The Physiochemical Responses of Stored Grain Insect Pests to Synthetic Amorphous Silica (SAS) Powders

This thesis is presented for the degree of Doctor of Philosophy at Murdoch University

By

Xin Du

College of Science, Health, Engineering and Education
Murdoch University
Perth, Western Australia
2021

Keywords

- Analysis of Insect Lipids
- DI-SPME-GCMS
- Hydrophilic SAS
- Hydrophobic SAS
- Inert Dust
- Insect Lipid
- Insect Respiration
- Metabolomics
- Model of Action
- Non-chemical Method for Management of Post-harvest Grain Insect Pests
- *Oryzaephilus surinamensis*
- *Rhyzopertha dominica*
- Phosphine Resistance Management
- Phosphine Resistance
- Physio-chemical Property of SAS
- Post-harvest Grain Biosecurity
- Post-harvest Grain Safety
- Stored Grain Insect Pests
- Stored Product Insect Pest Management
- Synthetic Amorphous Silica (SAS)
- *Tribolium castaneum*

Abstract

Fumigation is widely used for the disinfestation of stored grain products. Every loss of grain during storage is a loss of all the inputs that produced the grain in the first place. In many situations, fumigation is the only feasible process for pest control. Currently, phosphine is the only fumigant accepted by international trade for the disinfestation of grain and oilseeds. However, phosphine resistance now occurs worldwide and has challenged the continued use of phosphine in the grain industry. Food-grade synthetic amorphous silica (SAS) can act as a phosphine resistance breaker in storage systems. This thesis explored the mechanisms of SAS powder for controlling two phosphine-resistant stored grain insects, red flour beetle (Tenebrionidae: *Tribolium castaneum* (Herbst, 1797)) and lesser grain borer (Bostrichidae: *Rhyzopertha dominica* (Fabricius, 1792)).

Grain protection during storage is essential. Both contact grain protectants and fumigants leave toxic residue issues to humans and the environment. The world wants residue-free grain, especially countries where grain is a substantial proportion of the diet. A high sensitivity headspace-solid phase micro-extraction gas chromatograph-mass spectrometer (HS-SPME-GCMS) method was optimised and validated to determine the residues of eight fumigants simultaneously, including phosphine, methyl bromide, cyanogen, sulfuryl fluoride, ethylene oxide, propylene oxide, ethyl bromide and ethyl formate. A 2 cm long 50/30µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated SPME fiber was chosen based on its absorption performance. The food matrices included grain, oilseed, dried fruit, and nuts. The limits of detection (LODs) of the fumigants ranged between 0.03 to 1.99 ng/g. Responses to a range of diluted authentic standards gave significant ($r^2 > 0.9983$) linear regressions and the relative standard deviations (RSDs) were ≤ 8.7% at the 3 ng/g level of aged spiking standard, except for sulfuryