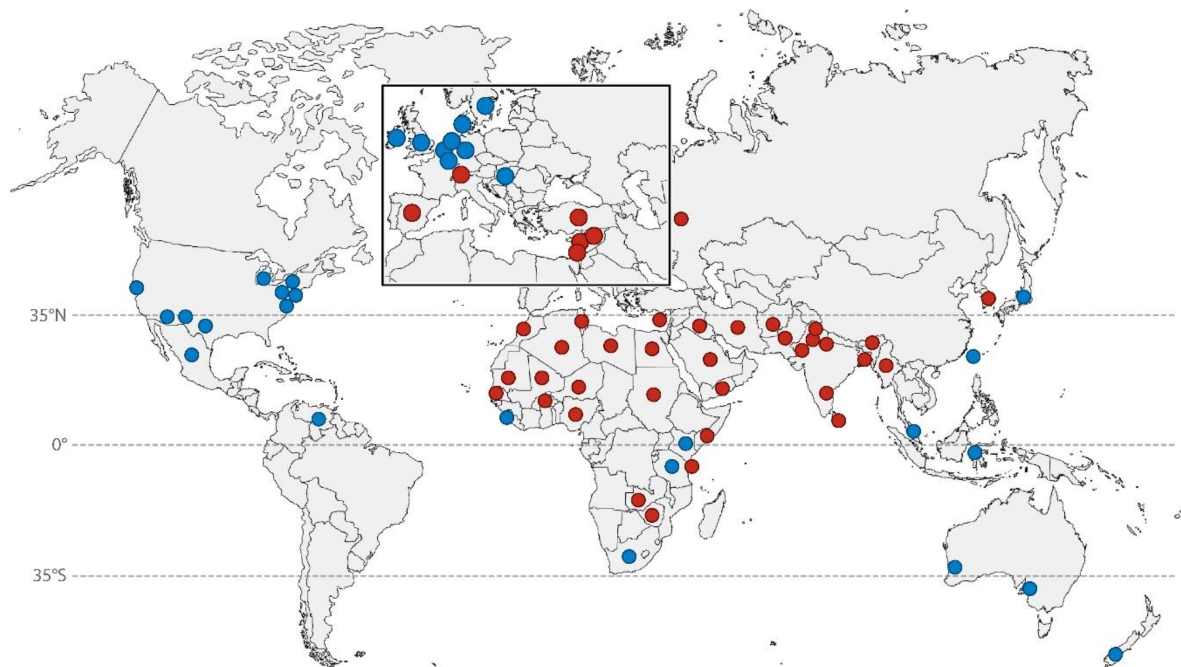

Figure 1-1. Map showing the current known world distribution of the khapra beetle countrywide.

1.1.1. Grains

A grain is a small, hard and dry seed harvested for human or animal consumption (Babcock, 1976). The two main commercial grain crops are cereals and legumes (pulses). The cereal grains include wheat, oats, rice, corn (maize), barley, sorghum and millet. Grains also include pseudo cereal (starchy) grains and oil seeds. Cereals constitute the most substantial proportion of crop production worldwide, with barley having the fourth highest production of the cereal crops (Didon, 2002). The grain industry is a large contributor to the Australian economy, with grains, pulses and oilseed production accounting for approximately 25% of Australia's gross value of agricultural production. Exports of summer and winter grain, pulses and oil seed represent an annual return of approximately AUD 6 billion and the industry is expected to steadily grow at a rate of 1.5% over the coming five years. The economic importance of grains and their contribution to the diets of humans and livestock cannot be disputed. Grains, especially wheat, is the most important crop worldwide and is considered as a major source of energy and starch. Wheat provides substantial amounts of several essential components, such as proteins, vitamins, dietary fibre and phytochemicals. The economic importance of wheat and its contribution to the diets of humans and livestock is obvious. Agricultural production began about 10,000 years ago, whereas food grain storage started approximately 4,500 years

ago to maintain food stocks for humans (Saxena et al., 1988). Storage is an important technique for maintaining the quantity and quality of commodities. Kiaya (2014) defined storage as the way to maintain the quality of agricultural materials and prevent them from deterioration for specific periods beyond their normal shelf life. However, insects are critical issues in storage products because storage areas provide an ideal environment for the development and growth of insects compared to the external environment (Rees, 2004; Nansen, 2008). This is particularly the case in warmer climates where the pressure of insects is high (Collins, 2006). For total farm production, the production of grains, oilseeds and pulse crops make up approximately 29% (AUD 18 billion), and approximately 30% of the total value of farm export income in 2016–2017. In a typical year, one-quarter of Australian agricultural businesses produce grains, oilseeds or pulses. In Australia, approximately 25 million tonnes of wheat are produced every year, accounting for 3%–4% of the world's wheat production and 10%–15% of global wheat exports. In addition, approximately 65%–75% of Australian total wheat production is exported each year, with Western Australia being the largest exporting state.

1.1.2. Stored grain insects

Insects are the largest group of animals. Insects (Class Insecta) are classified into 29 orders, including beetles (Coleoptera); flies (Diptera); bees, wasps, and ants (Hymenoptera); and moths and butterflies (Lepidoptera). The earliest records of insects associated with stored food products are those of a flour beetle which was found in an Egyptian tomb dating back to 2500 B.C. and of "beetles and weevils" that were found in the tomb of Tutankhamen (1390–1380 B.C.) (Munro, 1966). There are approximately 950,000 species of insects on Earth, and they feed on various items, including seeds, leaves and flowers (Samways, 1994). Of the known insect species, there are approximately 100 that are responsible for damage to stored products and, of these, about 20 are major pests with a cosmopolitan distribution. Stored product insects are infesting pests that are found in stored cereal grains, grain products and grain legumes is not a new problem. Stored grain insect pests can cause reductions in grain weight and quality, commercial value and seed viability. A major concern to the grain industry in Australia and overseas is the management of grain pests (Rees, 2004). The Australian grain industry, although well established, is facing new and significant pressures regarding insect management. Grain storage provides the ideal environment for insect and mould species to flourish. This is related to the structure of the storage units, as a full grain silo or storage unit provides a unique habitat that is in many aspects uniform (Nansen et al., 2008). The quality of grain resources

and the vast quantities in which they are stored provide an opportunity for several insect species to reproduce and reach densities that would not occur in the natural environment (Flinn et al., 2004; Nansen et al., 2004; Rees, 2004; Nansen et al., 2008).

Some insects associate with stored grains; however, they do not depend entirely on the grains themselves, rather feeding on other pests within the grain storages. Fungal feeders such as beetles in the family Latridiidae feed on moulds and cannot survive in clean grain, whereas predators, parasitoids and scavengers prey on other insect species and decaying organic material (Rees, 2004). There are approximately 300 species that infect stored products; however, only 18 are of primary economic importance. Such insects have adapted to infecting raw stored grain and grain products and pose continual threats to storage units worldwide (Boyer et al., 2012). Generally, beetles (Coleoptera) and moths (Lepidoptera) are the major insect pests that cause damage to stored products. Beetles are the most versatile and cause more significant losses to grains than moths. Both the larvae and adults of beetles attack stored food, whereas only the larvae of moths are harmful (Upadhyay and Ahmad, 2011). The most important species of insects that cause significant damage to stored products are *T. granarium*, *T. variabile, Rhyzopertha dominica* (F.), *Sitophilus granarius* (L.), *S. zeamais* (M.), *Tribolium castaneum* (H.), *T. confusum* (D.), *Callosobruchus chinensis* (L.), *C. maculatus* (F.), *Oryzaephilus surinamensis* (L.), *Prostephanus truncatus* (H.), *Acanthoscelides obtectus* (S.), *Lasioderma serricorne* (F.), and *Ephestia elutella* (H.) (Talukder and Howse, 1994; 1995; Benhalima et al., 2004). There are some insect pests such as cockroaches, silverfish, ants and termites that are spread in grain stores; however, they not cause damage directly, rather they cause food contamination and a bad smell, especially in wet areas (Upadhyay and Ahmad, 2011).

1.1.3. Management control of stored grain insects

The effective prevention and control of insect pests in stored commodities is the main goal of entomologists worldwide (Talukder, 1995). Many control methods exist; however, researchers are trying to develop safer and more economical techniques, and recently gases, radiation, pathogens, growth regulators and pheromones have been used to control storage product pests (Talukder, 2009).

1.1.3.1. Chemical control

In the past, different methods have been used to control stored product insect pests. The most frequent control measure of stored product insects has been the use of synthetic chemical insecticides and fumigations (Tebbets et al., 1986; Yokoyama et al., 1987; Banks, 1994; Zettler and Arthur, 2000). However, these have caused problems such as insecticide resistance (Beeman and Wright, 1990), chemical residues in foods and environmental pollution (Morallo-Rejesus, 1987). Insecticidal treatments such as malathion (Arthur and Zettler, 1992), deltamethrin (Arthur, 1997), cyfluthrin (Arthur, 1994, 1998), bioresmethrin (Ardley, 1976) and chlorpyrifos-methyl (LaHue, 1977) are also used to control stored product insect pests. However, problems associated with using these chemicals include resistance chemicals and negative effects on the environment and human health (Zettler and Haliday, 1989; Zettler and Cuperus, 1990; Arthur and Zettler, 1992). Fumigation is one method used to control grain insects. *Trogoderma* spp. are more resistant to fumigants than most stored product pests; however, using fumigations to control these species can produce high results. High concentrations of fumigants must be maintained over the fumigation period to allow penetration into all cracks and crevices. During an eradication program, fumigants and surface sprays are used in combination with preventive measures, such as good sanitation practices and exclusion (Harris, 2006). Currently, many countries are using fumigation as a chemical control of grain pests because it has shown promising results worldwide. Fumigation is a method of controlling pest insects by exposing them to gas or mixture of gases. Phosphine (PH3) and methyl bromide (CH3Br) are the most popular fumigants used (Quinlan and Mc Gaughey, 1983).

1.1.3.2. Biological control

To date, conventional pesticides have been used as the major tools for stored grain and food protection. However, there are many problems associated with conventional pesticides including toxic residues in the treated products, pesticide resistance, health hazards to operatives and pest resurgence, and handling hazards. Among these, resistance to conventional pesticides is a growing issue in stored product protection. Due to the problems associated with pesticides, there is interest in the development of alternative biorational strategies such as biological control agents, natural enemies, plant-derived materials and insect growth regulators (Talukder, 2009). The use of living organisms to manipulate the population of insect pests is

called biological control. Biological control agents use natural enemies (predators and parasitoids), nematodes, fungi and bacteria to control stored product insects. Biological control has increased in recent decades for the following reasons: reduced chemical pesticides, a large number of pests have developed resistance against pesticide action and the sensitivity of consumers towards pesticide residues (Frank, 2010). Biological control agents are being considered as supplements or alternatives to synthetic chemical insecticides, which are known to have toxic effects on non-target organisms, including animals and humans. Biocontrol agents have many advantages, i.e. they are safe to human health, and do not pollute the environment or accumulate in the ecosystem (Meikle et al., 2002; Schöller, 2010; Edde, 2012).

1.1.3.3. Physical control

Physical methods are important methods to control stored product insects. There are two main physical methods including heat and cold temperature. Firstly, temperature control is widely used in postharvest to slow down degradation of produce caused by physiological processes, pathogens, and insects. The first record of using heat to control a stored grain insect pest was the heating of grain to 69°C in France in 1762 to control *Sitophillus cerealella*. There are records of heated rooms used to raise the temperature of wheat to 57°C to control *Sitophilus* spp. in Ohio in 1835. Nowadays, the use of heat treatment continues, and a few flour mills or breakfast food processors have used heat in their facilities for over 50 years. The temperature and duration of exposure required to control different stored product insect pests have been studied extensively (Burks et al., 2015; Fields, 1992; Strang, 1992; Mason and Strait, 1998). Fields (1992) showed that most stored grain insects are controlled under the following time-temperature combinations: 30 s at 60°C, 1 min at 55°C, 5 min at 50°C, 12 h at 45°C and 24 h at 40°C. Short exposure to high temperatures (35-40°C) can increase insect survival to subsequently high temperatures. Secondly, cold treatment was used to control stored product insects. Recently, low temperatures have been extensively used for pest control in storage facilities (Imai and Harada, 2006; Arthur et al., 2015). The ability of insects to survive in cold temperatures varies based on their cryoprotectants (Andreadis and Athanassiou, 2017). In general, stored product insect pests are generally unable to reproduce below 18°C and they are unable to move below 5°C, except for *S. granarius*, which can reduce at temperatures as low as 15°C. Ideally, when outside temperatures are below -17°C for three days cold treatments took place in the winter, all accumulations of product were removed, the facility thoroughly cleaned, the water lines either drained or filled with antifreeze, sensitive equipment removed

or insulated, the equipment opened, drive belts loosened, windows opened and fans used to circulate air to ensure even cooling (Butler, 1999; Fields, 1992).

1.1.3.4. Modified atmosphere

Modified atmosphere technology controls the proportions of oxygen, carbon dioxide and nitrogen and is a recognised way to preserve grain storage and prevent postharvest insects and insect pests attacking grain (Emekci et al., 2002; Navarro and Donahaye, 1990; Bakr et al., 2013). The atmosphere is composed of more than 60% carbon dioxide, which can kill stored grain pests. There are many advantages for using carbon dioxide in the fumigant mixture, such as an increase in the toxicity of the fumigant, improvement in the distribution pattern, limitation of the levels of harmful residues in the treated commodity, and elimination of the flammable hazard of some fumigants (Navarro et al., 2004). Yehoshua (2005) reported that all stages of grain pests were killed when exposed to carbon dioxide at 26 c for four days. Many studies have been undertaken to determine whether the addition of carbon dioxide reduces the exposure period to phosphine/methyl bromide fumigations or decreases the effective dose to achieve a particular level of pest mortality. Nitrogen has many characters, which are colourless, odourless, tasteless and mostly diatomic non-metal gas. It has five electrons in its outer shell, so it is trivalent in most compounds.

On the other hand, the Earth contains approximately 78% nitrogen (Croswell, 1996). According to Ren et al. (2012), there are many advantages to using nitrogen in store product pest management, including the air is a free source of nitrogen, it is non-toxic, offers residue-free grain, no resistance problems, no reaction with construction materials and grains do not need ventilation before they can be marketed. Nitrogen molecules occur mainly in the air, in water and soils, with nitrogen found in nitrates and nitrites. All of these substances are a part of the nitrogen cycle and they are all interconnected (Bothe et al., 2007). The average nitrogen concentrations for controlling all stages of *S. oryzae* (L.), *Tr. castaneum* (Herbst), *R. dominica* (F.) and *T. variabile* (Ballion) in wheat, barley, oats, lupins and canola, and the adult stages of the ladybird and the bronzed field beetle (*Adelium brevicorne*) in canola at 20–30°C were 95%–99% balanced with oxygen (Ren et al., 2012).

1.1.4. Detection and identification

Over the last few years, 134 species of *Trogoderma* have been identified (Háva, 2011), with many still undescribed. Identification of *Trogoderma* eggs and pupae usually depend on external features, which is difficult because they possess limited external features and larvae identification is difficult and requires experience. The larval exuviate can be used for identification and adults are the easiest to identify. There are different methods to detect the infestation in stored grains (Table 1-1) (Neethirajan et al., 2007).

Table 1-1. Advantages and disadvantages of different detection techniques for stored-product insects in grain.

Insect detection methods	Pros	Cons
Grain probes and insect traps	Widely used, inexpensive, used for finding insect density	Labour intensive, limits the temporal availability of data, cannot detect internal insects, restriction in the placement of traps
Pheromones	Gives an indication of pest density	Environmental factors affect trap catches
Visual lures	Can be effective in indoor situations	Not very effective
Acoustical methods	Internal infestation can be detected	Cannot detect dead insects and infestations by early larval stages
Electrical conductance	Hidden internal infestation can be identified	Kernels with insect eggs and young larvae cannot be detected, efficiency is low compared to soft X-rays
Berlese funnel method	Cheap and commonly used method at elevators	Very slow and internal infestations cannot be identified
Near-infrared spectroscopy (NIR)	Rapid method, no sample preparation	Cannot detect low levels of infestation, sensitive to moisture content in samples, calibration of equipment is complex and frequent

Insect detection methods	Pros	Cons
Machine vision	Effective in detecting external insects	Cannot detect internal insects
X-ray imaging	Non-destructive, highly accurate, detect both internal and external insects, able to detect both live and dead insects inside grain kernels	

1.2. Literature review of *Trogoderma spp.* diagnostic and metabolic response to host grains

1.2.1. Damage caused by and economic importance of *Trogoderma spp.*

Trogoderma spp. is one of the world's most destructive pests of grain products and seeds. However, there are many countries, such as the USA, Australia, China, Kenya, Uganda and Tanzania that have specific quarantine regulations against the possible importation of these species. Grain stocks can be completely destroyed because massive populations of these insects might develop. *T. granarium* is one of the 100 worst invasive species worldwide and it is difficult to control because of its ability to remain without food for long periods. This species can survive under dry conditions and low-moisture food, and it also has resistance to many insecticides. Most storage insects that are found worldwide occupy various niches, based on grains and weather conditions, although several species are not found in some countries (e.g., *T. granarium* Everts is not present in Australia). The two main pest insects in grain storages are *T. granarium* and *T. variabile*, presumably because these species can survive in harsh conditions. Some species damage whole cereals, legumes and grains, or solid cereal products. Some are cosmopolitan pests of grain products, such as flour, damaged grain and dried fruit. Stored product insects consume these materials and contaminate them with insect fragments, faeces, webbing and a variety of microflora, which reduces their commercial value (Snelson, 1987). They can also increase the moisture content in grain locally, producing mould growth. Therefore, these insects constitute a major sanitation and quality control problem.

Immature stages of these insects develop inside the kernel and then the adults emerge and leave an exit hole, which produces an insect-damaged kernel. When insects develop inside the seeds, they consume the germ and endosperm of the seeds, with the amount consumed increasing with each larval stage. Insect damage to grains can lead to loss of weight and nutrients, which might affect its germination ability, and thus increasing its susceptibility to contamination by fungi (Singh et al., 2016). The infestation of cereal grains and seeds of beans and other plants could adversely affect germination as the germ is attacked (Partida and Strong, 1975). Insect infestation in wheat can result in diminished loaf volume, dense and inflexible crumbs, bitter taste, and off-flavours due to legal contamination limits (Edwards et al., 1991; Perez-Mendoza et al., 2003). Insect fragmentation is another significant concern. Internal feeding insects are the most damaging and hard to detect (Pearson et al., 2003). Because this pest cannot penetrate deep into the grain, the infestation occurs mainly in the superficial layers. The destruction of

the embryo end of the grain is the major damage caused by this pest; however, during heavy infestations, the entire grain is destroyed.

These insect species cause economic losses before and after harvest within a large range of 10% to 100%, especially in tropical countries (Mugisha-Kamatenesi et al., 2008). They also cause damage to stored products, leading to losses of 20% in developed countries and up to 50% in developing countries (Adam et al., 2006). Other losses result from injury that is down in the quality of the product, and the growth of mould contamination on the products (Hagstrum and Subramanyam, 2006). The young larvae of these insects can cause damage to seeds; however, the older larvae damage the entire grains. Analysis of wheat grain samples containing 5% to 100% of *T. granarium*-infested grains showed that they had decreased protein, gluten, crude fat, and ash, and an increased number of damaged grains, whereas the alcoholic stability and free fatty acids values increased. Damage caused a loss in weight averaging 16.36%–95%. *T. granarium* has also been shown to decrease the mineral content of maize (Jood et. al., 1992). Huge decreases have been found for crude fat, total carbohydrates, sugars, protein, nitrogen, starch contents, true protein contents, vitamins thiamine, riboflavin, niacin, total lipids, phospholipids, galactolipids, polar and non-polar lipids in many grains, such as wheat, maize and sorghum (Jood et al., 1993; 1996a).

The losses to Australian exports caused by *Trogoderma spp.* is approximately $46 million/year to $117 million/year, with these losses expected to increase over the last 30-year period from $200 million to $1.6 billion (McElwee, 2000). The costs associated with damage caused by the khapra beetle to Western Australia grains would be substantially less than the estimates quoted above. Conversely, even countries listed as containing khapra beetle might decrease the Western Australian wheat because they do not want a particular strain of the beetle to come to their country or because of market or political pressures. The khapra beetle can influence the environment by the destruction of grain products. Ingesting grain products potentially contaminated with insect body parts, setae and cast larval skins could result in gastro-intestinal irritation. Asthmatics and sensitised individuals are also at risk, as contaminants are highly allergenic. Because infestations are most likely confined to grain storage facilities and other buildings, this pest is not expected to have significant impacts on the natural environment or threatened species (Pasek, 1998).

While Australia is free of *T. granarium* at present, the closely related species *T. variabile* has succeeded in invading and establishing in this country. In 1977, *T. variabile* was first

discovered in Griffith, Australia, and has since spread throughout the remainder of Australia (Hartley and Greening, 1983). *T. variabile* and *T. granarium* are the species that cause high damage to grain stores worldwide, with *T. variabile* regarded as a minor and persistent pest in Australia (Rees et al., 2003). However, this species is of considerable concern because it could mask the presence of the more damaging khapra beetle because of the morphological similarity between these two species (Rees et al., 2003).

T. variabile larvae cause damage when they feed and damage all parts of the seed except for the shell. The damage caused includes loss of grain weight, reduction in quality, presence of larvae, masses of cast skins, live or dead insects and fine dust (Vincent and Lindgren, 1975). This pest has been reported infesting 119 different grains (Hagstrum et al., 2013). Most of the damage to stored products is caused by larvae, with adults reported as occasional feeders (Vincent and Lindgren, 1975).

1.2.2. Host range

Trogoderma spp. has a wide host range, having the ability to infect cereal products and grain, especially wheat, barley, oats, rice, flour, noodles and malt (Scholtz and Holm, 1985). According to Crop Protection Compendium (2005), the main hosts for these species are *Cicer arietinum* (garbanzo), *Avena sativa* (oat), *Hordeum vulgare* (barley), *Glycine max* (soybean), *Lens culinaris* (lentil), *Oryza sativa* (rice), *Pisum sativum* (garden pea), *Sorghum bicolor* (grain sorghums), *Triticum aestivum* (wheat), *Vigna unguiculata* (cowpea), *Zea mays* subsp. *mays* (corn), *Arachis hypogaea* (peanut), *Juglans* spp. (walnut), *Carya illinoensis* (pecan) and *Prunus dulcis* (almond). It can also infect animal food, including ground barley, corn, rice and dog food; rolled oats; dried orange pulp and cracked and ground wheat bran. These species sometimes prefer bread, dried coconuts, cornmeal, crackers, white and whole wheat flour, hominy grits, baby cereals, pearl barley and wheat germ. These pests can live under different conditions, for example, Pasek (1998) showed that the khapra beetle fed on grain and other product even when there was only 2% moisture. Dead mice, dried blood and dried insects can be developed by this pest. *T. variabile* is regarded as a persistent pest of grain storage because it is sometimes associated with residues. It can affect a wide host range of packaged goods. *T. variabile* is very close in appearance to the khapra beetle *T. granarium,* which is not present in Australia and is of quarantine concern (Rees et al., 2003). Thus, we cannot work in Australia with this latter species and hence we propose to use *T. variable* as our test insect. *T. variabile*

belongs to the order Coleoptera, superfamily Bostrichoidea, family Dermestidae (Myers et al., 2016).

1.2.3. Classification of target insects

T. variabile and *T. granarium* belong to the order Coleoptera, superfamily Bostrichoidea, and family Dermestidae (Belov, 2013; EOL, 2013; Myers et al., 2016):

Kingdom: Animalia (Animals)

Phylum: Arthropoda (Arthropods)

Subphylum: Hexapoda (Hexapods)

Class: Insecta (Insects)

Order: Coleoptera

Suborder: Polyphaga

Superfamily: Bostrichoidea

Family: Dermestidae

Genus: *Trogoderma*

Species: *variabile* (warehouse beetle), *granarium* (khapra beetle)

1.2.4. Life cycle

The life cycle of the warehouse beetle is completed between 30 and 40 days in suitable climatic conditions (30°C and 75% relative humidity). The warehouse beetle can survive up to six months without food. A mature female can lay more than 100-270 eggs over two weeks. The female lays her eggs on dried plants, animal material and grain. The eggs hatch after six days to larvae. The larvae start crawling in the food and feeding. They are highly active and move from one infested place to another, only infesting new food sources and preferring the dark. The larvae have six instars and shed their skin approximately ten times during growth. During pupation, if natural crevices are unavailable, they will force their way through materials such as wood and mortar. This stage takes approximately five days to complete. The pupation stage generally occurs near the surface of the food. Females usually begin laying eggs one day after emerging and continue to do so for three days. The adults live for one to five weeks. In difficult

living conditions, the adults enter a resting state called facultative diapause, where metabolism tapers off and growth comes to a halt. This period of dormancy can last up to two years. The entire life cycle is completed in approximately six months.

1.2.4.1. Eggs

T. variabile eggs are pearly-white, translucent, extremely fragile and covered with a sticky secretion. The eggs are approximately 0.7 mm long and 0.25 mm broad, are initially milky-white, and later a pale yellowish colour. They are typically cylindrically shaped, with only one rounded end and the other more pointed and bearing several spine-like projections, broader at the base and tapering distally (Rai, 2014). Loschiavo (1960) showed that there are five distinct brown spots or ocelli on each side at the anterior end of the egg, whereas the posterior end is dark brown owing to the coiled, long, simple hairs of the last abdominal segment.

1.2.4.2. Larvae

The length of the first instar is 1.6–1.8 mm, which consists of a long tail, made up of several hairs borne on the last abdominal segment. The body width is approximately 0.25–0.3 mm, with a yellowish-white colour, except for the head, which is brown or yellowish-brown. Moreover, the body hairs are brown. There is a short antenna with three segments on the head. There are two types of body hairs, which is a characteristic feature of the larvae. First are the simple hairs, in which the shaft bears many small, stiff, upwardly directed processes. Second are the barbed hairs, in which the shaft is constricted at regular intervals, and the apex consists of a barbed head. The larvae head is the longest part of the body and it is as long as the combined lengths of four of the preceding segments. Simple hairs are scattered over the body segments and dorsal surface of the head. The larvae tail consists of two groups of long simple hairs, borne on the 9th abdominal segment. Barbed hairs are found in pairs of tufts, borne on certain abdominal tergites. As the larvae increase in size, the colour changes progressively from the pale yellowish-white of the first instar larvae to a golden or reddish-brown. The density of the body hairs increases; however, these hairs and the tail become much shorter in proportion to the length and breadth of the larval body. The hairs give the appearance of four dark transverse bands in the 4th instar. The mature larvae are approximately 6 mm in length and 1.5 mm in breadth (Rai, 2014; Loschiavo, 1960).

1.2.4.3. Pupa

The larval skin splits at the last ecdysis; however, the pupa remains within this skin for its entire life. The pupa is of the exarate type. The pupa is wider than the larva and forces the skin apart, leaving the dorsal surface visible through the gap. It has a thin transparent skin that, at adult emergence, is passed to the posterior end of the larval skin. The male pupa is approximately 4.31 to 4.62 mm in length and 1.53 to 1.80 mm in width, whereas the female pupa ranges from 6.24 to 6.69 mm in length and 2.42 to 2.69 in width (Loschiavo, 1960).

1.2.4.4. Adult

Females are usually larger than males. The ratio of length to width is 1.8:1 in females and 1.7:1 in males. The adult beetles are capable of flight, similar to other *Trogoderma* spp. (Wright and Morton, 1995). The adults usually crawl, although they can spread their wings and fly in an almost vertical ascent. The adults only live for a short time and remain hidden and inaccessible most of the time but emerge for mating, oviposition and dispersal, termed the exposure period (Shapas and Burkholder, 1978; Wright and Morton, 1995). The flight behaviour of males is very different from that of females. *T. variabile* males fly for several hours after sunrise whereas females fly during daylight hours (Wright and Morton, 1995). Adults are less active at temperatures under 15°C. According to Loschiavo (1960), the adults of *T. variabile* do not require food to lay eggs. The overall sex ratio of adults (female: male) is approximately 1:1.2 (Partida and Strong, 1975).

1.2.5. Diagnostic tools for *Trogoderma* spp.

Over the years, the genus *Trogoderma* has been reported to include 117 species (Mroczkowski, 1968), 115 species (Beal, 1982), 130 species (Háva, 2003) and 134 species (Háva, 2011). The detection and monitoring of stored product insect pests are still a major issue and a variety of analytical methods for detecting insects and insect fragments in whole kernels and flour are currently applied. Among these are visual inspection, temperature and moisture monitoring, insect movement detection, pheromone traps, immunoassays, flotation and infrared spectroscopy-based methods. However, most of these are labour intensive and expensive, or they do not have enough sensitivity. New techniques for identifying grain insects could offer

easy, rapid solutions for the detection of grain insect pests, even in low densities. Using new techniques could help identify external and internal infestations so that decisive action can be taken as early as possible. The present study will focus on developing new diagnostic tools for *Trogoderma* spp. using SPME coupled with GC-MS and VNIH.

1.2.5.1. Morphology

Fundamental to the implementation of control measures is the correct identification of the organism. There are many problems associated with morphological identification especially when dealing with cryptic species in the invaded ecosystem or immature stages (Schutze et al., 2006). Hebert et al. (2003) states that it is hard to identify immature stages morphologically because these stages lack unique morphological characteristics to enable them to be distinguished from closely related species and morphological keys, which are used to identify adult and larvae samples, might be geographically limited and effective for only a particular life stage. While morphologically distinct characters are often not present in larvae, there are many instances where two or more adult species are unified under the same taxonomic identity (referred to as a cryptic species) but have different life cycles and host preferences (Walter, 2005). For instance, *Bactrocera tryoni* and *B. aquilonis* are two species that are morphologically similar but differ markedly in host specificity, locality and time of optimum daily activity (Wang et al., 2003). The presence of cryptic species can create problems for control methods and management strategies as each species may react quite differently to the treatments.

Increased international trade has led to an escalation in the introduction of economically and environmentally destructive invasive species worldwide (Hulme 2009). *Trogoderma* contains some of the world's most serious pests of stored grains and other food substances, including rice and barley (Beal 2003; Háva 2011). The larvae of most of these species are primarily generalist scavengers (Peacock 1993; Kiselyova and McHugh 2006; Zhantiev 2009).

T. variabile is morphologically similar to several other *Trogoderma* species and is the less destructive species, making it difficult to distinguish between species. The eggs and pupae are morphologically uninformative (Beal, 1954). Morphological keys are geographically limited, of minimal use without a confirmed voucher collection, and confusing due to inadequately described morphological characters, compounding identification difficulties (Rees 1943; Beal 1967, 2003). Larvae are difficult to identify because expert staff in identification are required

with skills in the dissection of small insects. According to Castalanelli (2011), *T. granarium* and *T. variable* can only be reliably identified by a limited number of highly skilled diagnosticians using traditional morphologically based keys. Delicate dissections of the genitalia and mouthparts are required to differentiate between these two species., the use of molecular markers in differentiating *Trogoderma* species was investigated to make the larvae identification more easily. Accurate identification is important to prevent false positives, false negatives and unnecessary implementation of expensive, time-consuming quarantines and treatments, or worse.

1.2.5.2. Molecular methods

The development of a diagnostic method for *Trogoderma* spp. is urgently required. Molecular technology permits species discrimination regardless of life stage (Darling and Blum 2007; De Marco et al., 2007; Castalanelli et al., 2011). DNA marker technology is increasingly used to solve identification problems (DeSalle, 2006; Gwiazdowski et al., 2006; Foottit et al., 2008; Carew et al., 2009). The main advantage of molecular methods is their ability to test insect fragments, damaged specimens and larvae that are almost impossible to identify morphologically (Byrne et al., 2018). Insect species can be identified using DNA extraction methods where it is impossible to diagnose them morphologically. Previous studies on 74 invasive insect species showed that molecular methods could provide valuable information regarding population structure, gene flow and dispersal pathways (Castalanelli et al., 2011). DNA taxonomy is the use of a DNA sequence to identify a species (Tautz et al., 2003). Hebert et al., (2003) showed that insect DNA taxonomy or population analysis are more specifically the use of the 5′ end of the cytochrome oxidase I gene, referred to as DNA barcoding. Molecular taxonomy is playing a significant role in understanding the phenotypic characteristics, identifying the morphonology features and revealing the presence of cryptic species (Hebert et al., 2003; DeSalle, 2006; Scheffer et al., 2006; Ros and Breeuwer, 2007; Foottit et al., 2008). We are unable to resolve morphological characters separating the new species because the method used for DNA extraction for 2001 to 2003 trapping specimens required the samples to be macerated; therefore, subsequent morphological examinations were impossible.

Several aspects should be considered when applying a molecular marker. First, different markers vary in their suitability for addressing particular questions. Second, molecular markers are species-specific and provide valuable information about the organism; however, the results

might not be reproducible between laboratories, across similar taxa and, to a lesser extent, within the same taxon (Vignal et al., 2002; Armstrong and Ball, 2005; Behura, 2006). In other instances, this can make it difficult to select the most appropriate molecular marker to discriminate between species or populations. While most molecular markers are selected using the criteria discussed above, three population genetic concepts must be considered before discussing the marker types, their usage and issues relating to their use. These concepts are based on founder effect, genetic bottlenecks and genetic drift. Each of these concepts influence the chance of survival by the pest species and on the sequence-based marker variability and subsequently on how the marker can be applied to analysing an invasive pest (Tsutsui et al., 2000; Puillandre et al., 2008; Kalinowski et al., 2010).

1.2.6. Hyperspectral imaging technique coupled with deep learning

1.2.6.1. Hyperspectral imaging technique

Hyperspectral imaging is a new technology that gathers and processes data from across the electromagnetic spectrum. The objective of hyperspectral imaging is to obtain the spectrum for each pixel in the image in sight to detect processes or find and identify materials. There are two types of spectral imaging: snapshot hyperspectral imaging, which generates an image and push broom scanner, which over time an image examination. Hyperspectral imaging applications are used in many fields such as environmental, physics, agriculture, food processing, mineralogy, surveillance, astronomy and chemical imaging (Hans, 2007). Hyperspectral imaging is non-invasive, non-contact and non-destructive. Furthermore, it does not require any sample preparation, which makes it a preferred choice as an analytical method compared to conventional analysis methods. Hyperspectral imaging is an economical analytical method due to savings in the aspects of labour, turnaround time, reagents and waste treatment (Elmasry and Sun, 2010; Gaston, 2010). Despite the advantages of hyperspectral imaging, its limitations must be considered as well as the cost of the software and data analysis. Hyperspectral imaging is an indirect analytical method that requires standardised calibrations and model transfer procedures to be performed before the analysis can be conducted (Elmasry and Sun 2010). Recently, insect classification based on computer imaging has been developed as a safer identification method (Al-saqer et al., 2011). Near-infrared hyperspectral imaging has been used for the detection of insects and insect parts in whole grain and ground samples and to evaluate the quality of many cereal grains (Maghirang et al., 2003; Singh et al., 2006). Hidden

insects and internal damage can be found using hyperspectral imaging because it provides spectral information in a spatially resolved manner (Gowen et al. 2007). According to Singh et al. (2009), hyperspectral imaging can be transferred out in reflectance, transmission or fluorescence modes. Hyperspectral imaging has been used for the detection of coleopteran pests of rice and wheat (Dowell et al., 1998; Huang et al., 2013). Cao et al. (2015) identified stored product insect pests using hyperspectral imaging. Hyperspectral data for geographical strains of two insect species, the rice weevil (*S. oryzae*) and the maize weevil (*S. zeamais*), were collected and analysed. The overall recognition rates of the classification model for insect species were 100% and 98.13% for the calibration and prediction sets, respectively, whereas the rates of the model for geographical strains were 94.17% and 86.88%, respectively. Singh et al. (2009) investigated whole wheat kernels and wheat kernels that were damaged by the rice weevil (*S. oryzae*), lesser grain borer (*R. dominica*), rusty grain beetle (*Crytolester ferrugineu*), and red flour beetle (*Tr. castaneum*) in the wavelength range of 1000–1600 nm using hyperspectral imaging. Bhuvaneswari et al. (2011) conducted image analysis for detecting insect fragments of *Tr. castaneum* (Coleoptera: Tenebrionidae) in semolina. Furthermore, Wang et al. (2011) identified external insect damage in jujube fruits in the spectral region of 400–720 nm with a hyperspectral reflectance imaging approach. Additionally, Voss et al. (2017) acquired external hyperspectral imaging data (77 spectral bands, 389–89 2nm) from the ventral and dorsal sides of an individual pupa of two species of blowfly (Diptera: Calliphoridae), *Calliphora dubia* Macquart 1855 and *Chrysomya rufifacies* Macquart 1842.

1.2.6.2. Deep learning

Statistical learning methods can be utilized to deal with hyperspectral data which are highly dimensional and heterogeneous. However, it is hard to know which features are essential for the classification task because there are various substances described (Makantasis et al., 2015). Deep learning tools automate the process of feature construction through assembling high-degree attributes from low-level ones, to learn a scale of characteristics. Moreover, deep learning edifice is apt to solve the classification problem when faced with big datasets and large images with extremely high spatial and spectral resolution. Hence, deep learning detects objects, such as man-made ones (Mnih & Hinton, 2012, Montavon et al., 2012), in addition to classifying hyperspectral data (Chen et al., 2014). Deep learning has made many improvements in agriculture through the progress of science and research, such as leaf diseases identification and insect recognition (Zhang et. al. 2018; Xie et. al. 2018; Cheng et. al. 2017). In addition,

deep learning is used to classify the feature that vectors from insect image features based on the generalized learning ability of big data to quickly identify the categories of different insects. Meanwhile, the target detection technology based on deep learning can automatically learn and generalize the characteristics of large picture data. Image detection also has notably high research value in the field of stored grain pest detection. Nowadays, deep learning models such as Convolutional Neural Networks (CNN) and Capsule Network have become growingly popular in many applications for hyperspectral pest image classification.

1.2.6.2.1. Convolution neural network (CNN)

The Convolution neural network (CNN) module that has a large learning capacity. However, this module is helping to learn thousands of objects from millions of images. Because of this module have lots of prior knowledge to compensate for all the data we do not have, so the immense complexity of the object recognition. Furthermore, Convolutional neural networks (CNNs) constitute one such class of models (Fukushima et. al., 1983; Krizhevsky and Hinton, 2010; LeCun et. al., 2004; Lee et. al. 2009). While this mosdule make strong and mostly correct assumptions about the nature of images and also their capacity can be controlled by varying their breadth and depth. Convolutional Neural Networks (CNNs) are a special type of neural network inspired by the cognitive mechanism of biological vision. The core of the convolutional neural network is the convolution operation. Therefore, a convolutional neural network has excellent performance in image classification object detection and other computer vision tasks. Without complex image preprocessing, a convolutional neural network can automatically extract the effective features from a large number of original input data, which makes image feature extraction simple and efficient (Shi, et. al., 2020). In recent years, deep learning models (DL) based on CNN are extensively used as a powerful class of models for classification of images in a variety of problems in agriculture field such as plant disease recognition, fruit classification, weed identification and crop pest classification (Kamilaris et. al., 2018). Convolutional neural network models were developed to diagnose and identify plant diseases from the leaf images of healthy and diseased plants (Ferentinos, 2018). Rice diseases identification method was proposed by Liu et al. (2017) based on deep CNN (DCNN) techniques to identify ten common rice diseases, which increases both the convergence speed and recognition accuracy. Later, transfer learning was introduced to fine-tune the pre-trained deep networks to improve learning efficiency. Recently, Too et al. (2019) reported the analysis of state-of-the-art deep learning models for plant disease identification.

1.2.6.2.1. Capsule networks

Capsule Network is one of the specified Deep Learning models that developed by Hinton and his colleagues to overcome the deficiencies of CNN (Sabour et. al. 2017). However, Capsule Network are commonly used for the researching areas that CNN achieved well enough classification and segmentation performances in a few years. Capsule Networks has been identified as a promising concept that has demonstrated vast potential in outperforming other deep learning modles in various domains including computer vision and natural language processing. The main advantage of this module is able to discover and preserve the relative spatial and hierarchical relations among objects within an input (Sabour et al., 2017). Capsule networks use dynamic routing to model spatial and ierarchical relations among objects in an image (Sabour et al., 2017; Sabour et al., 2018).Furthermore, Capsule Network were applied in different area such as classification, character recognition and computer vision (Jayasundara et al., 2019; Zhao et al., 2018). Xinyi and Chen (2018) mentioned that Capsule Network has been recently proposed to classify biological and social network graphs, yet, has not been applied to trees for programming languages processing yet. Capsule networks use similar approaches as CNNs to visualize different, and direct, stimuli to specialist capsules (modules). A capsule network adds layers within each hidden layer, rather than adding more layers (Sabour et. al. 2017).

1.2.7. X-Ray

X-ray imaging is the non-destructive, encouraging technique utilized and direct method that can detect insect infestations in grain kernels (Yacob et al., 2005; Karunakaran et. al., 2003). Karunakaran et al. (2003) correctly identified wheat kernels infested by Sitophilus oryzae (L) larvae and pupae-adults with more than 97% accuracy from the soft X-ray images. However, they identified sound kernels with 99% accuracy and also indicated that in the future an automated line-scan X-ray system could inspect 1 kg grain in about 15 min compared to 5–6 h using a Berlese funnel. Karunakaran et al. (2003, 2004 a,b) showed that soft X-ray detected several stored-product insects and achieved an identification accuracy of 84% and 98%. However, *Sitophilus oryzae* (L.) were detected in wheat using soft x ray with more than 95% accuracy. High accuracy percent were achieved between 98% and 86% to detect internal infestation of *R. dominica* and *T. castaneum* larva respectively (Karunakaran et al., 2004 a,b).

Fornal et al. (2007) showed that the models developed from soft X-ray images accurately detected *Sitophilus granaries* (L.) eggs and other internal stages in wheat kernels from 5 days after oviposition. Moreover, the soft X-ray method also has the ability of detecting fungal infections in stored wheat kernels (Narvankar et al., 2009). Karunakaran et. al. (2003) used the soft X-ray method in detecting *Cryptolestes ferrugineus* (Stephens), *T. castaneum* (Herbst), Indian meal moth (*Plodia interpunctella*), *S. oryzae* (L.), and *R. dominica* (F.) in wheat kernels. The X-ray method is widely used as a test reference method. Thus, all modifications of the existing procedures making this method more accurate and easier to use are of great importance. The existing X-ray techniques enable the classification of at least four stages of insect development by measuring the area occupied by the insect, and an accurate classification is also possible based on visible insect morphology (Pearson et al., 2003). The soft X-ray technique has already been investigated and has shown very high accuracy for detection of sprout damage and vitreousness (Neethirajan et al., 2004) and internal and external insect infestations (Karunakaran et al., 2004a, Karunakaran et al., 2004b) in wheat.

1.2.8. *Trogoderma spp.* metabolic response to host grain

1.2.8.1. Solid phase microextraction (SPME)

SPME was developed by Arthur and Pawliszyn (1989) for rapid sample preparation under laboratory conditions and on-site arrangements, and to provide an efficient method towards the integration of sample preparation with detection systems and separation (Arthur and Pawliszyn, 1990; Holt, 2001; Wady et al., 2003). SPME is a new technique that is rapid, inexpensive and good for heat sensitive materials (Richter and Schellenberg, 2007). SPME is a very common technique that is available for rapid and selective sample preparation. SPME has several advantages such as sensitivity and provides linear results for a wide range of concentrations and analytes (Nerín, 2009).

The main principle of SPME is to expose a precoated surface to the sample matrix of interest (Camarasu, 2000). The coating on the exposed surface extracts the compound of interest, and once equilibrium is reached between the sample matrix and the coating on the surface, the extracted compounds are transferred to a sensing device. This sensing device can be a GC or other analytical instrument, which can sense the introduced sample, and identify them (Figure 1-2). The objective of SPME is concerned only with attaining equilibrium as rapid as possible

(Zhang and Pawliszyn, 1993) and not exhaustive extraction (Camarasu, 2000). After equilibrium is reached, further exposure of the fibre does not increase the amount of compound extracted. Hence, using SPME, sample extraction and pre-concentration processes can be achieved in one single step (Davoli et al., 2003; Tuduri et al., 2003). SPME is a solvent-free adsorption and desorption technique where desorption occurs in the GC injector. Headspace analysis of SPME involves the insertion of a coated silica fibre above the sample, which allows the adsorption of VOCs for a specific period. Concentrated VOCs can be obtained without interference from food matrices and other non-volatile compounds from the headspace (Richter and Schellenberg, 2007). After that, the SPME needle is removed and inserted into the GC placed into the GC inlet and heating causes the VOCs, adsorbed by the fibre, to be released into the GC column. Finally, the VOCs are separated and characterised by GC or GC-MS (Martos and Pawliszyn, 1998; Reineccius, 2002; Turner, 2006). SPME employs a small volume of polymeric extracting phase coated on the outside of a metal alloy solid support or fused silica (Pawliszyn, 1997). SPME is applied for the isolation of analyses from a liquid matrix and purified extracts. Solid-phase extraction procedures are used to remove the interfering components of the complex matrices to obtain a cleaner extract containing the analyses of interest (Żwir-Ferenc and Biziuk, 2006). The identification of VOCs by headspace or thermal desorption GC uses different columns in combination with appropriate detection methods: MS, a FID, a flame photometric detector, an infrared analyser or a photoionisation detector (Dettmer-Wilde and Engewald, 2016). The efficiency and sensitivity of the SPME method depends on the extraction time and temperature. High temperature and long extraction times favour the collection of more analytes (Laopongsit et al., 2014). Senthilkumar (2010) showed that SPME has an absorptive layer that absorbs solutes above or from liquid or solid samples in both static and dynamic headspaces. The desorption of the solutes can be achieved with both thermal and liquid desorption methods.

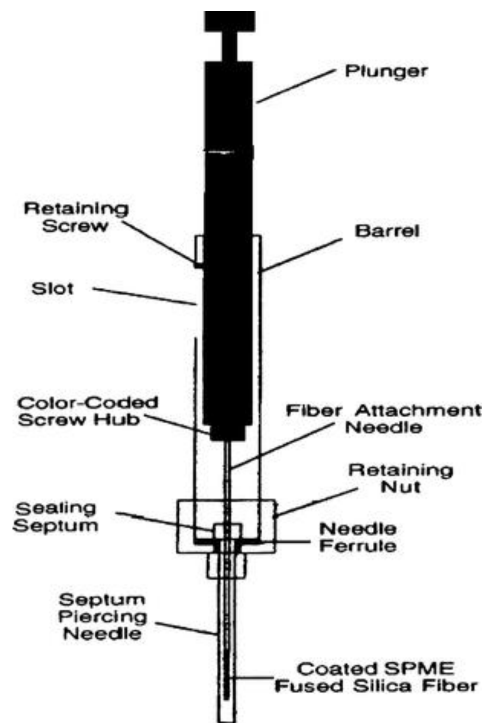

Figure 1-2. Solid phase microextraction device. (Aulakh et al., 2005b)

1.2.8.2. Gas chromatography-mass spectrometry (GC-MS)

GC-MS is a contributory technique, in which complex mixtures of chemicals can be separated, identified and quantified. GC-MS has the ability to analyse hundreds of relatively low molecular weight compounds found in environmental materials (Natural Environment Research Council, 2002). GC-MS is sensitive and highly effective, even with a small quantity of sample and this detector can be used to identify the analytes in chromatograms from their mass spectrum (Skoog et al., 2013). GC-MS has many advantages. For example, GC-MS analysis is a very useful technique for analysing VOCs. GC-MS has many applications including the identification and quantification of unknown samples and contaminants and it can identify trace elements in samples, gases in a sealed environment and residual solvents (Mathias, 2014). Gas chromatography is one of the most powerful detectors which can add to the GC system and are capable of structure identification (Frysinger and Gaines, 1999; Mostafa et al., 2012).

GC-MS is an analytical technique that can be considered as a GC with an MS as the detector or as an MS with a GC as the molecule separator in a mixture before the ionisation of separated molecules. The GC-MS results are more accurate than that of separate GC and MS machines because any compounds that have similar physical and chemical properties come out of the GC at the same time, with both compounds recognised as a similar compound; however, the presence of the MS eliminates this problem via the production of a mass spectrum (Senthilkumar, 2010). The operating molecular mass range of GC spans the interval 2–1500 atomic mass units (amu) (Biniecka and Caroli, 2011) (Figure 1-3).GC has facilitated the unravelling of the trace-level composition of complex mixtures such as petrochemical analysis (Frysinger and Gaines, 1999) and pheromone analysis (Kalinová et al., 2006). Environmental analysis has been undertaken for polychlorinated biphenyls (Focant et al., 2004), fatty acids (Quinto Tranchida et al., 2008), amino acids (Halitschke et al., 2001; Michaud and Denlinger, 2007), lipids (Vrkoslav et al., 2010) and other chemicals. The structural information and selectivity available from the MS made the combination of MS with a GC the most effective technique for the analysis of complex mixtures. The separation of the phase ions is achieved within the MS using electrical and magnetic fields to differentiate ions. The products are ionised before analysis in the MS. The GC-MS analysis produces an enormous amount of data; therefore, processing is essential. Chromatograms are visualised with the x-axis corresponding to retention times at the first dimension (Hites, 1997). GC is a rapid technique for the separation and validation of chemicals (Łaniewski et al., 2003). VOCs with mass of up to 200 amu can be

separated using this technique (Biniecka and Caroli, 2011). Phillips and Beens (1999) showed that low molecular weight chemicals can be separated using GC compared with other conventional instruments. GC-MS results in enhanced peak capacity high sensitivity due to multiple separation dimensions (Van Geem et al., 2010).

Figure 1-3. Gas chromatography-mass spectrometry

1.2.8.3. Flame ionisation detector (FID)

There are more than 15 different types of detectors for GC instruments; however, the most common detectors used in insect volatile analysis are FIDs. The FID is one of the most successful and important techniques used to analyse VOCs because it is fast and can detect the narrow GC peaks (Marriott and Shellie, 2002; Shellie et al., 2003). The use of the FID as a universal GC detector has produced impressive results in terms of sensitivity, resolution, high acquisition rate, robustness, user friendliness, reliability and stability (Von Mühlen et al., 2006). The FID rapidly became a popular technique, overtaking several other ionisation detectors proposed at the same time (Ettre and Zlatkis, 1974). The FID is inexpensive, high linearity, linear dynamic range and sensitive for carbon-containing compounds, which make it the universal detector of choice. The FID passes the sample and carrier gas from the column through a hydrogen-air flame and then the hydrogen-air flame alone creates a few ions. However, this ion increases when an organic compound is burned. The FID detector works only for hydrocarbon and organic-containing compounds because of the ability of the carbons

to form cations and electrons upon pyrolysis, which generates a current between the electrodes (Harris, 1999; Higson,2004). Therefore, the importance of FID comes from its ability to produce narrower peaks than other detectors. Other positive aspects of the FID include a very large linear range 10^6 or 10^7 depending on the system. According to Holm (1999) and Amirav (2001), GC-FID is the most popular method for signal detection. FID was the first detector applied for GC (Liu and Phillips, 1991). Shellie et al. (2003) and Marriott and Shellie (2002) showed that GC-FID was the only universal detector that presents an acquisition rate up to 200 Hz, which is fast enough to measure the fast GC × GC peak at the end of the 2D column. The FID detector works because burning of carbon compounds produces ions in the flame. The FID system is often referred to as a carbon counting device because hydrocarbons give ionisation responses in proportion to the number of carbon atoms (Holm, 1999). Thus, the ion current generated is proportional to the amount of C-compound present, and the ion-generation reaction is fast. However, the chemical nature of the sample molecule influences the effectiveness of the carbon atom in producing a flame ionisation response (David, 1974). This is reflected in the varying response factors found for different compounds; however, in many cases there is an approximately equal response factor for a given class of compounds. The response factor can be better referred to by considering the effective carbon number, where individual functional groups contribute a certain response magnitude to the total molecule response, compared with that of a carbon atom (Carin et al., 2006).

1.2.8.4. Volatile organic compounds (VOCs)

Insect detection in stored grains is an important measure of quality and deterioration for grain producers and it can significantly help to decrease quantitative loss, severe physical damage, off-odour and contamination caused by infestation (Bulla et al., 1978; Seitz and Saucer, 1996). There are several detection techniques available, both commercially and non-commercially. Manual sampling, traps and probes are generally the most basic commercial tools used on farms, whereas manual inspection, sieving, flotation and Berlese funnels are more advanced techniques used in grain handling facilities (Neethirajan et al., 2007). Moreover, odour detection techniques for insect infestation and grain quality evaluation are gaining popularity. Analysing VOCs released into the airspace surrounding stored products is a potential method of diagnosis and species identification (Laopongsti et al., 2014; Qiu et al., 2014). The measurement of VOCs facilitates the early detection of an infestation, storage age determination and varietal discrimination of food grains. Many studies have focused on the

detection of insects in grain by measuring their VOCs. One of the most successful techniques for identifying grain insects VOCs is HS-SPME coupled with GC-MS and gas chromatography-flame ionisation detection (GC-FID) (Schmidt and Podmore, 2015) (Figure 1-4). SPME coupled with GC-MS has been widely used to examine volatile secretions in Coleoptera, e.g., the isolation and identification of the rhinoceros beetle and cerambycid beetle pheromones (Rochat et al., 2000; Ginzel and Hanks, 2005). It has also been used to detect the aggregation pheromones and other volatile metabolites from grain insects, such as *R. dominica* (F.) and *T. castaneum* (Seitz and Ram, 2004; Arnaud et al., 2002). Senthilkumar et al. (2012) detected *T. castaneum* and *C. ferrugineus* using HS-SPME coupled with GC-MS. Niu et al. (2016) used SPME coupled with GC-FID and GC-MS to establish relationships between storage period and grain quality, and grain quality and insect infestation of *R. dominica* in wheat. Some compounds from wheat infested by lesser grain borers were identified in their experiment. However, limited studies have used SPME to detect insect infestation in grain.

Abuelnnor et al. (2010) identified distinct VOCs from infested wheat flour and wheat grain with *T. confusum* and *S. granaries*, respectively, using SPME coupled with GC-MS. Larval and adult insects secreted distinct VOCs that were useful for the early monitoring of the infestation. This is the first chemical method for the estimation of volatile quinones proposed as an indicator of *T. castaneum* infestation. Previous studies have identified VOCs in the headspace above *Tribolium* spp. (Villavarde et al., 2007) and in the headspace above the lesser grain borer, *R. dominica* (L.) (Seitz and Ram, 2004).

The present study was undertaken to discover new VOCs and confirm the presence of compounds previously reported using SPME to collect and concentrate the headspace volatiles above the samples, with subsequent analysis with GC-MS. VOCs contribute significantly to food flavour and can be used as indicators of quality, age of storage and hygiene condition of stored products.

The VOCs in the headspace of three different samples, healthy wheat, *R. dominica* and wheat with *R. dominica*, were analysed at 25°C by SPME coupled with GC-FID and GC-MS. Several researchers have used HS-SPME coupled with GC-MS methods to analyse the VOCs of stored wheat and *R. dominica*. Most of these studies have chosen non-polar columns to separate VOCs (Seitz and Ram, 2004) and many low molecular weight organic compounds emitted from stored grains have been identified (Niinemets et al., 2004).

To identify VOCs produced by *T. variabile* and other grains, it is necessary to develop a repeatable, sensitive, easy to operate, cost-effective and rapid method. To date, no studies have examined the use of the HS-SPME technique for *T. variabile* infestation. The SPME method has been excessively used for the analysis of VOCs. The HS-SPME technique is a new, rapid, simple, eco-friendly and solvent-free sample preparation technique for the extraction of VOCs (Najafian and Roewshan, 2012; Bicchi et al., 2000). The HS-SPME technique provides tens or hundreds of possible VOCs simultaneously and improved results when GC is combined with either MS or FID; however, it must be optimised for the VOCs being targeted (Dorea et al., 2008; Jelen et al., 2012).

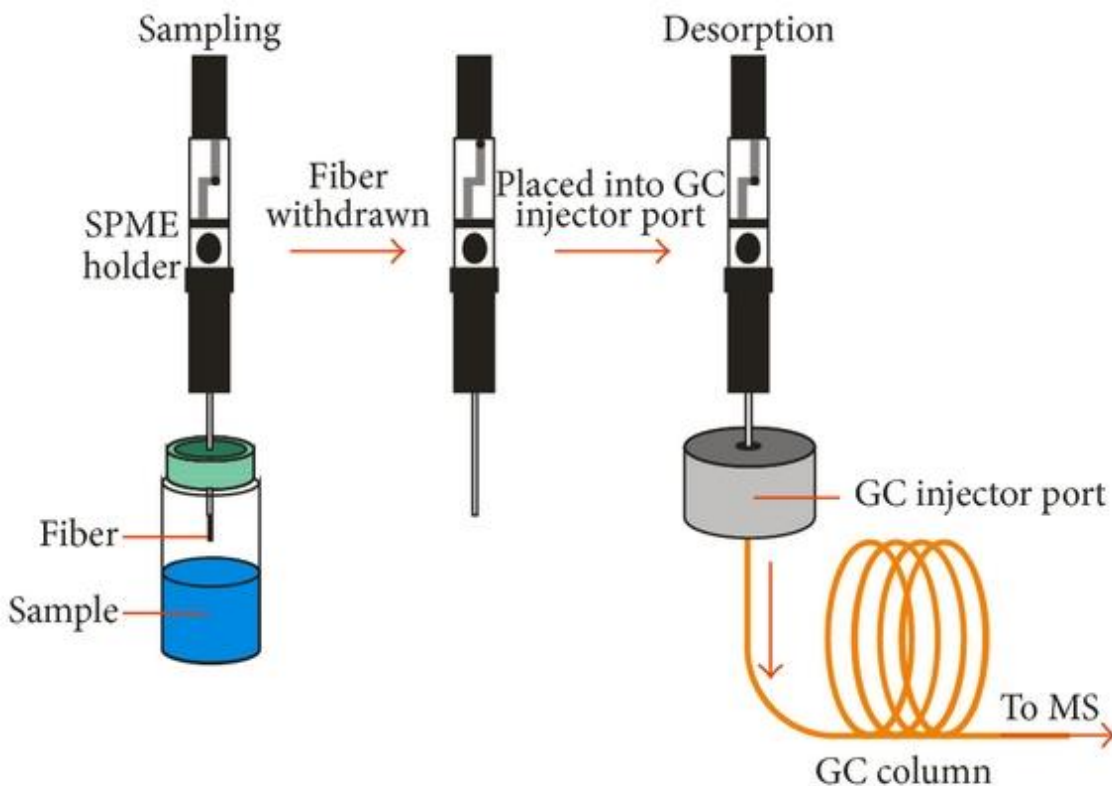

Figure 1-4. Diagram showing extraction and analysis of compounds using solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) (Schmidt and Podmore, 2015)

1.2.8.5. Insect Lipids

Lipids are compounds that are naturally excreted in animals and plants (Cerkowniak et al., 2013). Lipids are essential to insects because they provide a significant role for their energy needs (Downer and Matthews, 1976). Insect lipids vary in quality and composition and depend on the species and developmental stage. Insect lipids contain wax, fatty acid, triacylglycerols, aldehydes, alcohols, ketones and hydrocarbons (Blomquist and Jackson, 1979; Gibbs, 1998; Gołębiowski et al., 2011; Cohen and Moussian, 2016). Insects commonly contain a high content of lipids, around 50%–75% of the dry weight of some insect are lipids (Bursell, 1970; Rumpold and Schlüter, 2013; Pino Moreno and Ganguly, 2016). Several methods have been applied to extract insect lipids. SPME has been used as an alternative to solvent extraction for studying the lipid composition of insects (Peeters et al., 1999; Roux et al., 2002; Lacey et al., 2004). SPME has been used since 1989 and it is identified as a rapid and efficient method to detect chemicals using detection and separation systems (Arthur and Pawliszyn, 1990). Furthermore, one of the SPME method is direct immersion (DI-SPME), where the fibre is directly immersed in the liquid samples (Aulakh et al., 2005). The SPME technique is cheaper, easier, faster and more reproducible (Malosse et al., 1995).

Various separation instruments have been used to identify insect lipids. The most commonly used techniques are GC, high performance liquid chromatography, and combined techniques such as GC-MS and liquid chromatography-mass spectrometry. Buckner et al. (1999) gas chromatography (CGC) combined with mass spectrometry (Cerkowniak et al. 2013). GC-FID has been used to identify hydrocarbons from *Periplaneta* species (Saïd et al., 2005). SPME coupled with GC-MS was used to identify *Drosophila cuticular* compounds (Everaerts et al., 2010). GC-MS was used to analyse the cuticular hydrocarbons extracted from the pupal exuviae of necrophagous flies (Ye et al., 2007). However, the significant components of insect lipids are hydrocarbons (Gołębiowski et al., 2010). In the case of insect lipids are form the surface protective layer, and also are responsible for the interaction between species. In addition, surface waxes protect against various insect pathogens, such as fungi (Gołębiowski et al., 2008; Szafranek et al., 2012). The main function that the lipid layer provides is protection to the insect from desiccation and abrasion, and acts as a medium for communication (Lockey, 1988; Howard and Blomquist, 2005).

Lipids are a useful biochemical characteristic that can be used as a taxonomic tool for the identification and differentiation of species (Cohen and Moussian, 2016). CHCs of pupal exuviae are chemotaxonomically diagnostic for *Aldrichina graham* (Aldrich), *Chrysomya*

megacephala (F.), *Lucilia sericata* (Meigen), *Achoetandrus rufifacies* (Macquart), *Boettcherisca peregrina* (Robineau-Desvoidy), and *Parasarcophaga crassipalpis* (Macquart) with 100% correct identification by cross-regression analysis based on discriminant functions (Ye et al., 2007). Hydrocarbons include straight-chain saturated, unsaturated and methyl-branched hydrocarbons, which are predominate in the cuticular lipids of most insect species; fatty acids, alcohols, esters, ketones, aldehydes, and trace amounts of epoxides, ethers, oxoaldehydes, diols, and triacylglycerols. Monnin et al. (1998) used SPME to analyse cuticular hydrocarbons from ants. Some insects are extremely similar to other species such as *T. granarium* and *T. variabile* and identifying these species is challenging for taxonomists. Successful diagnosis of insect species would help in successful pest control, habitat management and nature conservation projects (Paterson, 1991; Besansky, 1999; Copren et al., 2005; Garros et al., 2006). Some studies showed that the lowest values had been found in Orthoptera and Odonata (less than 20%), whereas the highest total fat values were reported in Lepidoptera and Coleoptera (up to 30%) (Rumpold and Schlüter, 2013). Fat content depends on rearing conditions and feed composition and it also varies depending on the developmental stage. However, higher fat content was found in the preimaginal stages (larvae and pupae) than in the adult stages (Stanley et al. 1988; St-Hilaire et al. 2007; Chen et al. 2009). Fatty acids that synthesise in the insect body are stored in fat bodies as a form of triglyceride (Ad et al., 1985). Fatty acids are vital sources of energy and are required to build up cell membranes. Therefore, they are an important element for organism growth, differentiation, reproduction and homeostasis (Carballeira, 2008). Essential fatty acids are fatty acids that are essential to the growth and development of animals (Simopoulos, 1999). Free fatty acids store energy and usually bond with other compounds to build more composite lipids such as triglycerides (Desbois and Smith, 2010). Analysis of the cuticular lipids of *Calliphora vicina, Dendrolimus pini* and *Galleria mellonella* showed that the larval cuticle contains three main groups, including free fatty acids, hydrocarbons and triacylglycerols (Gołębiowski et al., 2008).

1.3. Research Gaps and aim of the study

Currently, no research is known in using volatile organic compounds (VOCs) to diagnose *Trogoderma granarium* and *Trogoderma variabile* by using Solid phase micro extraction technique (SPME), Gas Chromatography GC/MS and Visible/near-infrared hyperspectral (VNIH) technique. Therefore, the objective of this study is:

1- Study the feasibility of the Solid phase microextraction (SPME) technique for identification of volatile organic compounds (VOCs), hydrocarbons and the insect metabolism.

2- Develop new diagnostic tool for *Trogoderma granarium* and *Trogoderma variabile* identification using different life stages, body fragments and larvae skin based on visible near infrared hyperspectral imaging.

Statement of Contribution

Title of Paper	Optimization and Validation for Identification of Volatile Organic Compounds (VOCs) released from *Trogoderma variabile* Ballion using Headspace Solid Phase Microextraction (SPME) Coupled with GC-FID/MS
Publication Status	Submitted to Current Analytical Chemistry Journal
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Principal Author	
Name of Principal Author (Candidate)	Thamer Alshuwaili
Contribution to the Paper	Methodology, collected data, writing original draft
Overall Percentage (100%)	65%
Signature	Date 25 / 11 /2020

Co-Author Contributions

By signing the statement of contribution, each author certifies that:

- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated Contribution

Name of Co-Author	Xin Du
Contribution to the Paper	Data analysis and set up the equipment
Overall Percentage (100%)	10%
Signature	Date 25 / 11/2020
Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Data curation, editing and supervision
Overall Percentage (100%)	15%
Signature	Date 25/ 11/2020
Name of Co-Author	Yonglin Ren
Contribution to the Paper	Editing and supervision
Overall Percentage (100%)	10%
Signature	Date 25/11 /2020

Chapter Two

Optimization and Validation for Identification of Volatile Organic Compounds (VOCs) released from *Trogoderma variabile* Ballion using Headspace Solid Phase Microextraction (SPME) Coupled with GC-FID/MS

2.1. Abstract

Volatile organic compounds (VOCs) emitted by *Trogoderma variabile* at different life stages (larvae, adults including female and male) can help us to understand the chemical signals that are released by the beetle which can serve as biomarkers for diagnostic purpose. There are several factors that affect the optimization of VOC extraction including number of insects, duration of extraction, and gas chromatography (GC) conditions. This study used headspace solid phase microextraction (HS-SPME) fibre coupled with flame ionization detection (FID) and gas chromatography with mass spectrometry (GC-MS) to determine the optimal method for accurate, rapid and cost-effective extraction and identification of VOC from different life stages of *T. variabile*. The HS-SPME technique and the analytical conditions with GC and GC-MS were optimized and validated for the determination of VOCs released from *T. variabile*. Selection of the number of insects was based on the height and the number of peaks. Results showed that 25 and 20 larvae and adults respectively gave the best number of peaks. Sixteen hours were optimized as the best extraction time for larvae and adults to get the maximum number of emitted VOCs. Some of the VOCs compounds identified from this insect that can be used as biomarkers are pentanoic acid; diethoxymethyl acetate; 1-decyne; naphthalene, 2-methyl-; n-decanoic acid; dodecane, 1-iodo- and m-camphorene identified from larvae. However, butanoic acid, 2-methyl-; pentanoic acid; heptane, 1,1'-oxybis- 2(3H)-Furanone, 5-ethyldihydro-; pentadecane, 2,6,10-trimethyl-; and 1,14-tetradecanediol VOCs, were found in male while pentadecane; nonanic acid; pentadecane, 2,6,10-trimethyl-; undecanal and hexadecanal were identified from female.

2.2. Introduction

Grains, especially wheat is the most important crop around the world. The economic importance of grains and its contribution to the diets of humans and livestock cannot be disputed. However, there are many problems with grains especially in the storage process; of which pest problem is a critical issue (Ress, 2004; Nansen et al., 2008). One of these insects is Trogoderma spp.; which has more than 134 species including *T. granarium*, *T. glabrum, T. inclusum* and *T. variabile* (Banks, 1994). In Australia, there are over 50 Trogoderma described species including *T. variabile* which is morphologically closest to *T. granarium. T. granarium*, is a quarantine pest in Australia. Suspected *Trogoderma* specimens found in grain products are usually the larvae which are difficult to diagnose morphologically (Banks, 1994). Adult

specimens are usually scarce and damaged and need expert dissection for identification (EPPO, 2012; IPPC, 2012). Diagnostically the warehouse and khapra beetle can only be reliably identified by a limited number of skilled taxonomists. Many times, *T. variabile* and a comprehensive range of WA's native *Trogoderma* species and related Dermestid species could potentially be mistaken for *T. granarium*. Misidentification of *Trogoderma* and related Dermestids has the potential to seriously compromise Australian grain exports (Szito, 1997). Early monitoring and detection of insects in the stored food grains become necessary for applying corrective actions. The capability of in-situ early detection, monitoring, reliability, cost, and labor requirements are the major factors that considered during for selection of the method. Detection of hidden infestation, whose population may be many times higher than the free-living insects is an important concern to mitigate the losses in bulk storage warehouses, so as to enable the early actions for fumigation or to dispose of the grain (Banga, 2018). Several detection techniques have been developed for the internal and external detection of insects in stored food grains such as detection probe, staining of the kernel, Berlese funnel method, acoustic techniques, uric-acid method, X-ray imaging, nuclear magnetic resonance imaging, thermal imaging and solid-phase micro-extraction method (Neethirajan, 2007). Some of these techniques are time-consuming, expensive, have potential health hazard, and less efficient. Manual sampling traps and probes are the most common methods used on farms, while manual inspection, sieving, and Berlese funnel method are used in grain storage and handling facilities (Neethirajan, 2007). The HS-SPME technique is a new, fast, simple, and highly sensitive and solvent-free sample preparation technique for the extraction of volatile compounds (Prosen and Lujcija, 1999; Wardencki et al., 2004; Mohammed et al., 2017; Villaverde et al., 2007). SPME is an establish technique to identify and analyses compounds released by insects (Zhang et al. 2007; Kudlejova et al., 2012; Senthilkumar et al., 2012). The solid phase microextraction (SPME) technique coupled with GC-MS has been used in other studies to collect volatile from grain or horticulture insects, such as fruit fly, rhinoceros beetle and cerambycid beetle pheromones (Al-Khshemawee et al., 2017; Rochat et al., 2000; Ginzel et al., 2005); it was also used to detect the aggregation pheromone and other volatile metabolites of the lesser grain borer, *Rhyzopertha dominica* (F.) and the red flour beetle *T. castaneum* (Seitz and Ram 2004; Arnaud et al., 2002). Gas chromatography (GC) combined with flame ionization detection (FID) or mass spectrometry (MS) are known methods in detecting food flavors or insect metabolites using HS-SPME technique (Jelen et al., 2012; Niu et al., 2016). In the recent years, studies showed that using head-space solid phase microextraction (HS-SPME) coupled with gas chromatography–flame ionization detection (GC-FID), gas chromatographic

electroannographic detection analysis (GC-EAD) and gas chromatography–mass spectrometry (GC-MS) are a good technique for identifying volatiles in stored grains to detect infestation with insects (Pavia et al., 2005; Pureswoaran et al., 2004). Using SPME fibre combined with GC-FID and GC-MS technique gives us an accurate, rapid, efficient and non-destructive method to extract volatile organic compounds from insects (Al-kshemawee et al., 2017; Mohammed et al., 2017). Many factors might affect the optimization of extraction conditions which include an optimum extraction time and the correct fibre for capturing the whole range of VOCs, the temperature during extraction and the fibre absorption time from the headspace (Nonogonierma et al., 2006). Identification of the volatile organic compounds emitted by grain insects in future can be used in early detection of insects in stored grains using headspace analysis (Niu et al., 2016; Senthilkumar et al., 2012). Based on previous studies, the aim of this study is to focus on developing optimal condition to collect volatile organic compounds from *Trogoderma variabile* including a number of insect and extraction time, followed by identification of unique peaks which can be used as early infestation detection tool for *T. variabile*.

2.3. Materials and Methods

2.3.1. Insect culture

T. variabile was obtained from the Post-Harvest Plant Biosecurity laboratories, College of Science, Health, Engineering and Education, Murdoch University, Western Australia. To get different stages of *T. variabile*, around 150 adults were added into one kg jar containing 450 g of canola seeds covered with a meshed lid. Prior to use, canola seeds were sterilized at -20°C for a week and stored at 3°C until further use. The insects were reared in a controlled room with 29 ±2°C and 70±2% relative humidity. The jars were kept in the culture room for 1-2 months to get the required number of insect population (larvae and adults) used for this study.

2.3.2. Apparatus and equipment

Solid phase microextraction (SPME) fiber Divinyl benzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS fibre, 50/30 µm (Sigma-Aldrich Australia, catalog number 57299-U), was used in this study to collect volatile organic compounds (VOCs). An Agilent Technologies gas chromatograph 7829A (serial number CN14272038) fitted with an HP-5MS column (30 m x 0.25 mm, film thickness 0.25 µm, RESTEK, catalogue number 13423) non-polar, with a flame

ionization detector (FID) was used. For identification of VOC's GC Agilent GCMS 7820A equipped with a DB-35ms column (30 m × 250 µm × 0.25 µm) and MS detector 5977E (Agilent Technologies, USA) (Santa Clara, CA 95051, USA) was used. GC-MS operation conditions were as follows: Injector port temperature 250°C. The initial oven temperature kept at 50°C with an increase to 250°C (increment of 5°C/min). The flow rate of the column was 1.1 ml/min, while the split less mode was 20 ml/min at 1.5 min. The run time of GC-MS was 46 min. The glass vials 5 ml with screw tight cap with septa (SUPLICO, USA Lot: 82742) was used for collection of *T. variabile* VOCs by SPME. Three experimental replicates were taken during the optimization process and identification of peaks.

2.3.3. Optimization of number of insects

Different number of insects (15, 20, 25, and 30) were tested for each life stage to get the optimal number of insects. Larvae (mixed instars) and adults (mixed male and female). The insects were placed into five ml glass vial (SUPLICO, USA Lot: 82742) and kept at 35°C in thermostatic and humidity chamber (HWS, Ningbo southeast Dongnan Instrumental Ltd) for four hours to enhance the release of VOCs.

2.3.4. Optimization of extraction time

Four different extraction time (4, 8, 16 and 24h) were used to collect VOCs from warehouse beetle larvae and adults (mixed males and females). Solid phase microextraction (SPME) fibre was exposed to the headspace of 5 ml jar containing 15 insects for 4 hrs extraction times. After that, SPME fibre was injected into gas chromatography-flame ionization detector (GC FID) for 10 min for desorption of the volatiles from fibre to GC column. Same procedure was repeated for 8, 16 and 24h. Each treatment was replicated three times.

2.3.5. Analysis and identification of real samples using optimized method

Once the optimal conditions were selected, the optimal conditions were applied on GC-MS to identify the emitted VOCs from two different stages of *T. variabile* (larvae and separate male and female). Each of the above test was replicated three times.

2.3.6. Data analysis

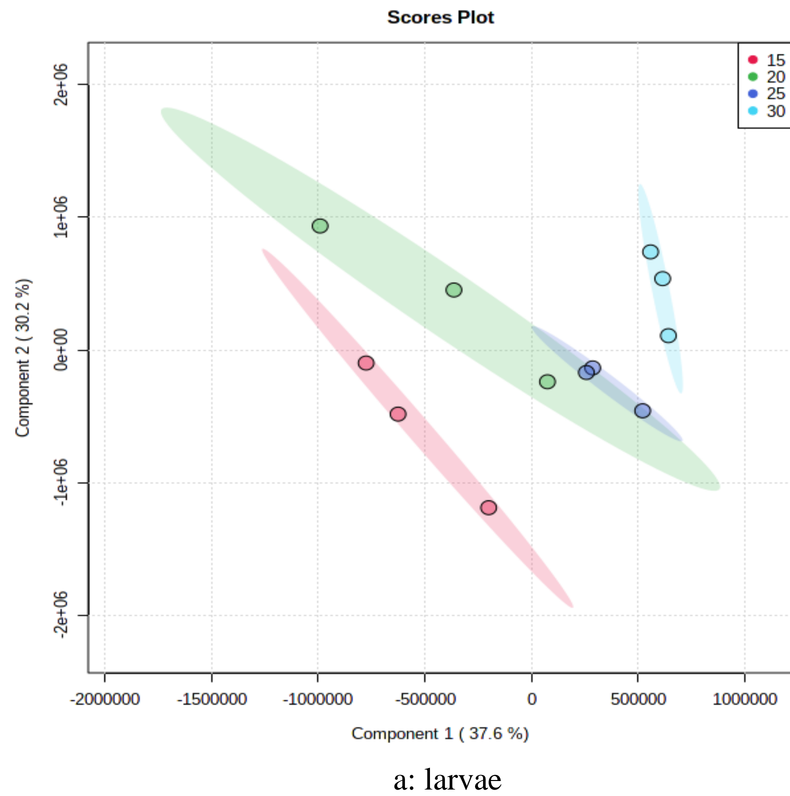
The GC data including peak area and retention time were collected and integrated into the chromatography software Agilent Chemstation (Mass Hunter Quantitative Analysis Software, B.07.00), and then the data was exported to Microsoft Excel for further analysis. The repeatability of replicates from the same sample was verified by checking the chromatogram pattern features such as detected peak areas and retention times. The averages of compound areas were statistically performed by Metaboanalyst 4.0 using Partial Least Squares - Discriminant Analysis (PLS-DA).

2.4. Results and Discussion

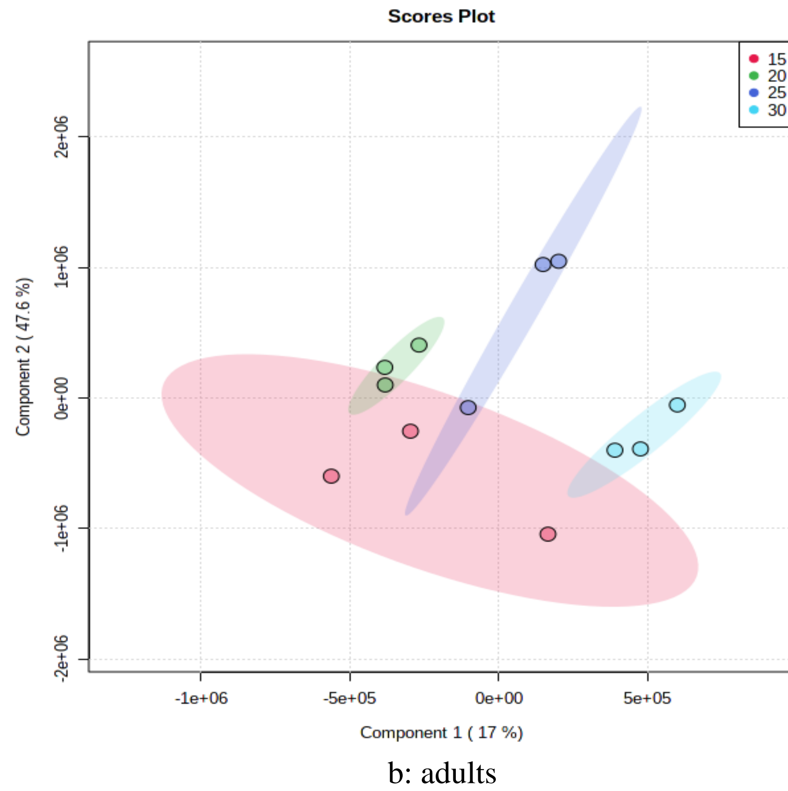
2.4.1. Insect densities

The number of GC peaks from different insect densities (15, 20, 25, and 30) for two life stages, including larvae and adults (mix male and female) were compared. The compounds peak areas were analyzed using Partial Least Squares Discriminant Analysis (PLS-DA). The result shows the scores plot between the selected PCs (Figure 2-1a and b). The model showed good separation between 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 25 larvae had the highest abundance of most metabolic products, including those which had high intensity in the GC-FID chromatogram like FID-18.442, FID-14.173, FID-5.136, FID-3.466, FID-20.011 and FID-23.693 (Figure 2-2a). However, results in Figure 2-2b showed that nine compounds out of 15 compounds gave the higher concentration of VOCs extracted from 20 adults based on GC-FID data such as FID-38.426, FID-11.275, FID-27.163, FID-39.899, FID-36.915, FID-19.157, FID-18.422, FID-24.854, and FID-40.800. These results proved that this number of insects has an optimal density to produce abundant amount of VOCs that can be collected by SPME. Twenty insects in 8 ml glass jar were chosen as the optimum insect density to implement further optimization. Different number of insects can affect the amount of volatile released by these insects. This result showed that the less numbers of *T. variabile* insects gives more volatile compared with the high numbers and that could be due to the overcrowding in the small vial (8 mL). The overcrowding might have caused a reduction in the metabolism of insects due to an increase in the CO_2 quantity which has a critical effect on the biological and physiological processes of insects (Guerenstein and Hildebrand, 2008; Nicolas and Sillans,

1989). Additionally, the amount of sample strongly affects the amount of the extracted analyte (Jelen et al., 2000).



a: larvae



b: adults

Figure 2-1a and b. Partial Least Squares Discriminant Analysis (PLS-DA) shows volatile profiles from different *T. variabile* stages in different extraction time based on VOCs with three biological replicates.

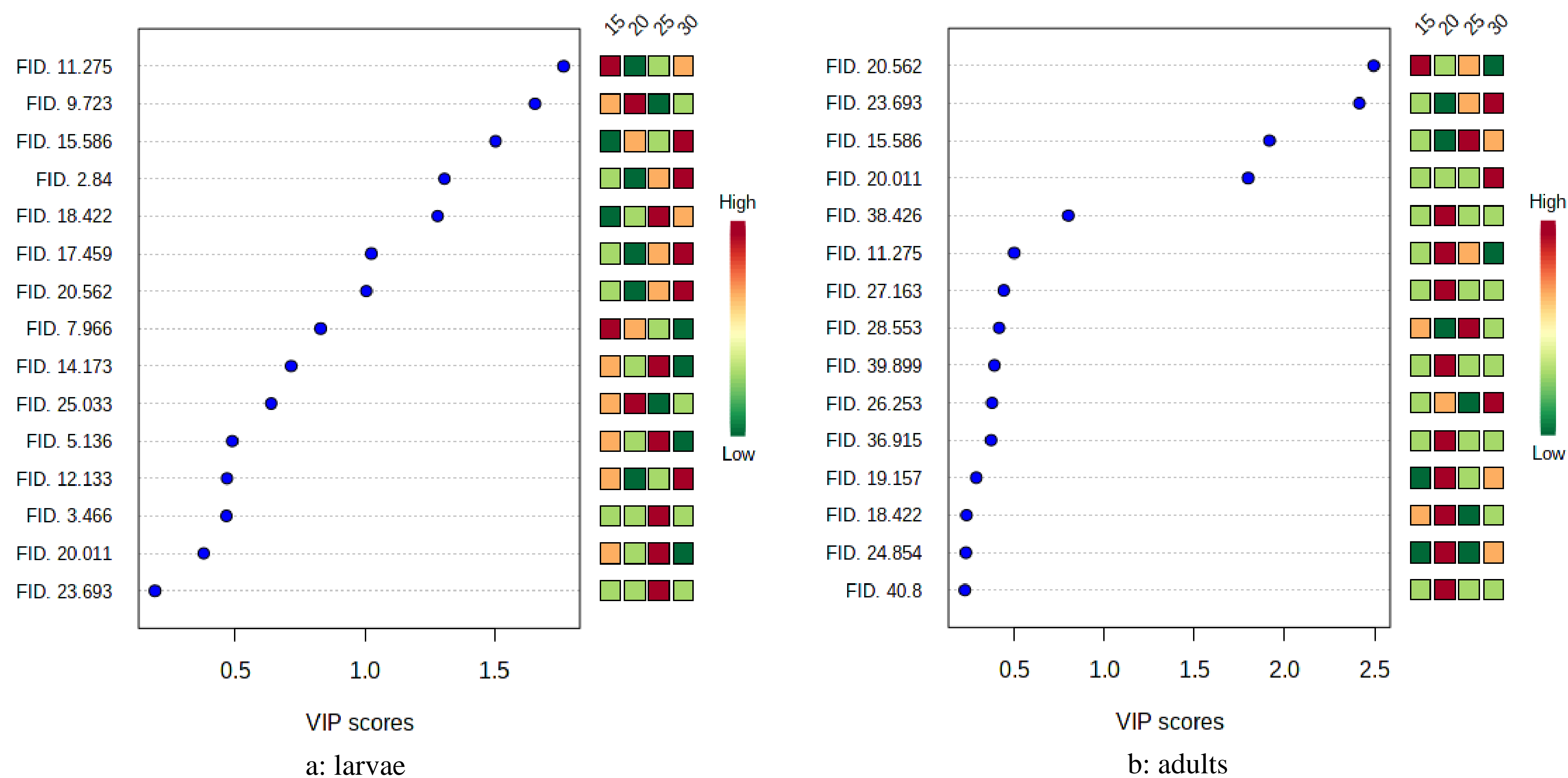
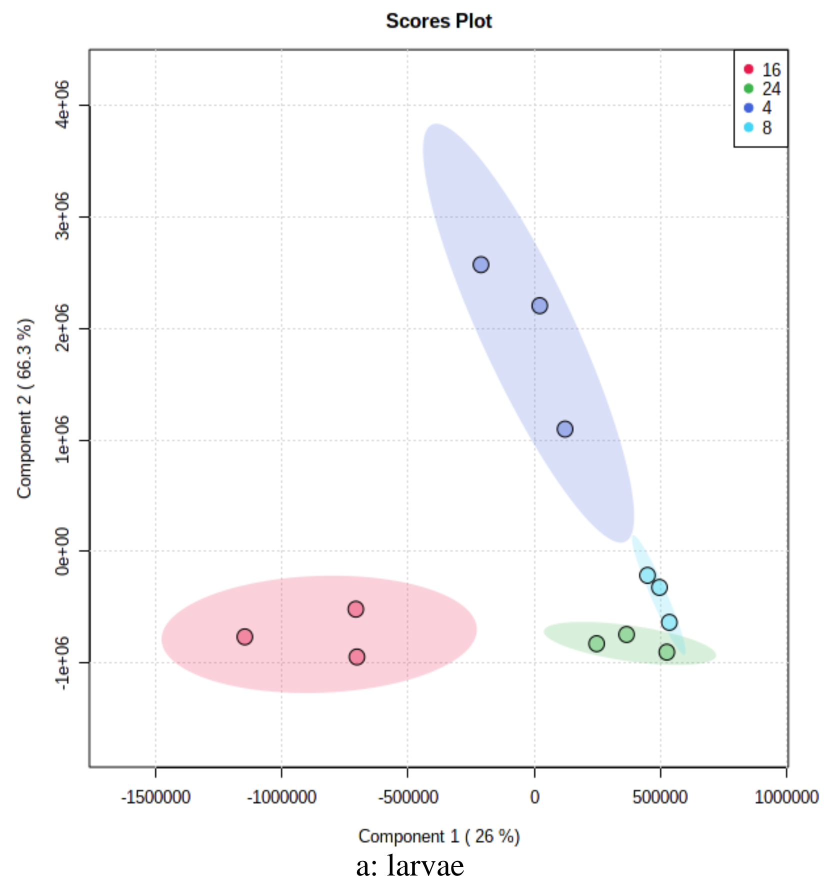


Figure 2-2a and b. Variable Importance in Projection (VIP) shows important features identified by PLS-DA from data of different insect densities based on Volatile organic compounds (VOCs) with three biological replicates. Codes in left side are the metabolic ID (FID indicates GC-FID detector and numbers indicate the retention times (min) while the right side indicate the high relative abundance of the corresponding metabolites in each group under study.

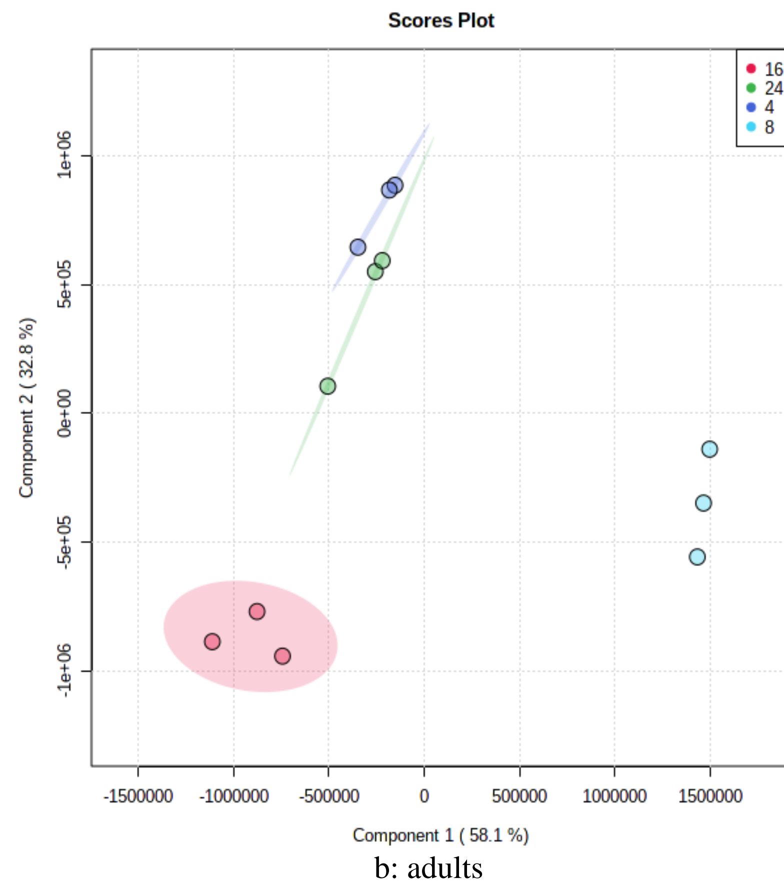
2.4.2. Extraction time

Once the insect densities were selected, the effect of extraction time on the VOCs emission from *T. variabile* (larvae and adults) was studied. In this study, four different extraction times (4h, 8h, 16h, and 24h) were tested to collect volatile organic compounds from two different *T. variabile* stages larvae (mixed instars) and adults (mix male and female). The areas of the 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 has a significant effect on the VOCs emission from the larvae and adults of *T. variabile* (Figure 2-3a and b). Furthermore, results in Figure 2-4a and b showed that Variable Importance in Projection (VIP) scores play a significant effect on the metabolites that contributed in the PLS-DA model. However, the intensities of chromatogram peaks increased significantly after increasing the extraction time from 4 to 8, 16 and 24 h (Figure 2-4a and b). In case of larvae, at 16 h, some of the early compounds had higher abundance than other extraction periods like a compound at FID-20.562, FID-3.466, FID-2.840, FID-20.011, FID-5.136, FID-5.804, FID-14.173, FID-18.422 and FID-24.050. However, the abundant metabolic compounds (main compounds) in VIP score like FID-9.723, FID-24.661, FID-11.275, FID-24.854, FID-20.562, FID-19.157, FID-39.899, FID-27.163, FID-25.618, FID-35.903 and FID-34.805 were found to be higher at the 16 h extraction period than other extraction periods. Based on this result, the optimum time selected was 16 hours for adults.

Extraction time is a significant parameter in head space solid phase microextraction. It is an important step to determine extraction time using SPME fibre method (Senthilkumar et al., 2012; Dorea et al., 2008). The amount of extracted volatile depends on the sampling method, such as extraction time (Arnaud et al., 2002; Qazi et al., 1998). 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 (Kudlejova et al., 2012; Laopongsti et al., 2014; Niu et al., 2012). The temperature and extraction time significantly affect HS-SPME methodology because they effect equilibrium during extraction of volatile organic compounds (Zhang et al., 2007).



a: larvae



b: adults

Figure 2-3a and b. Partial Least Squares Discriminant Analysis (PLS-DA) shows volatile profiles from different *T. variabile* stages in different extraction time based on VOCs with three biological replicates.

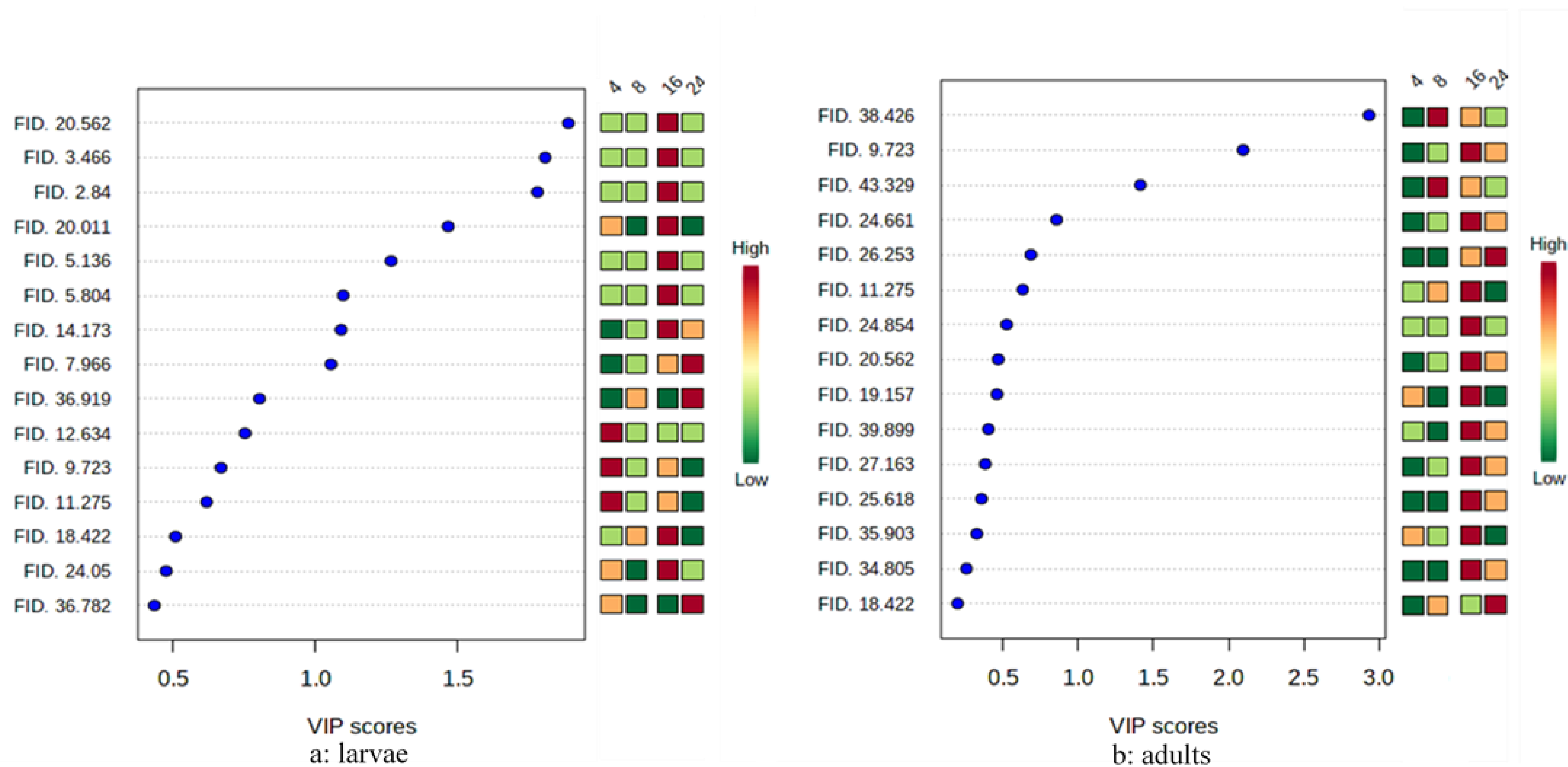


Figure 2-4a and b. Variable Importance in Projection (VIP) shows important features identified by PLS-DA from data of different insect densities based on Volatile organic compounds (VOCs) with three biological replicates. Codes in left side are the metabolic ID (FID indicates GC-FID detector and numbers indicate the retention times min) while the right side indicate the high relative abundance of the corresponding metabolites in each group under study.

2.4.3. Analysis and identification of real samples using optimized method

The GC-MS was used to identify volatile compounds in larvae and adults. Results showed that there is difference in the amount of each chemical. Volatile organic compounds were identified based on their retention index and mass spectra in comparison with external n-alkane standards. Each value in the diagram represent three replicates. The lowest relative abundance of the compounds was specified as light color while the dark color represent the height of the peak. There were 12 compounds identified in larvae stage which was pentanoic acid; oxime-, methoxy-phenyl; diethoxymethyl acetate; 2(3H)-furanone,5-ethyldihydro naphthalene, 2-methyl-; -; nonanal; 1-decyne; decanal; naphthalene, 2-methyl-; n-decanoic acid; dodecane,1-iodo-; n-hexadecanoic acid and m-camphorene (Figure 2-5a). Compounds identified from *T. variable* male were different from those identified from female such as butanoic acid, 2-methyl-; heptane, 1,1'-oxybis-;1,14-Tetradecanediol and n-hexadecanoic acid (Figure 2.5a and b). Four compounds were identified in all three different stages which were oxime-, methoxy-phenyl, 2(3H)-Furanone, 5-ethyldihydro- and nonanal (Figure 2-5a, b and c). The compounds detected only from *T. variabile* larvae were identified as pentanoic acid; diethoxymethyl acetate; 1-decyne; naphthalene, 2-methyl-; n-decanoic acid; dodecane, 1-iodo- and m-camphorene. Four compounds exclusively detected from female were pentadecane; nonanic acid; undecanal; hexadecanal and dodecane (Figure 2-5b). In case of male, three compounds can also be detected and identified which are butanoic acid, 2-methyl; heptane, 1,1'-oxybis- and 1,14-tetradecanediol (Figure 2-5c). Hence these compounds can act as biomarkers and as diagnostic compounds for the infestation stages. Identification of the volatile organic compounds released by insects can be used to detect insects' in stored grains (Senthilkumar et al., 2012). There was an attempt to identify the VOCs compounds released by *Cryptolestes ferrugineus* (rusty grain beetle) and *Tribolium castaneum* (red flour beetle) by headspace analysis. According to the available literature there has not been a detailed study of the VOCs produced by *T. variabile* different stages. Some of the reported compounds in this study were identified on different insects such as oxime-methoxy-phenyl-_; decanal; nonanal; dodecane, pentadecane and nonanoic acid (Niu et al., 2016; Alnajim et al., 2019; Niu et al., 2015; Alnajim, 2020). It is therefore significant, that from larvae pentanoic acid; diethoxymethyl acetate; 1-decyne; naphthalene, 2-methyl-; n-decanoic acid; dodecane, 1-iodo- and m-camphorene were identified. However, butanoic acid, 2-methyl-; pentanoic acid; heptane, 1,1'-oxybis- 2(3H)-Furanone, 5-ethyldihydro-; pentadecane, 2,6,10-trimethyl-; and 1,14-tetradecanediol VOCs, were found in male while pentadecane; nonanic acid; pentadecane, 2,6,10-trimethyl-;

undecanal and hexadecanal were identified from female. These too might also be potential biomarkers.

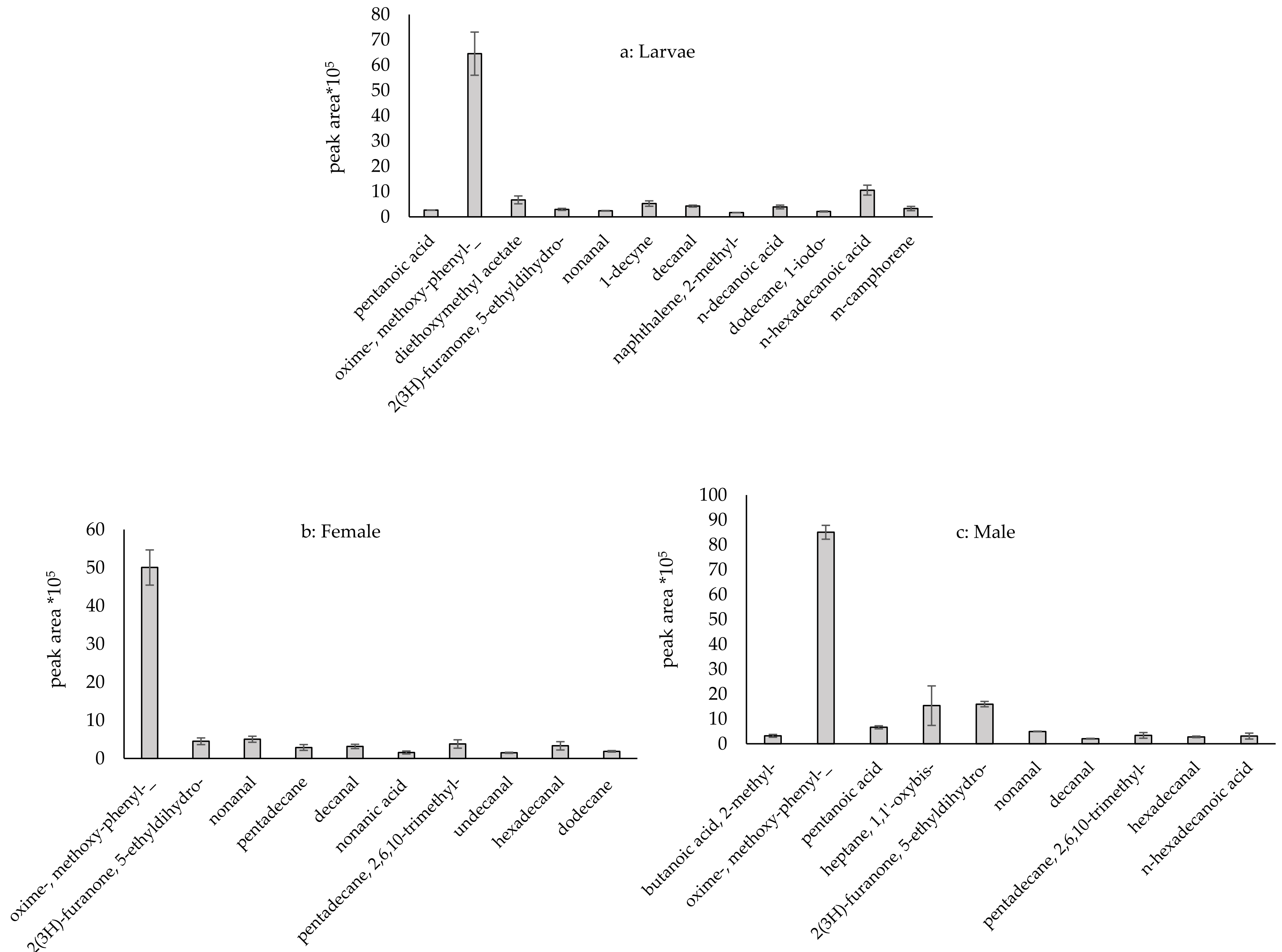


Figure 2-5a, b and c. Volatile organic compounds (VOCs) detected from *T. variabile* different stages using headspace microextraction technique and gas chromatography mass spectrometry.

2.5. Conclusions

HS-SPME fibre coupled with GC-MS and GC-FID can be used to detect volatile organic compounds from different *T. variabile* stages (adults and larvae). This study showed that the optimal number of insects were 20 and 25 for adults and larvae respectively. Also, the best extraction time was 16 hours for both adults and larvae. The optimized method was used for the identification of volatile organic compounds from the insects using GCMS. Identified VOCs compounds from larvae, female, and male of *T. variabile* can further be explored to develop a sensitive method for early and timely detection of infestation or development of lures.

Statement of Contribution

Title of Paper	Study on metabolic response of male and female *Trogoderma variabile* (Ballion) on different host grain using Direct Immersion Solid-Phase Microextraction (DI-SPME) Coupled with Gas Chromatography Mass Spectrometry (GC-MS)
Publication Status	Submitted to molecules journal
Publication Details	
Principal Author	
Name of Principal Author (Candidate)	Thamer Alshuwaili
Contribution to the Paper	Methodology, Collected Data, Writing- Original Draft Preparation, Conceptualization and Validation
Overall Percentage (100%)	65%
Signature	Date 25 /11/2020

Co-Author Contributions

By signing the statement of contribution, each author certifies that:

- The candidate's stated contribution to the publication is accurate (as detailed above).
- Permission is granted for the candidate to include the publication in the thesis
- The sum of all co-author contributions is equal to 100% less the candidate's stated Contribution

Name of Co-Author	Manjree Agarwal
Contribution to the Paper	Data curation, editing and supervision
Overall Percentage (100%)	15%
Signature	Date 25 /11 /2020
Name of Co-Author	Ihab Alnajim
Contribution to the Paper	Data analysis and editing
Overall Percentage (100%)	5%
Signature	Ihab Alnajim Date 25 / 11/2020
Name of Co-Author	Xin Du
Contribution to the Paper	Data analysis and set up the equipment
Overall Percentage (100%)	5%
Signature	Date 25 /11 /2020
Name of Co-Author	Yonglin Ren
Contribution to the Paper	Editing and supervision
Overall Percentage (100%)	10%
Signature	Date 25 / 11 /2020

Chapter 3

Study on metabolic response of male and female *Trogoderma variabile* (Ballion) on different host grain using Direct Immersion Solid-Phase Microextraction (DI-SPME) coupled with Gas Chromatography Mass Spectrometry (GC-MS)

3.1. Abstract

The purpose of this work is to use the technique of gas chromatography coupled to mass spectrometry (GC–MS) to study the metabolite profile of *Trogoderma variabile* using different host grains including canola, oats, wheat, and barley. Also, as a part of the results, it is suggested that hydrocarbons profiling can be used as a chemo-taxonomical tool for insect species identification especially for very morphologically similar species like *T. granarium*. Samples from different *T. variabile* genders (female and male) were used in this experiment. *T. variabile* were reared on the four various commodities. For sample preparation insects were subjected for extraction with acetonitrile. Direct immersion-solid phase microextraction (DI-SPME) was employed, followed by gas chromatography mass spectrometry analysis (GC-MS) for the collection, separation and identification of compounds. Results showed that insect host grains have a significant effect on the gender specific insect chemicals that were identified from *T. variabile* adults such as fatty acid and hydrocarbons. There were 23 compounds identified from adults reared on canola and wheat. However, there were 26 and 28 compounds detected form adults reared on oats and barley respectively. Results also showed that 11-methylpentacosane; 13-methylheptacosane; heptacosane; docosane, 1-iodo- and nonacosane were the most significant compounds that identified form *T. variabile* male reared on different host grains. However, the main compounds that were identified from female cultuerd on different host grains include docosane, 1-iodo-; 1-butanamine, N-butyl-; oleic acid; heptacosane; 13-methylheptacosane; hexacosane; nonacosane; 2-methyloctacosane; n-hexadecanoic acid and docosane.

3.2 Introduction

Trogoderma variabile (Ballion) or warehouse beetle, (Coleoptera: Dermestidae), is an internationally significant invasive pest that attacks wide range of packed goods and stored grain (Castalanelli, et al., 2011). Nowadays, *T. variabile* has been regarded as a persistent pest of grain storage and handling structures. Warehouse beetles are primary voracious feeders that infect variety of products such as cereal products, candy cocoa, corn, corn meal, dog food (dried and 'burgers'), fishmeal, flour, oatmeal, milk powder, spaghetti, spices, peas, wheat, barley and pollen. In grain, they cannot feed on whole grain, but can feed on broken kernels that are usually present in the store (Mason, 2003). Larvae of *T. variabile* can infest 119 of different kinds of commodities (Hagstrum et al., 2013).

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 and Matthews, 1976). Lipids composition occurs naturally, performing an essential role in the metabolism of insects and plants (Cerkowniak et al., 2013; Ad et al., 1985). Insects commonly contain a high content of lipids, making up 50-75% of the dry weight in some insect (Pino et al., 2016; Rumpold and Schlüter, 2013). Studies mentioned that the season of field collection, geographical origin of strain, genetic background, and number of generations has effect on lipid content of lesser grain borer, *Rhyzopertha dominica* (Cohen and Moussian, 2016). These factors affect the composition of different types of compounds, such as long chain hydrocarbons, waxes, alcohols, aldehydes and free fatty acids. Lipid types and content in insects vary according to the life stages and insect species. Total lipid content for grasshoppers and other related species (Orthoptera) is a relatively low; ranging from 3.8 g to 5.3 g/100 g fresh insects. In contrast, caterpillars (Lepidoptera) ranges from 8.6 to 15.2 g/100 g fresh (Bukken,1997). Other studies observed that the fat content of yellow mealworms was strongly affected by the different protein and starch content of their diets, suggesting that larvae fed with a low nutritional quality diet probably use fat reserves for energy, thereby reducing fat content (Van Broekhoven et al., 2015; Arrese and Soulages, 2010). Long chain fatty acids, such as palmitoleic, palmitic, stearic, linoleic, and oleic acids have been found in the cuticular extracts and exocrine secretions of many insects (Lockey, 1988).

The development of analytical technology with powerful qualitative and quantitative capabilities, as well as high specificity, is essential for the study of metabolic samples. Previous studies showed that solid-phase microextraction (SPME) coupled with GC has been used because it provides an efficient method to detect chemicals (Al-Khshemawee et al., 2017; Najafian and Rowshan, 2012; Bicchi et al., 2000). Proving that SPME technique is a cheaper, easier and faster, so it can be used as an alternative extraction method (Malosse et al., 1995). Also, SPME has been used to extract cuticular hydrocarbons from ants (Monnin et al., 1998). The SPME technique coupled with GC-MS has also been used to detect long-chain free fatty acids from insect exocrine glands (Maile et al., 1998).

This study investigates the feasibility of using high-resolution direct immersion-solid phase microextraction (DI-SPME) coupled gas chromatography mass spectrometry analysis (GC-MS) for profiling of *T. variabile* adults. DI-SPME is more sensitive compared with HS-SPME,

and it is the method of choice for the analysis of clean aqueous samples (Menezes et al., 2010). The two extraction modes were evaluated and, despite being less sensitive than HS-SPME in the case of the more volatile compounds, direct immersion DI-SPME mode successfully extracted 16 pesticides, compared to HS-SPME which was able to extract only 12 compounds (Arthur and Pawliszyn, 1990). In previous studies, eight solvents were used to extract lipids *Tribolium castaneum* and *Rhyzopertha dominica* and acetonitrile extract showed the highest peak numbers with 41 compounds; including some of the fatty acids and hydrocarbon waxes (Alnajim et al., 2019).

Numerous tools have been used to identify *Trogoderma spp.,* such as genetic tools, morphological and taxonomic keys. However, these methods are expensive and inefficient because it takes time for identification and need professional taxonomic staff. Also, insect hydrocarbons could be used as an alternative method when the taxonomical identification of the insect is not feasible due to its damaged condition or if its DNA is too degraded (Braga et al., 2013).

The aim of this paper is to use the technique of gas chromatography coupled to mass spectrometry (GC–MS) to study the metabolism of *T. variabile* that reared on different host grains including canola, oats, wheat, and barley and use the hydrocarbons chemicals for insect identification.

3.3. Materials and Methods

3.3.1. Insect culture

Trogoderma variabile were obtained from the Post-Harvest Plant Biosecurity and Food Safety laboratory, School of Science, Health, Engineering and Education, Murdoch University, Murdoch, Western Australia, Australia. To get adult females and males of *T. variabile*, 150 adults were added into 1-L plastic jars containing 450 g of sterilized canola, oats, wheat, and barley separately and then the jars were covered with a meshed lid. Prior to usage the insect food was sterilized by keeping it at -20°C for five days using 4 L glass jars and then maintained the jars at 4°C until used. Before using the insect food for culture, it was thawed at room temperature. The insects were reared in a controlled room with 29 ± 2°C and 70 ± 2% relative humidity.

3.3.2. Apparatus and equipment.

Gas chromatography GC-MS 7890B equipped with a 5977B MSD mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), with an Agilent HP-5MS column (30 m, 0.25 mm, 0.25 µm film thickness) were used in the experiments. Helium was used as a carrier gas with 99.99% *v/v* purity (BOC, Sydney, Australia). GC-MS operation conditions were as follows: injector port temperature was 270°C. The initial oven temperature was 60°C with an increase to 270°C (increasement of 5°C/min) MS Quad at 150°C; MS source at 230°C; pressure at 10.2 psi. The flow rate of the column was 1:1 ml/min, while the split less was 30 ml/min at 1.2 min. The total run time of GC-MS was 54 min.

3.3.3. The extraction and analysis method

Adults of *Trogoderma variabile* reared on different grain (canola, oats, wheat, and barley) were used in the trials. One adult male or female from each host grains was separately transferred into 2 mL plastic microtube (Benchmark Scientific, From Sigma-Aldrich, lot no.3110, USA). Then, two milling balls were added. After that, 200 µL of acetonitrile ≥ 99.9 *v/v* (HPLC grade, fisher chemical scientific, Glee, Belgium) was added to the microtube using micropipette and homogenized for two minutes using BeadBug microtube homogenizer. The extract was centrifuged at 8150× g for three minutes by Dynamica mini centrifuge (Model no. velocity 13µ), and then was transferred to 300µl insert glass (Thermo scientific micro- insert, 31x6mm clear glass, 15 mm top) placed into 2000 µL clear screw HPLC vial (Agilent Technology, China) using micropipette. Finally, solid phase microextraction (SPME) fibre 50/30 µm with 2cm DVB/CAR/PDMS coating (Sigma-Aldrich, Bellefonte, PA, USA) was inserted into extracted samples for 16 hours in the room temperature (25 ± 2°C). After that, the fibre was withdrawn and removed from the vial and immediately introduced into the GC-MS injector port for thermal desorption.

3.3.4. Data collection and analysis

The GC–MS signals were collected by the Mass Hunter Acquisition software (Agilent Technologies, Santa Clara, CA, USA). The National Institute of Standards and Technology (NIST) mass spectra library was used to identify chemical compounds. The retention index was used to assist identification. The experiment was repeated three times to confirm the chemicals. The area, which represents each peak in the chromatogram, was extracted using Mass Hunter

Acquisition software (quantitative analysis) B.06.00 (Agilent Technology, USA). After selecting the compounds, peak area of each compounds was generated to Microsoft Excel 2016, which was also used for data arrangement and sorting. Data were statistically analyzed using MetaboAnalys version 4
https://www.metaboanalyst.ca/MetaboAnalyst/upload/StatUploadView.xhtml

3.4. Results and Discussion

3.4.1. Effect of insect gender of *T. variabile* on the compound production

Results in Table 3-1 showed that *T. variabile* cultured on canola produced overall 23 compounds from male and female. Also, differences in the number of compounds are gender specific. Female yielded 20 compounds while male yielded 22 compounds. Sixteen compounds showed a significant difference which were 1,2-benzisothiazole; 2-decenal, (E)-; heptadecane; methoxyacetic acid, 2-tridecyl ester; 1-decanol, 2-hexyl-; n-hexadecanoic acid; oleic acid; docosane; tetracosane; heptadecane, 9-octyl-; pentacosane; 11-methylpentacosane; 2-methylhexacosane; hexacosane; heptacosane; docosane, 1-iodo-; 13-methylheptacosane; 2-methyloctacosane and nonacosane. Some compounds were only detected in male including heptadecane; methoxyacetic acid, 2-tridecyl ester and docosane, 1-iodo- while 1-decanol, 2-hexyl- were identified from female.

Furthermore, results in Table 3-1 showed that rearing insects on oats affected the quantity, quality, and number of the compounds produced by female and male. DI-SPME and GC-MS method extracted and detected overall 26 compounds from both genders. Results showed that 22 and 23 compounds were identified from the female and male respectively. Statistical analysis revealed that there were significant differences in the GC-MS response (peak areas) using insect samples collected from oat such as nonanal; decanal; 2-decenal, (E)-; 2-undecenal; dodecanal; caryophyllene; 1-decanol, 2-hexyl-; pentadecanoic acid; oleic acid; docosane; heptadecane, 9-hexyl-; tetracosane; heptadecane, 9-octyl-; pentacosane; 11-methylpentacosane; hexacosane; heptacosane; 13-methylheptacosane; 2-methyloctacosane and nonacosane. However, some compounds were identified from male which were nonanal; decanal and caryophyllene compared with 2-decenal, (E)-; 2-undecenal; dodecanal and pentacosane while nonanal and decanal were only detected from female reared on oats and not from male (Table 3-1).

There were 23 compounds obtained from both female and male reared on wheat. Fourteen compounds were significantly different between these two genders including tetradecanoic acid; n-hexadecanoic acid; nonadecanoic acid; oleic acid; tricosane, 2-methyl-; tetracosane; 11-methylpentacosane; 2-methylhexacosane; hexacosane; heptacosane; docosane, 1-iodo-; 13-methylheptacosane; 2-methyloctacosane and nonacosane (Table 3-1).

In the case of female and male reared on barley, results in Table 3-1 showed that there were differences among compounds for each gender. Some of the compounds detected in female, were found to be absent in male. From 28 compounds in total detected from *T. variabile* adults reared on barley, 23 compounds produced by the female. Many compounds were detected in male but not in female and these included hexadecanes; decanoic acid, hexyl ester; 2-hexadecanol; heptadecane and 1-decanol, 2-hexyl-. However, 22 compounds showed a significant different such as 1-butanamine, N-butyl-; 2-decenal, (E)-; hexadecane; decanoic acid, hexyl ester; 2-hexadecanol; heptadecane; 1-decanol, 2-hexyl-; pentadecanoic acid; nonadecanoic acid; oleic acid; docosane; heptadecane, 9-hexyl-; tricosane, 2-methyl-; heptadecane, 9-octyl-; pentacosane; 11-methylpentacosane; 2-methylhexacosane; hexacosane; docosane, 1-iodo-; 13-methylheptacosane; 2-methyloctacosane and nonacosane. This study has focused on the metabolism of *T. variabile* adults, which reared on different host grains including canola, oats, wheat and barley.

Our results confirmed that there was a significant difference in the chemical compounds between female and male. this finding was agreed with data that collected by Howard (1992) where their study confirmed that there were significant differences between *T. variabile* genders lipids content. Furthermore, differences in lipid content of insects were found between adult males and females (Lease and Blair, 2011).

The current study showed that male tend to produce more compounds than female. Results showed that 22,23,23,28 compounds were detected from male reared on canola, oats, wheat and barley respectively. Our data is incosistent with Kinn et al. (1994) study where they found that females of *Dendroctonus frontalis* were heavier, had more lipid. Where Kinn et al (1994) confirmed that lipids content between genders varied based on their activity such as flying. Beetles that tend to fly have more lipids compared with others lowest lipids content (Perez-Mendoza et al., 1999).

Our data showed that chemical compounds identified from female and male were qualitatively similar, while showing appreciable quantitative differences between them. Previous studies marked that females and males, had similar chemicals components but in different proportions (Nelson et al., 2003). In addition, insect lipid allocation was varied between female and male and that agreed with the results that collected by Lease and Blair (2011). Furthermore, the chemicals components profiles especially hydrocarbon of male and female *Bagrada hilaris* were qualitatively equal but marked sex-specific quantitative differences were observed for some of the linear alkanes (De Pasquale et al., 2007).

As hydrocarbons used in many previous studies as a reliable chemotaxonomically tool for classification of insect species (Barroso et al., 2014; Kather and Martin, 2012) therefore we propose that the results of chemical compounds that identified in this study especially hydrocarbons might be useful as a taxonomy tool between *T. variabile* and other species like *T. granarium*, however, no data is available for comparison because of unavailability of *T. granarium* live culture in Australia.

The identification of the insect's species according to their hydrocarbon composition demonstrates that this is a highly reliable tool in insect taxonomy and play an important role in chemotaxonomy (Kaib et al., 1991; Nowbahari et al., 1990). The lipids considered a successful diagnostic tool for the identification of insect, especially hydrocarbons which are biochemical characteristics and chemotaxonomic tools for identification of insects (Lockey,1988; Braga et al, 2013; Yi et al., 2013; Pradesh, 2011). Soares eta al. (2017) investigated that some compounds were identified in three species of Mischocyttarus (Hymenoptera: Vespidae) *Mischocyttarus consimilis*, *M. bertonii*, and *M. latior* and these compounds include heneicosane, docosane, pentacosane, octacosane, hexacosane, 2-methylhexacosane, 2-methyloctacosane. The compounds of henicosane, oleic acid, docosane, tricosane, tetracosane, pentacosane, hexacosane, octacosane, 2-methylhexacosane, 13-methylheptacosane and nonacosane were reported in *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (Alnajim et al., 2019). Oleic acid was also identfied to be the primary fatty acid in the larvae of Oryctes rhinoceros, *Imbrasia belina*, and *Rhynchophorus phoenicis* (Ekpo et al., 2009; Raksakantong et al., 2010).

Table 3-1. Compounds peak areas (10^5) detected from *T. variabile* male and female reared on canola, oats, wheat, and barley

compounds	feature ID	RI NIST	RI	Canola		Oats		Wheat		Barley	
				Female	Male	Female	Male	Female	Male	Female	Male
1-butanamine, N-butyl-	7.59_129.15	1015	948.6	51.86±20.49	50.32±1.16	77.68±14.93	55.12±13.99	37.00±7.97	43.03±5.32	292.29±0.00	50.11±6.56*
nonanal	15.72_142.13	1104	117.7	n.d.	n.d.	4.46±0.93	n.d.*	n.d.	n.d.	2.37±0.23	1.87±0.12*
decanal	19.58_156.15	1204	1164.2	4.92±0.61	1.98±1.13*	3.49±0.45	n.d.*	n.d.	n.d.	n.d.	n.d.
1,2-benzisothiazole	20.21_135.01	1208	1200.4	2.04±0.73	6.41±1.19*	2.50±0.24	4.35±1.14	n.d.	n.d.	n.d.	n.d.
2-decenal, (E)-	21.53_154.25	1212	1202.1	n.d.	n.d.	n.d.	4.46±0.61*	n.d.	n.d.	7.98±0.15	0.77±0.20*
2-undecenal	24.42_168.15	1311	1325.8	n.d.	n.d.	n.d.	2.50±0.07*	n.d.	n.d.	n.d.	n.d.
dodecanal	25.77_184.18	1402	1408.8	n.d.	n.d.	n.d.	1.91±0.35*	n.d.	n.d.	n.d.	n.d.
caryophyllene	25.95_204.18	1494	1489.7	n.d.	n.d.	1.86±0.58	n.d.*	3.34±0.61	2.32±0.80	2.30±0.59	2.00±0.87
hexadecane	30.38_226.26	1612	1560.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.24±0.16*
tetradecanal	30.67_212.21	1601	1601.3	4.52±0.62	2.72±1.17	n.d.	n.d.	4.33±1.00	3.03±0.21	n.d.	n.d.
decanoic acid, hexyl ester	31.27_256.24	1779	1629.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.60±0.28*
2-hexadecanol	32.11_242.26	1774	1704	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.72±0.06*
heptadecane	32.65_240.28	1711	1669.4	n.d.	1.35±0.11*	n.d.	n.d.	n.d.	n.d.	n.d.	2.27±0.18*
tetradecanoic acid	34.05_282.20	1769	1778.8	n.d.	n.d.	17.89±11.38	4.80±0.25	6.97±0.69	2.21±0.02*	4.72±1.02	2.55±1.25
methoxyacetic acid, 2-tridecyl ester	34.69_272.23	1791	1780.3	n.d.	2.17±0.56*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1-decanol, 2-hexyl-	34.85_242.26	1790	1854.1	2.67±0.97	n.d.*	1.98±1.15	5.16±0.56*	5.98±1.72	4.32±0.65	n.d.	4.04±0.28*
pentadecanoic acid	36.16_242.22	1869	1890.3	n.d.	n.d.	36.19±6.10	11.19±4.27*	4.58±1.45	4.18±0.31	9.27±0.69	1.22±0.06*
n-hexadecanoic acid	38.24_256.24	1968	2012.3	27.6±5.53	32.77±5.96	136.64±17.45	123.27±28.93	118.54±13.51	91.26±4.32*	130.46±27.33	73.33±2.42*
nonadecanoic acid	40.14_188.22	2266	2209.9	n.d.	n.d.	n.d.	n.d.	5.20±2.49	25.31±3.82*	1.74±0.25	4.98±4.40
oleic acid	41.58_282.25	2175	2171.9	5.20±0.76	34.38±1.65*	273.15±11.20	21.40±7.94*	57.02±3.32	41.62±0.54*	44.31±3.00	28.04±5.68*
docosane	44.07_310.35	2228	2230.2	57.2±7.49	89.62±9.38*	27.49±2.31	138.22±1.31*	183.01±32.86	226.47±9.73	65.55±9.92	181.15±7.82*

heptadecane, 9-hexyl-	44.76_324.37	2413	2308	69.3±8.18	62.54±2.55	37.93±8.43	180.55±14.86*	158.62±22.55	130.85±16.11	135.41±17.92	214.12±3.40*
tricosane, 2-methyl-	45.35_338.39	2343	2398.7	n.d.	n.d.	n.d.	n.d.	28.05±2.19	6.60±3.73*	26.77±3.41	18.58±4.25
tetracosane	45.77_338.39	2407	2412.6	10.6±0.66	20.46±3.11*	28.20±2.21	19.01±2.75*	31.24±5.77	27.27±2.75	33.39±1.98	38.64±1.26*
heptadecane, 9-octyl-	46.22_352.40	2442	2449.9	20.4±3.54	22.83±0.64	10.36±6.20	56.43±7.38*	37.71±10.26	52.16±8.11	30.41±1.67	89.77±4.93*
pentacosane	47.17_352.40	2506	2501.6	17.2±4.84	103.99±2.68*	n.d.	165.10±26.26*	44.56±12.62	187.61±10.4*	41.52±5.36	209.31±9.82*
11-methylpentacosane	47.85_366.42	2542	2533.7	72.5±10.6	585.63±13.16*	59.56±8.55	5.71±0.17*	222.97±8.95	1353.±28.4*	171.41±7.45	931.22±15.7*
2-methylhexacosane	48.42_380.43	2641	2566.3	51.9±11.8	9.46±2.90*	13.58±4.41	20.37±12.44	154.95±22.33	39.15±12.5*	118.15±16.60	25.11±2.40*
hexacosane	48.98_366.42	2606	2610.4	427.±26.4	48.71±21.85*	282.85±18.33	108.90±3.54*	745.58±22.50	83.89±6.87*	597.08±15.63	117.95±11.6*
heptacosane	50.16_380.43	2705	2666	179.±7.28	130.61±11.37*	94.72±1.53	119.56±6.89*	439.06±25.03	661.69±5.26*	329.46±25.75	343.69±17.7
docosane, 1-iodo-	50.25_436.25	2622	2611.5	n.d.	217.95±11.37*	112.64±34.64	132.84±9.18	412.46±20.80	127.75±2.26*	172.70±12.53	272.96±15.8*
13-methylheptacosane	50.67_394.45	2740	2692.5	147.±27.9	76.67±11.44*	9.00±0.55	540.74±14.85*	385.43±9.02	351.7±18.3*	170.53±20.57	235.55±11.52*
octacosane	51.13_394.45	2840	2718.6	54.20±14.44	57.21±8.73	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2-methyloctacosane	51.31_408.46	2840	2723.6	76.1±10.6	54.55±10.13	47.45±6.47	97.60±14.70*	177.90±19.17	132.77±14.65*	195.80±18.67	77.75±15.4*
nonacosane	52.91_408.46	2904	2846.1	123.±10.7	78.90±2.06*	42.01±5.31	364.20±4.86*	249.27±14.01	155.79±8.16*	198.61±3.39	43.67±13.2*

*Significant different between male and female in each host grain. Feature ID includes retention time (min) and m/z ratio; RI NIST is retention index from National Institute of Standards and Technology database (NIST); RI is retention index calculated by running n-alkane standard C7-C40; n.d is not detected.

3.4.2. Effect of host grains (canola, oats, wheat and barley) on the compound production

The PCAs showed the effect of host type on quality and quantity of the chemical compounds (Figures 3-1a and b and 3-2a and b). The PCs in score plot describe the differentiation among the host grain (Figures 3-1a and 3-2a). According to the graph, the separation was obvious among all the diet types. However, the most intensive differentiation in female and male samples was between oats and other grain types. The loading plots in Figures 3-1b and 3-2b show the most important compounds that significantly participated in the differentiation among the diet types. Results showing that docosane, 1-iodo-; 1-butanamine, N-butyl-; oleic acid; heptacosane; 13-methylheptacosane; hexacosane; nonacosane; 2-methyloctacosane; n-hexadecanoic acid and docosane in the female samples (Figure 3-1b). While 11-methylpentacosane; 13-methylheptacosane; heptacosane; docosane, 1-iodo- and nonacosane were the most significant compounds that identified form *T. variabile* male (Figure 3-2b). The results showed that insect host grains have a significant effect on the chemical compounds such as fatty acid and hydrocarbons. The number of extracted compounds from different host grains varied, whereas barley produced the highest compound number compared to the other host grains. In addition, the host grains influenced the peak area of some compounds.

Results in Table 3-2 showed the number of compounds detected from female and male reread on different diets. In female, results showed that there were 15 compounds detected in all kind of host grains from both genders 1-butanamine, N-butyl-; n-hexadecanoic acid; oleic acid; docosane; heptadecane, 9-hexyl-; tetracosane; heptadecane, 9-octyl-;11-methylpentacosane; 2-methylhexacosane; hexacosane; heptacosane; docosane, 1-iodo-; 13-methylheptacosane; 2-methyloctacosane and nonacosane. Results also showed that octacosane and methoxyacetic acid, 2-tridecyl ester were identified from the *T. variabile* reared on canola compared with two compounds detected from oats that include 2-undecenal and dodecanal. Furthermore, there were four compounds identified from *T. variabile* reared on barley such as nonanal; hexadecane; decanoic acid, hexyl ester; 2-hexadecanol.

However, three compounds were detected in canola which is not detected in other grains, such as decanal; methoxyacetic acid, 2-tridecyl ester and octacosane while two detected in oats, for example, 2-2-undecenal and dodecanal. In case of barely, our results in Table 3-2 showed that

three compounds were detected in barley including Hexadecane; decanoic acid, hexyl ester and 2-hexadecanol.

Our findings consistent with the data collected in previous studies that showed the significant effect of different host grains on the lipids content of *T. garanarium* larvae (Mohammadzadeh and Hamzeh, 2018). Also, our results agreed with other previous studies where the extracted lipids of insects strongly affected by their vary host grains (Paul et al., 2016; Xin et al., 2018). The diet of insects is mainly responsible for the variations in the lipids and fatty acids (FAs) composition of the insects (Barroso et al., 2014; Henry et al., 2015). Other studies showed that diet appears to be another factor that influences the fat content of insects. A comparison of the fat content of the wild orthopteran *Heteracris littoralis*, at 8.2%, with captive-bred orthopterans (*Acheta domestica*, *Gryllus assimilis* and *Locusta migratoria*), with a higher proportion of fat, suggests that diet could affect lipid content (Barroso et al., 2014; Paul et al., 2016). The data obtained in this experiment agree with (Justi et. al. 2003) who showed that fatty acids content of insects is more dependent on diet. Other studies showed that different diet can lead to differences in lipids profile in some species (Etges et al., 2014; Liang and Silverman, 2000).

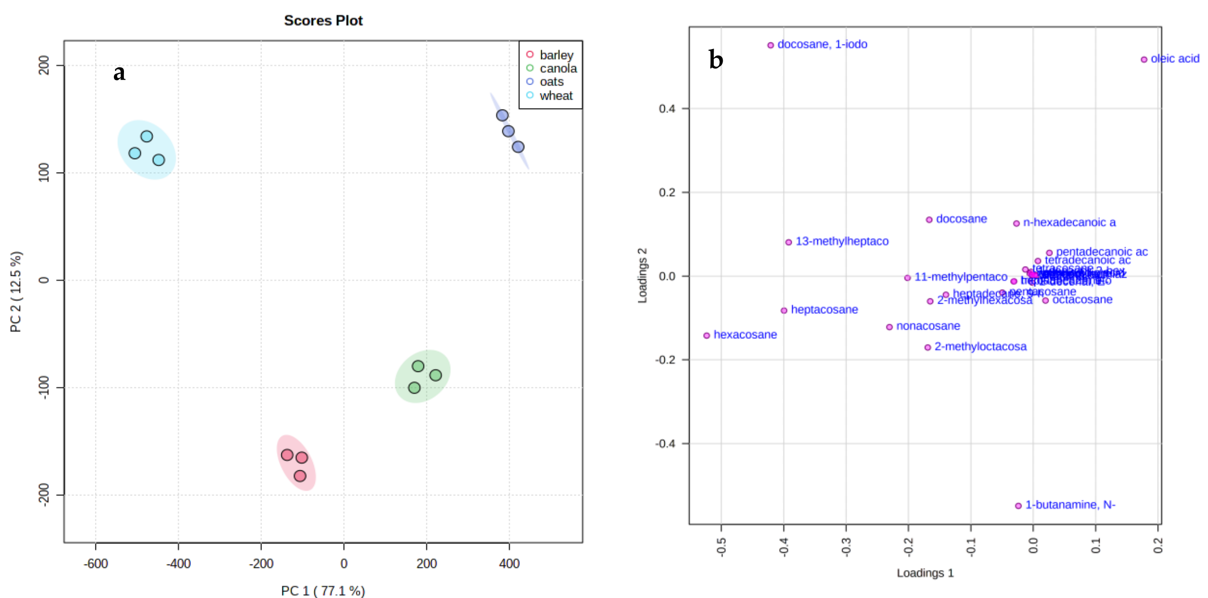


Figure 3-1a and b. a. Score plot of principle components analysis (PCA) for chemical compounds obtained from *T. variabile* female reared on different host grains (canola, oats, wheat and barley), b. loading plot shows the most significant compounds that participated in the differentiation.

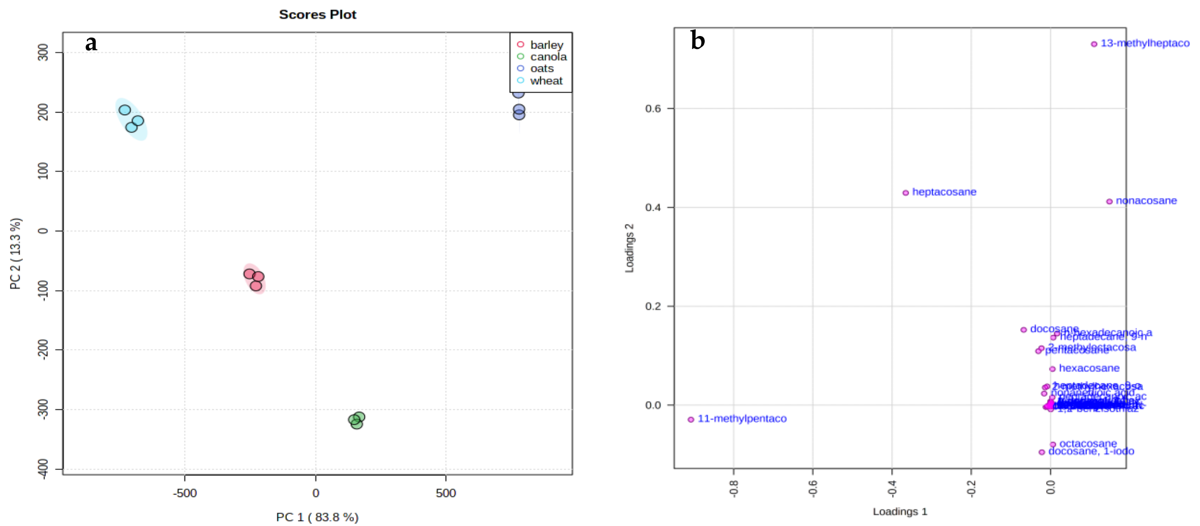


Figure 3-2a and b. a. Score plot of principle components analysis (PCA) for chemical compounds obtained from *T. variabile* male reared on different host grains (canola, oats, wheat and barley), b. loading plot shows the most significant compounds that participated in the differentiation.

Table 3-2. Compounds that were detected and not detected from *T. variabile* female and male reared on canola, oats, wheat and barley.

	Female				Male			
Chemical compounds	Canola	Oats	Wheat	Barley	Canola	Oats	Wheat	Barley
1-butanamine, N-butyl-	+	+	+	+	+	+	+	+
Nonanal	-	+	-	+	-	-	-	+
Decanal	+	+	-	-	+	-	-	-
1,2-benzisothiazole	+	+	-	-	+	+	-	-
2-decenal, (E)-	-	-	-	+	-	+	-	+
2-undecenal	-	-	-	-	-	+	-	-
Dodecanal	-	-	-	-	-	+	-	-
Caryophyllene	-	+	+	+	-	-	+	+
Hexadecane	-	-	-	-	-	-	-	+
Tetradecanal	+	-	+	-	+	-	+	-
decanoic acid, hexyl ester	-	-	-	-	-	-	-	+
2-hexadecanol	-	-	-	-	-	-	-	+
Heptadecane	-	-	-	-	+	-	-	+
tetrdecanoic acid	-	+	+	+	-	+	+	-
methoxyacetic acid, 2-tridecyl ester	-	-	-	-	+	-	-	-
1-decanol, 2-hexyl-	+	+	+	-	-	+	+	+
pentadecanoic acid	-	+	+	+	-	+	+	+
n-hexadecanoic acid	+	+	+	+	+	+	+	+
nonadecanoic acid	-	-	+	+	-	-	+	+
oleic acid	+	+	+	+	+	+	+	+
Docosane	+	+	+	+	+	+	+	+
heptadecane, 9-hexyl-	+	+	+	+	+	+	+	+
tricosane, 2-methyl-	-	-	+	+	-	-	+	+
Tetracosane	+	+	+	+	+	+	+	
heptadecane, 9-octyl-	+	+	+	+	+	+	+	+
Pentacosane	+	-	+	+	+	+	+	+
11-methylpentacosane	+	+	+	+	+	+	+	+
2-methylhexacosane	+	+	+	+	+	+	+	+
Hexacosane	+	+	+	+	+	+	+	+
Heptacosane	+	+	+	+	+	+	+	+
docosane, 1-iodo-	-	+	+	+	+	+	+	+
13-methylheptacosane	+	+	+	+	+	+	+	-
Octacosane	+	-	-	-	+	-	-	-
2-methyloctacosane	+	+	+	+	+	+	+	+
Nonacosane	+	+	+	+	+	+	+	+

\+ detected compounds; - not detected compounds

3.5. Conclusions

In this study, identified chemicals were used to study *T. variabile* adult's metabolism. As hypothesized, there should be difference in metabolites based on the gender of *T. variabile* and the commodity the insects were reared upon. This difference can be used as developing future diagnostic methods. The result from this study support this hypothesis. SPME coupled with GC-MS could be performed successfully to identify lipids such as fatty acid and hydrocarbons from *T. variabile* male and female. Also, results showed that there was a significant difference between adults fed on four different host grain. Thus, the chemical hydrocarbons could be used for comparison as taxonomic tool to identify different *T. variabile* adults including female and male from other *Trogoderma sp*.

Statement of Contribution

Title of Paper	Identification and diagnosis of whole body and fragments of *Trogoderma granarium* and *Trogoderma variabile* using visible near infrared hyperspectral imaging technique coupled with deep learning
Publication Status	Published
Publication Details	Computers and Electronics in Agriculture journal 173(2020)
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Chapter Four

Identification and diagnosis of whole body and fragments of *Trogoderma granarium* and *Trogoderma variabile* using visible near infrared hyperspectral imaging technique coupled with deep learning

4.1. Abstract

The khapra beetle, *Trogoderma granarium* Everts, is the most critical biosecurity pest threat which threatens the grains industry worldwide. To prevent incursion of the khapra beetle, very accurate and reliable diagnostic tools are required to differentiate the khapra beetle from other morphologically, closely related *Trogoderma sp.*, in particular the larvae stage. However, at present, it can only be identified by highly skilled taxonomists. Furthermore, often suspected *Trogoderma sp.* found in grain products are the body fractions such as larval skins or fragmented adult, which are impossible to diagnose morphologically. This work explored the combination of visible near infrared hyperspectroscopy (VNIH) and deep learning tools to identify the khapra beetle. About 2000 hyperspectral images were acquired under this study. Images of *T. granarium* and *Trogoderma variabile*, adult, larvae, larvae skin, fragments of adult and larvae images, were subjected to two deep learning models; Convolutional Neural Networks (CNN) and Capsule Network for analysis. Overall, above 90% accuracy was obtained with both models, whereas Capsule Network achieved a higher accuracy of 96%. For whole adult body and adult fragments, the accuracy achieved was 96.2% and 91.7%, respectively. For whole larvae, larvae skin and larvae fragment, accuracies of 93.4%, 91.6%, and 90.3% were achieved. Ventral orientation gave better accuracy over dorsal orientation of the insects for both larvae and adult stages. Based on the above results, VNIH imaging technology coupled with appropriate machine learning tools can be used to identify one of the most notorious stored grain pests, the khapra beetle, from other morphologically similar *Trogoderma sp.* like *T. variabile*. Particularly, the technology offers a new approach and possibility of an effective identification of *Trogoderma sp.* from its body fragments and larvae skins, which are otherwise impossible to diagnose taxonomically.

4.2. Introduction

The Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) is the most critical biosecurity pest threat worldwide. It remains as the number one most dangerous stored grain pest and is devastating if it is accidentally introduced to any country. Once this pest establishes in a country, the export restrictions have to be applied. This pest not only damages stored food commodity, but also makes it unfit for consumption because of contaminants like larvae exuviae, skin, dead bodies or their fragments. The exuviae is also known to cause irritation of mucous membranes in the respiratory tract amongst people working in warehouses

and on-board ships (Maliński et al., 1986). *T. granarium* may also result in contamination by *Aspergillus flavus* (Sinha and Sinha, 1990). With the increase of exportation, interception numbers of the khapra beetle have increased world-wide; including Australia, United States and other non khapra countries. In Australia, interceptions of the khapra beetle have been reported in 2007 and in 2016 (Day and White, 2016). In USA, initially, the number of interceptions were about 11 between 2007 and 2009, but rose to 100 in 2011 (Customs, 2011). Between 1957 and 1973, 46 to 131 interceptions were detected every year in commodities entering England (Day and White, 2016). Eradication is very costly. For example, an interception in Western Australia in 2007 alone cost AUD 207,685 for its eradication (Day and White, 2016). The introduction of *T. granarium* to the countries which do not have khapra beetle would be economically disastrous, and hence accurate confirmation of the species is exceedingly critical. There are over 134 described *Trogoderma* species worldwide (Háva, 2012) and, many yet to be discovered. Apart from the khapra beetle, there is the less significant pest, the warehouse beetle, *Trogoderma variabile* Ballion, which has already established in Australia and many other countries. To diagnose the khapra beetle from other morphologically similar *Trogoderma* species, highly experienced personnel with exceptional skills in dissection of small insects are required. This becomes more difficult when border security inspectors usually get incomplete specimens with missing body parts and without morphological features, thus making it impossible to identify morphologically (ISPM, 2012).

Identification through eggs and pupae is still not possible as they possess very few external features. Hence, larvae and adults are the only stages that are used for identification purposes. Skilled personnel can identify adults by dissecting out the genitalia and examining them under the stereomicroscope, which is a cumbersome and time-consuming procedure. For larval identification, mouthparts need to be dissected by trained personnel and observed under 400× to 800× magnification for satisfactory identification (ISPM, 2012) However, most of the time, adults received in cargo are broken into pieces or are brittle and have a high chance of fragmenting when handled. Sometimes the body fragments don't even have the required diagnostic parts, which makes the taxonomic identification procedure impossible. Identification methods have also been developed using immunological and molecular techniques but are still not reliable enough to be used as quarantine diagnostic techniques (ISPM, 2012).

Given these drawbacks, an alternative technique is to use a hyperspectral imaging system. Hyperspectral images are high-resolution images which are used by experts in geolocation identification, plant species identification, and identifying pest damages on plants (Camps-Valls et al., 2014; Singh et al., 2010; Cao et al., 2015). As hyperspectral imaging combines the properties of imaging and spectroscopy, it can attain both spatial and spectral information from an object, making it more sensitive and reliable. The hyperspectral image acquired consists of hundreds of continuous wavelengths for each spatial position of the target object. Consequently, each pixel contains an individual spectrum, enhancing the sensitivity of the image over a normal RGB image. The technique can more sensitively and simultaneously measure multiple parameters, including internal structure characteristics, morphological information, and chemical composition in comparison to a single machine vision technology or spectroscopy analysis technology (Gowen et al., 2007). In the past, the technique has been used for inspection of fruit quality and ripeness (Lu, 2003; Polder et al., 2002). It has also been used in the identification of internal infestations and quality in wheat kernels (Ridgway and Chambers, 1998; Singh et al., 2009). Moisture and oil content in corn kernels have been studied using near infrared hyperspectral imaging as well (Cogdill et al., 2004).

Once the hyperspectral images are captured, for accurate analysis, it is required to establish reliable machine learning models (Krizhevsky et al., 2012). Machine learning approaches have been used on hyperspectral images with promising results (Ebrahimi et al., 2017). Recently, in the area of machine learning, deep learning models have become growingly popular in many applications. In this paper, the Convolutional Neural Networks (CNN) and Capsule Network were investigated for hyperspectral pest image classification. CNN has the capability of handling non-linear classification (LeCun et al., 1989). Capsule Network uses the concepts of a combination of nested capsules (layers) of neurons to perform classification. The lower levels of capsules are capable of learning the sections of an image separately as a capsule and pass the prediction to a higher-level capsule, which would make an overall decision for the set of capsule predictions. The model would learn section by section, allowing the learning model to learn sections faster than other models (Sabour et al., 2017). The high definition of hyperspectral images would produce an in-depth image representation. The in-depth representation has a potential to support the Capsule Network's learning capability.

Thus, the objective of this paper is to address biosecurity surveillance and identification gaps for the khapra beetle by establishing proof of concept and an effective hyperspectral pest image classification system based on deep learning algorithms which can be used in the future by biosecurity personnel to accurately and timely identify exotic khapra beetle from other *Trogoderma sp.* that is *T. variabile.*

4.3. Materials and Methods

4.3.1. Insects

Stock culture of *T. variabile* was reared on non-fumigated canola whole seeds. Initial culture was taxonomically confirmed from Department of Primary Industries and Regional Development (DIPRD), South Perth, Western Australia. Canola seeds were disinfested by keeping at -20 °C for one week and then thawing at room temperature for 24 h before use. Once the canola seeds reached room temperature, about 800 g of seeds were added to the insect culture box with mixed age larvae and then covered with a wet paper towel to maintain the moisture. After that, the boxes were closed with meshed lids and kept in culture room with temperature and relative humidity at 30°C and 60%, respectively. The frequency of change of culture was done once in 3 months. Using same way, *T. variabile* was also reared on wheat, barley, rice, maize, and oats. Dead Specimens of *T. granarium* adults both males and females, larvae (mixed instars) and larvae skins in ethanol were procured from Spain, Greece, and Pakistan. All the specimens were provided to us by taxonomist in DPIRD after taxonomic confirmation.

For body fragments, adults and larvae specimens were damaged by cutting adults down the dorsal midline or by removing appendages or mouth part from the specimen. For larvae, the body was cut into the head or upper region, and the lower or tail region.

4.3.2. Hyperspectral imaging system

Visible near infrared hyperspectral imaging system at State Agricultural Biotechnology Centre (SABC), Murdoch University was used. The system consists of spectral imager (Applied

Science Imaging, Model: CCD-1300DS, Germany) with two light sources to illuminate the samples: 1) a 150 W quartz halogen illuminator, and 2) a Light Emitting Diode (LED) bulb (Philips 470 lm-D65). Since the current spectral imager can cover spectral range only up to 800 nm, so halogen lamp with a higher spectral power was used to provide an extended wavelength range from 800 to 1000 nm. The imager was hooked up to a microscope with 10 times magnification. Spectra Cube was used as the spectral imaging acquisition system.

4.3.3. Spectral sample preparation

Insects were killed by immersing in 100% ethanol for 24 h and then transferring the dead ones on Whatman™ filter paper 1 to soak up all the ethanol. Once all the ethanol has dried up, the insects were transferred on to their respective slides for imaging. Glass slides with the black background made by permanent marker ink was used as the image background color to avoid overexposure.

4.3.4. Image acquisition

About 2000 images were acquired (Table 4-1), comprising *T. granarium* from Spain (colony material), Greece, Pakistan and Germany (colony material) and *T. variabile*, cultured on rice, wheat, canola, barley, maize and oats, along with wild *T. variabile* which were collected and identified through National Trogoderma trapping program by the DPIRD in Western Australia. The images were acquired for adults, larvae, larvae skin, fragmented adult and larvae, and for both dorsal and ventral orientation.

To acquire the image, individual insect was kept on black background glass slide with brush and imaged under the image analyzer through 10× magnification. Image capture parameters used were 256 frames and 45 steps to get the best resolution image between 400 and 1000 nm. Exposure time used were 24 and 26 ms^{-1} for the dorsal and ventral position, respectively. The parameters were kept consistent for all the images. The resulting hyperspectral images was a special block of 704 × 1248 × 80 reflectance image, representing a 3-D image with X-axis and Y-axis coordinate information and the other representing the spectral information at 80 different wavelengths after ten spectral binning operations. This information was stored for subsequent analyses.

Table 4-1. Number of images acquired for each insect species, including specimen type and orientation.

Insect species	Specimen type	Geographical location and commodity	Number of images	
			Ventral	Dorsal
TG	Whole Adults	Germany	29	29
TG	Whole Adults	Pakistan	17	17
TG	Whole Adults	Spain	29	29
TV	Whole Adults	Canola	59	59
TG	Whole Larvae	Germany	55	55
TG	Whole Larvae	Pakistan	20	20
TG	Whole Larvae	Spain	63	63
TV	Whole Larvae	Barley	59	59
TV	Whole Larvae	Canola	60	60
TV	Whole Larvae	Oats	58	58
TV	Whole Larvae	Rice	56	56
TV	Whole Larvae	Wheat	60	60
TG	Larvae Skin	Germany	28	28
TG	Larvae Skin	Pakistan	18	18
TG	Larvae Skin	Spain	21	21
TV	Larvae Skin	Barley	60	60
TV	Larvae Skin	Wheat	30	30
TV	Larvae Skin	Canola	69	69
TG	Fragmented adults	Greece	27	27
TV	Fragmented adults	Wild	20	10
TV	Fragmented adults	Canola	9	9
TG	Fragmented adults	Spain	25	25
TV	Fragmented adults	Barley	28	28
TG	Fragmented larvae	Spain	34	34
TV	Fragmented	Canola	26	26

TG stands for *T. granarium* and TV stands for *T. variabile*.

4.3.5. Deep learning model for pest classification

CNN based approach and Capsule Network based approach were used. The data set was randomly divided into training dataset (80% of the images) and testing dataset (20% of the images).

4.3.5.1. Dataset description

The available hyperspectral pest images are mainly divided into *T. granarium* and *T. variabile*. The whole hyperspectral image including the background was used by CNN and Capsule Network based approach.

4.3.5.2. Convolutional Neural Network (CNN)

CNN was initially developed for identifying hand-written zip code recognition. This application showed that CNN is capable of learning the features of the whole image well when properly trained. The images are passed on to the CNN pre-processing. The images are rescaled to 1/255, and the images are zoomed to 0.2. In order to generalize the CNN, the images were randomly flipped (augmented).

The CNN has 5 convolutional blocks and the final layer was a fully connected dense layer to classify the images (Figure 4-1). The CNN has the activation function relu with max pooling. The fully connected dense layer contains a relu activation function with a dropout rate of 0.5. Thousand epochs were used to train the CNN. CNN classifier was implemented in Python using Keras package. An overview of the procedure is given below.

The input image is converted into a vector,

$$\boldsymbol{ui = vector\ (input)}$$

Then the vector is transformed using affine function,

$$\widehat{\boldsymbol{U_{j|i}}} = \boldsymbol{W_{ij}\, u_i}$$

The weighting sum of the network is defined as,

$$\boldsymbol{s_j = \sum_i c_{ij}\ \widehat{u_{j|i}}}$$

while the nonlinear activation function is,

$$v_j = \frac{||s_j||^2}{1 + ||s_j||^2} \frac{s_j}{||s_j||}$$

and the final output is defined as:

output = vector (vj)

where: $u_i$is the image converted into the vector. W_{ij} is the weight for each vector value u_i. $c_{ij}$is the bias and *sj* being the sum of the network. v_j is the activation function generated from the s_j.

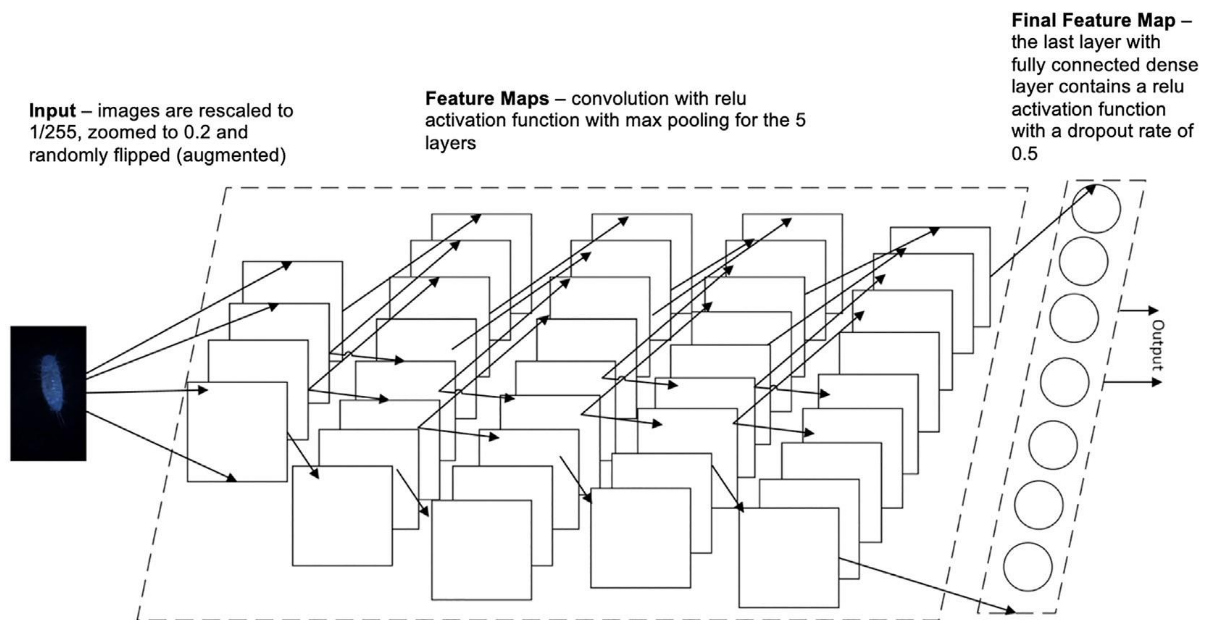

Figure 4-1. The CNN architecture used for the pest image classification. CNN contains 5 layers and the last layer consists a dense (fully connected) network.

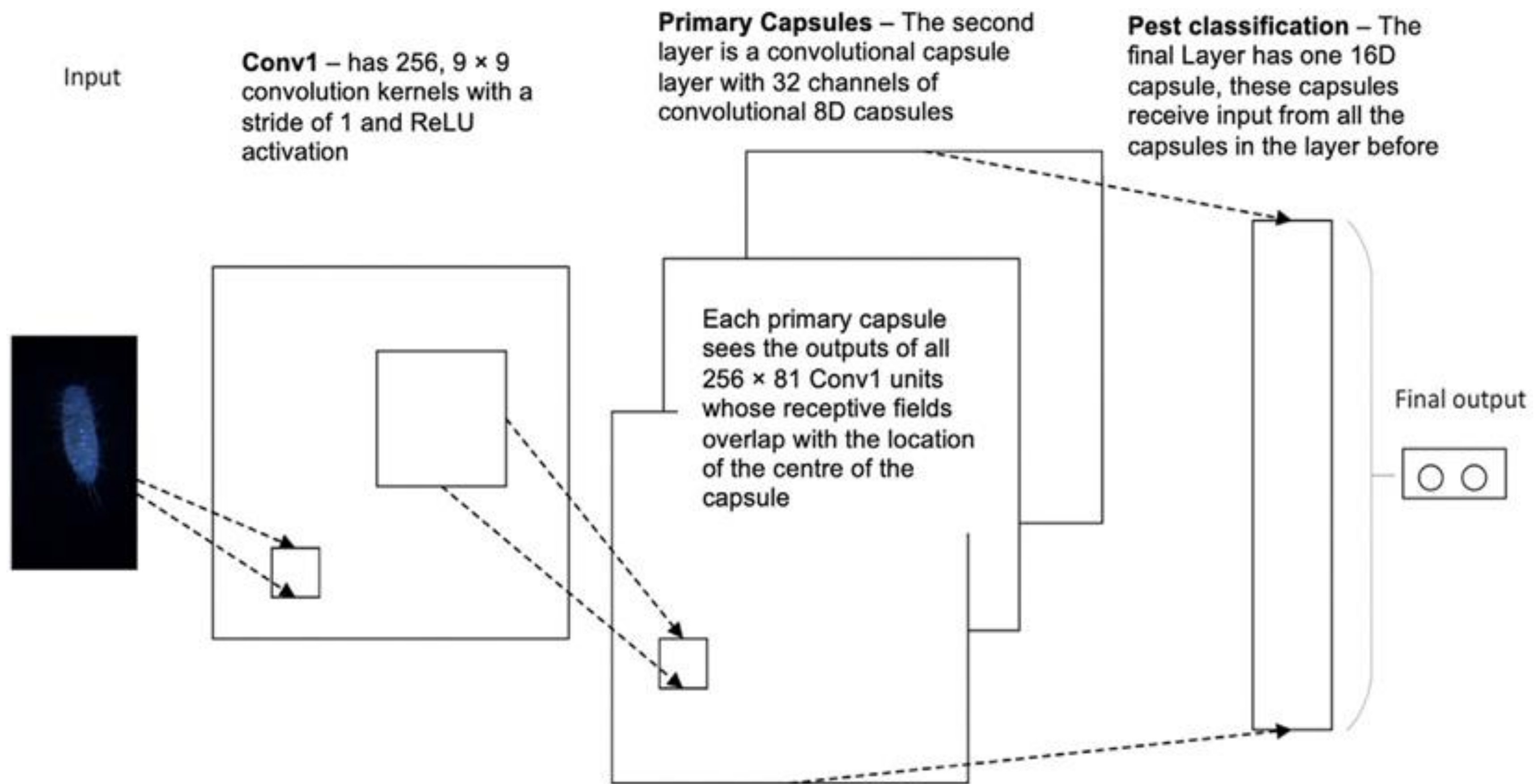

Figure 4-2. Schematic diagram showing Capsule Network, which has 2 convolutional layers and a fully connected layer to perform the pest classification.

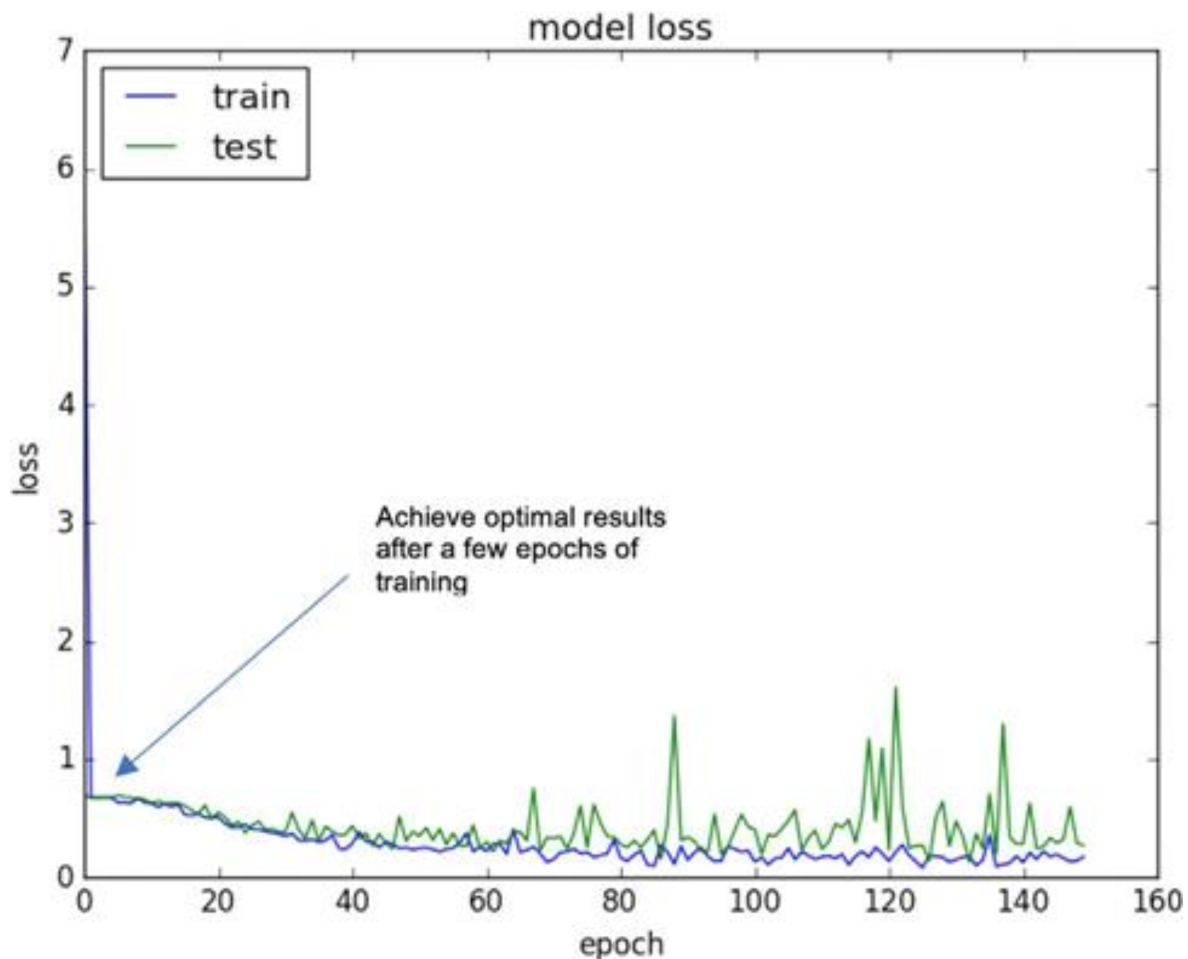

Figure 4-3. Capsule Networks loss plot for the training and testing datasets. The plot demonstrates the Capsule Networks adaptability to the dataset. The graph shows that the Capsule Network trains and achieve the optimal loss values within a few epochs of training.

4.3.5.3. Capsule Network

Capsules are a group of neurons selected to perform a particular task in order to combine as a unit to produce the final result (Sabour et al., 2017). Active capsules are at a level that makes predictions and passes results to the higher-level capsules. When multiple predictions agree, a higher level of capsule activates. These higher-level capsules yield the final results. Capsule Network has been shown to achieve the best results in MNIST dataset. Capsule Network has been applied to CIFAR10 dataset and shown to increase accuracy with an added final convolutional layer (Xi et al., 2017). Capsule Network considers images as capsules (sections)

and performs classification separately and integrates each capsule output to generate the final classification results (Figure 4-2). The Capsule Network is defined as follows. The input image is defined as;

$$x_i = scalar\ (input)$$

weighting sum is defined as,

$$aj = \sum_i w_i\ x_i + b$$

nonlinear activation function is, $h_j = f\ (a_j)$

Output is, output = scalar (h_j)

Where: x_i is the scaled value for the input, the w_i is the weight and b are the bias. a_j weighted sum. f is the non-linear activation function generating h_j. The final output is scales the h_j.

4.4. Result and discussion

4.4.1. Training of algorithms

Two deep learning models (Capsule Network and CNN) were investigated, and the Capsule Network performs slightly better than the CNN. Figure 4-3 shows the loss graph for the Capsule Network. The graph demonstrates that the Capsule Network can achieve the optimal loss values within a few epochs of training. It also shows that the loss value is very small, which clearly indicating that the Capsule Network is capable of adapting faster to the new images and generate accurate results when comparing to the CNN model. Figure 4-4 demonstrates the CNNs loss curve graph during training. Although the Capsule Network outperforms the CNN, Figure 4-4 indicates that the CNN is also able to be trained for the new dataset, in which generalisation can be observed. However, comparing the loss curve of CNN and Capsule Network (Figures 4-3 and 4-4), CNN tends to give slightly larger loss values and higher error rate when compared to Capsule Network. In CNN, the initial layers are used to learn the basic features of the objects, and the deeper layers are used to learn more complex features. All the learned features are then used to generate the final prediction. However, as

CNN does not retain much spatial information, it does not hold invariant information well, such as the position of the pest. CNN learns to identify khapra from warehouse beetle using the training set. If new images with different orientations of the pest were given to CNN, the accuracy of CNN could be affected. To fully utilise the benefits of a CNN, normally a large number of training image data is required. Capsule Network can alleviate this problem. Capsule Network creates an equivariance between capsules. The orientation of the features is generated and learned as capsules. The information was detected and passed from capsules to capsules at different layers. Each capsule detects sections of the image separately and integrates them into producing the final output classification. In this way, each capsule can be trained to identify individual features of the image. Given the limited number of training images in our study, the Capsule Network could outperform CNN and should be used as an alternative method.

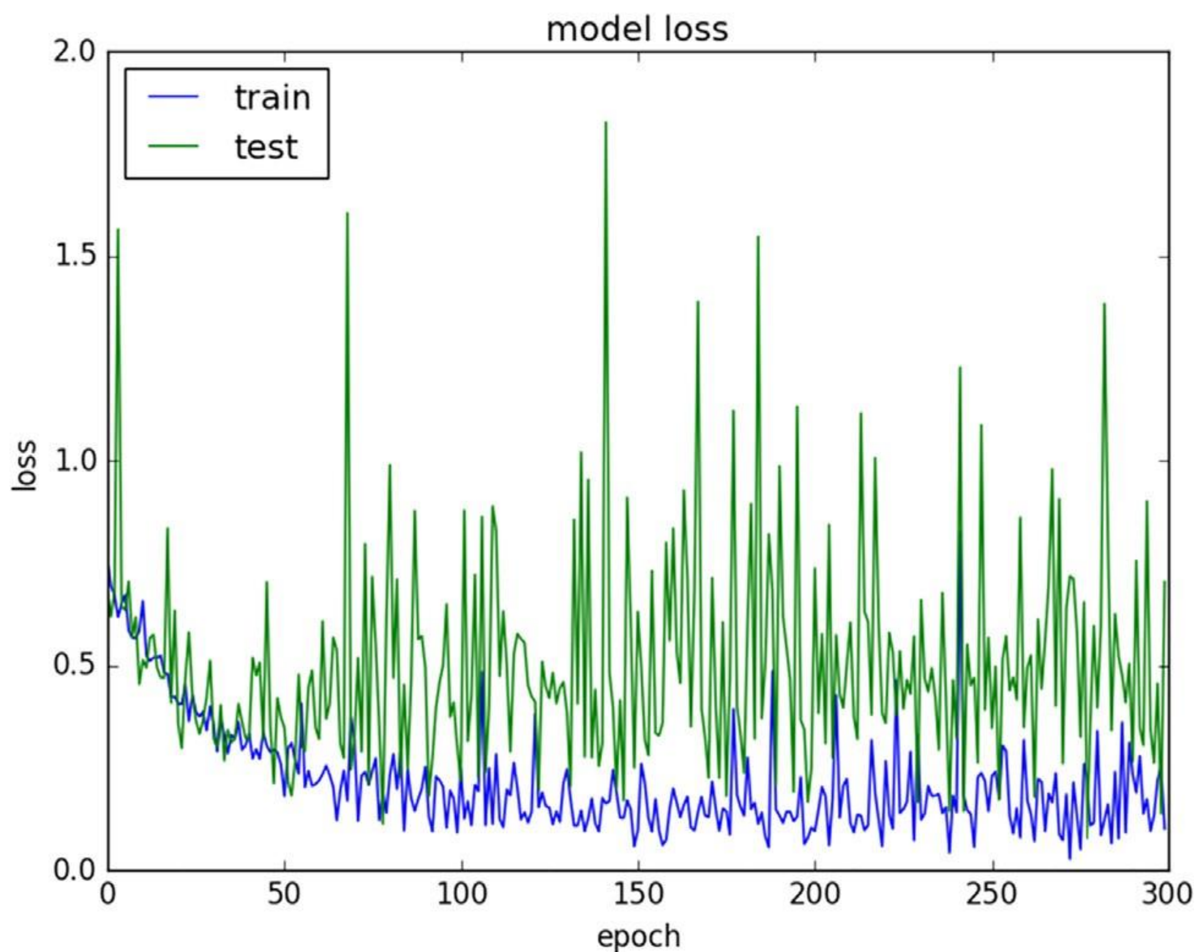

Figure 4-4. Loss graph for CNN for binary classification. The CNN's training and testing aligns with each other which indicates that the CNN has not over trained.

4.4.2. Optimisation of larvae and adult orientation

A binary classification was conducted between *T. granarium* and *T. variabile* for both larvae and adult stages using both dorsal and ventral orientations. As shown in Table 4-2, ventral orientation has better accuracy results as compare to dorsal orientation for both larvae and adults using both CNN and Capsule Network. In the case of larvae, the accuracy of 93.4% and 93.0% respectively for ventral and dorsal orientation was observed using Capsule Network and the accuracy of 89.8% and 88.7% respectively, was observed with CNN. In the case of adults, the difference in the accuracy between ventral and dorsal was more significant. Capsule Network gave an accuracy of 96.2% and 92.5%, respectively for ventral and dorsal orientation, while CNN gave an ac- curacy of 92.5% and 88.9% respectively for ventral and dorsal orientation. Based on the outcome, the best orientation to be used for subsequent experiments is ventral images for the larvae and adult fragments. Better accuracy with ventral orientation could be because of more discriminating features on the ventral side of larvae and adults, like denser setiferous punctures on setae of the adults (ISPM, 2012). Voss et al. (2017), have also found 89% accuracy with ventral orientation and 82% accuracy with dorsal orientation for two blowfly puparia species: *Calliphora dubia* Macquart 1855 and *Chrysomya rufifacies* Macquart 1842, using hyperspectral imaging (Voss et al., 2017). Based on our results, ventral orientation was applied as the most preferred orientation for developing the models for larvae skin, fragmented adults, and larvae.

Table 4-2. Binary Classification between *T. granarium* and *T. variabile* for identification accuracy using both CNN and Capsule Network for larvae (ventral and dorsal), adult (ventral and dorsal), larvae skin, fragments of adults and larvae.

Insect specimen	Insect body Tissues	Accuracy using CNN (%)	Accuracy using Capsule Network (%)
Larvae	Ventral	89.8	93.4
	Dorsal	88.7	93.0
Adult	Ventral	92.5	96.2
	Dorsal	88.9	92.5
Fragment	Larvae skin	88.2	91.6
	Adult	89.1	91.7
	Larvae	88.9	90.3

Table 4-3. Confusion metrics using CNN and Capsule Network for both larvae and adult with ventral and dorsal orientation

Insect stage	Deep learning tool	Species and orientation	Predicted TG	Predicted TV
Larvae	CNN	TG (ventral)	25	3
		TV (ventral)	6	52
		TG (dorsal)	24	4
		TV (dorsal)	6	52
	Capsule Network	TG (ventral)	26	2
		TV (ventral)	4	54
		TG (dorsal)	26	2
		TV (dorsal)	3	55
Adult	CNN	TG (ventral)	13	2
		TV (ventral)	0	12
		TG (dorsal)	13	2
		TV (dorsal)	1	11
	Capsule Network	TG (ventral)	14	1
		TV (ventral)	0	12
		TG (dorsal)	14	1
		TV (dorsal)	1	11

TG stands for *T. granarium* and TV stands for *T. variabile*

4.4.3. Classification using larvae and adults of *T. granarium* and *T. variabile*

As shown in Table 4-2, both CNN and Capsule Network have shown to achieve classification accuracy above 90% in discriminating *T. granarium* and *T. variabile* larvae. Table 4-3 depicts the confusion matrix using optimal CNN and Capsule Network for larvae. For this total 86 test insect images including 28 images of *T. granarium* larvae, and 58 images of *T. variabile* larvae were used. From these generated confusion matrices with CNN for *T. granarium*, out of 28 images, 25 were identified as *T. granarium*, and 3 and 4 were misidentified as *T. variabile* for ventral and dorsal orientation respectively. For confusion metrics generated using Capsule Network out of 28 images of *T. granarium*, 26 were identified correctly, and two were identified as false positives. Thus, both methods performed well. In contrast, Capsule Network gives 2 false negatives and CNN gives 3 and 4 larvae false positives for ventral and dorsal orientation respectively for *T. granarium*. Similarly, for *T. variabile* out of 58 images classified 52 were identified correctly, and 6 were misidentified for CNN, while 4 and 3 were misidentified in case of Capsule Network for ventral and dorsal orientation, respectively (Table 4-3). With adults, Capsule Network outperformed in terms of accuracy of 96.2% and 92.5% in comparison to 92.5% and 88.9% for CNN with ventral and dorsal orientation, respectively

(Table 4-2). Detailed recognition results using 27 test adult insect images, through confusion matrices (Table 4-3) shows that in case of identification of *T. granarium* using CNN for both ventral and dorsal orientation 2 *T. granarium* were false identified as *T. variabile* but in case of *T. variabile* 100% were identified as *T. variabile* using ventral orientation with no false positive. Using Capsule Network, out of 15 *T. granarium* only one *T. granarium* adult was identified as *T. variabile* (false negative) for both the orientation. Similar to CNN, in Capsule Network also, with ventral orientation no false positive *T. variabile* was identified (Table 4-3).

The identification of insect species using visible near infrared hyperspectral technique is based on the difference in reflectance and absorbance spectra in the region between 400 and 1000 nm. The surface of the insect body is covered with cuticle, which is further made up of many layers made up of lipids. Hydrocarbons constitute the main part of cuticular lipids (Maliński et al., 1986). The cuticular lipids give peaks corresponding to the C-H overtones in near infrared region (700–1100 nm) (Baker et al., 1999; Ridgway et al., 1999). This could be the reason of difference in the spectra of the hyperspectral images be- tween *T. granarium* and *T. variabile*. The reason how *T. granarium* can be identified from *T. variabile* with an accuracy of more than 96.2% is consistent with this hypothesis. As cuticle are well developed in adults, so from the result, it shows that identification accuracy with adults is far better than larvae. The results are also consistent with previous research in which adults of *Sitophilus oryzae* (Linnaeus) (rice weevil) and *S. zeamais* Motschulsky (maize weevil) were differentiated with more than 98% accuracy and puparia of two species of blowfly *Calliphora dubia* and *Chrysomya rufifacies* where distinguished with the ac- curacy of 92% using similar technology (Cao et al., 2015; Voss et al., 2017). Researchers in the past have used cuticular hydrocarbons in insect recognition system, as qualitative and quantitative composition of the insect cuticular hydrocarbon depending on the order, group, subgroup, species and even sex of the insects (Jackson, 1976).

4.4.4. Classification between larvae skins, fragments of adults and larvae of *T. granarium* and *T. variabile*

Since ventral orientation gave better accuracy for both larvae and adults, ventral orientation images of larvae skins, fragments of adult and larvae were used to find the accuracy between *T. granarium* and *T. variabile* using optimised CNN and Capsule Network. From the current model, more than 90% accuracy can be achieved for the body fragments and larvae skin by

using Capsule Network (Table 4-2). This again supports the hypothesis stated above, that cuticular hydrocarbon peaks corresponding to the C-H overtones in near infrared region (700–1100 nm) (Baker et al., 1999; Ridgway et al., 1999) could be the reason of difference in the spectra even for the body fragments or the larvae skin of *T. granarium* and *T. variabile*. The presence of larvae skins in commodities is a common indicator of infestation by either *T. granarium* or *T. variabile*, but with larvae skin it is not possible to differentiate the species taxonomically. When trading the commodities be- tween the countries, it is also common to get broken specimens and larvae skin. At the time of quarantine inspection, identification of in- sects especially khapra beetle becomes very difficult, as most of the time the identifying morphological features are lost, hence visible near infrared hyperspectral technology can be an effective diagnostic tool in that scenario.

4.5. Conclusion

This paper introduces hyperspectral imaging technique coupled with appropriate machine learning tools to identify one of the most notorious stored grain pest khapra beetle from other morphologically similar *Trogoderma sp.* that is *T. variabile*. This otherwise, can be identified only by experienced and trained personnel following detailed dissecting protocol on insect whole bodies. With respect to the whole insect body, the identification accuracies achieved by the hyperspectral imaging technique using Capsule Network was 96.2% and 93.4% for adults and larvae respectively. The ventral orientation of the insect body gave better accuracy over dorsal orientation. Additionally, this technique can effectively differentiate between the two species with accuracies of 91.6, 91.7 and 90.3% for larvae skin, adult fragments, and larvae fragments, respectively. The taxonomic identification of these two species becomes impossible if larvae skin or fragmented adult or larvae bodies are available. The technology thus offers a new approach and possibility of an effective identification of *Trogoderma sp.* from its body fragments and larvae skins, which are otherwise impossible to diagnose taxonomically.

For future work, we intend to further improve the accuracy of the algorithms by generating and collecting more image profiles of *T. granarium* and other *Trogoderma* species from different geographical locations. In addition, we will expand this to other desired stored grain species. The outcomes of these research will then help to incorporate the technology into actual quarantine practice.

Chapter Five

General Discussion

5.1. General discussion

Australian grains are globally regarded for their high quality and reliability, both as bulk commodity exports and as value-added products. It was valued at approximately $22.8 billion in 2013–14 (Sarina 2014). The total amount of wheat grain grown across Australia was 25 million tonnes per year (AEGIC 2015) and WA was the largest wheat grain exporter in Australia. The value of WA grain exports in 2014/2015 was worth over $ 5.1 billion with $3 billion of this value from wheat (Department of Agriculture and Food WA 2016). *Trogoderma spp.* has been identified as the most notorious stored grain insects throughout the world. Grains infested by these insects could be destroyed because of the massive populations of the insect which may develop. Furthermore, they cause the biggest economic losses and *T. granarium* has been recognized as a quarantine pest in some countries. Khapra beetle can affect on environment by destruction of grain products. Those pests have been reported infesting 119 different commodities. Many damages can be caused by *T. variabile* and *T. granarium*, such as loss of grain weight, reduce in quality, the presence of larvae, masses of cast skins, live or dead insects, and fine dust. The important of these two species comes from the close morphological features which can make identification uncertain. In order to achieve this aim, three experiment chapters were conducted. These experiments progressed for understanding (1) the optimal conditions to collect volatile organic compounds and then apply these optimal conditions to collect volatile organic compounds from different *T. variabile* life stages (Chapter two); (2) to understand *T. variabile* metabolism reared on different host grains such as canola, oats, wheat, and barley and to identify chemical hydrocarbons can be used as a chemo-taxonomical tool for insect species identification especially for very morphologically similar species (Chapter three); and (4) visible near infrared hyperspectral imaging coupled with deep learning can be used to identify one of the most world destructive pests, *T. granarium*, from other morphologically similar *Trogoderma sp*. like *T. variabile* (Chapter four).

Briefly, the major aims from this research were:

- Study the feasibility of the Solid phase microextraction (SPME) technique for identification of volatile organic compounds (VOCs), hydrocarbons and the insect metabolism.

- Develop new diagnostic tool for *Trogoderma granarium* and *Trogoderma variabile* identification using different life stages, body fragments and larvae skin based on visible near infrared hyperspectral imaging.

The aim of the second experimental chapter (Chapter 2), was to determine the best conditions for extracting VOCs from *T. variabile* different stages including larvae and adults. The SPME technique coupled to the GC FID/MS was found to be a robust, rapid and reliable method to analyse VOCs. These findings agree with Villaverde et al., (2007) who used an SPME fibre to extract VOCs from *Tribolium castaneum* (Herbst). Therefore, different parameters were optimized for the analysis of emitted VOCs to ensure maximum release of VOCs from the fibre without compromising the composition of VOCs released. Four different insect densities (15,20,25, and 30) were tested to analysis VOCs from two different stages including larvae and adults (male and female). Moreover, the results proved that different insect densities can affects the VOCs amount emitted from insects. This result showed that less densities of *T. variabile* gave more VOCs. This could be attributed to the overcrowding which might have caused a reduction in the metabolism of insects due to an increase in the CO_2 quantity which has a critical effect on the biological and physiological processes of insects (Guerenstein and Hildebrand, 2008; Nicolas and Sillans, 1989) Furthermore, Jelen et al. (2000) mentioned that the amount of sample has a significant effects on the amount of the extracted analyte. The second aspect in this chapter was to focus on the using different extraction time including (4h, 8h, 16h, and 24h) to provide the optimal parameters for determining VOCs from *T. variabile* larvae and adults (male and female). Previous studies showed that extraction time play a significant parameter in headspace solid phase microextraction. In this regard, other studies focused on the importance of extraction time, Senthilkumar et al. (2012) showed that it is an important step to determine extraction time using SPME fibre method. The amount of extracted volatile depends on the sampling method such as extraction time (Arnaud et al., 2002; Qazi et al., 1998). Finally, it would be appropriate to determine if the fibres used in the present study can be inserted inside silos and left for various periods of time, before being processed by HS-SPME together with GC/FID and GC/MS to accurately monitor VOCs produced by storage Insects. In addition, the fibres could be used to detect VOCs in other insects such as *T. castanium* and *R. dominica*. Diversity profiling and VOCs profiles are both potentially useful tools to confirm the presence of grain stored insects such as *T.varibaile* and *T. garanarim* at an early stage of contamination of grain during storage. Furthermore, *T. variabile* is of

considerable concern because it could mask the presence of the more damaging *T. granarium* because of the morphological similarity between these two species.

The aim of the third chapter was to study *T. variabile* adult's metabolism on different hosts, separate and identify *T. variabile* adults' hydrocarbons with possibility of using these compounds as taxonomic tool for this insect. To analyse *T. varibaile* adults lipids, DI-SPME method coupled with GC-MS was used to detect lipids which include hydrocarbons and fatty acids. Host grains have a significant effect on the insect lipids such as fatty acid and hydrocarbons. Different number of compounds were extracted from *T. variablile* adults. There were 23 compounds that identified from adults reared on canola and wheat compared 26 and 28 compounds detected form adults reared on oats and barley respectively. Several other studies explain the impact of host grains on the lipids, and lipids might vary based on the insects host grains (Mohammadazadeh,2018; Paul, 2016; Xin et. al., 2018). Results showed in females the major compounds are docosane, 1-iodo-; 1-butanamine, N-butyl-; oleic acid; heptacosane; 13-methylheptacosane; hexacosane; nonacosane; 2-methyloctacosane; n-hexadecanoic acid and docosane.. While 11-methylpentacosane; 13-methylheptacosane; heptacosane; docosane, 1-iodo- and nonacosane were the most significant compounds identified form *T. variabile* male.

The aim of the fourth experimental chapter (Chapter 5) is to address biosecurity surveillance and identification gaps for the khapra beetle by establishing proof of concept and an effective hyperspectral pest image classification system based on deep learning algorithms which can be used in the future by biosecurity personnel to accurately and timely identify exotic khapra beetle from other *Trogoderma sp*. that is *T. variabile.* Due to high similarity between two of *Trogoderma sp.* Including *T. granarium* and *T. variabile*, a modern technique was used which is visible near infrared hyperspectroscopy (VNIH). The hyperspectral imaging technique therefore has the ability to rapidly and simultaneously monitor morphological characteristics. It is also non-destructive and reagent less analytical technique (Cogdill et. al. 2004 and Migdall et. al., 2009). *T. granarium* from different geographical locations were obtained while *T. variabile* were reared on different host grains. Different stages (adult, larvae, larvae skin, fragments of adult and larvae) from both species were used in this study. Results were applied using two different models including Convolutional Neural Networks (CNN) and Capsule Network for analysis. In comparison between these two models, Capsule Network achieved a higher accuracy of 96% compared with 90% obtained from CNN model. This technique showed ability to identify whole insect body as well as fragment samples. The percent of accuracy were 96.2% and 93.4% for whole adults and larvae respectively. In case of fragment

and skin, the accuracy was 91.7%, 90.3% and 91.6% for fragment adult, fragment larvae and larvae skin respectively. Our results have proved that the hyperspectral imaging technique is superior for distinguishing between very similar sibling insect species such *T. granarium* and *T. variabile*. The need of using VNIH technique in quarantine is increased because it saves time and cost. Cao et. al. (2015) showed that a hyperspectral imaging technique could potentially be developed to identify stored-product insect species and geographical strains.

5.2. Conclusion

To conclude, the series of experiment reported in this thesis time of extraction and number of insects are effect on the VOCs of *T. variabile*. SPME coupled with GC-FID and GC-MS is a useful technique to collect VOCs form different stages of warehouse beetle. As well as this technique can be used to study *T. variabile* metabolism. Identifying the volatile organic compounds and chemical hydrocarbones from *T. variabile* can help quarantine facilities to identifying this species from other *Trogoderma sp.* such as *T. granrium*. The current study can be used to establish database of warehouse beetle that could potentially be used for the comparison of Khapra beetle VOCs, metabolism and chemical hydrocarbons. Finally, VNIH coupled with deep learning could be to used identify khapra beetle from other similar morphology *Trogoderma sp.* Such as warehouse beetle.

Recommendations for future research and development includes developing these dosgnostic technology for grain silos as early doiagnostic tool for timely management of stored grain pests. We encourage further evaluation of interaction between VOCs of *T. variabile* and other grain storage insects.

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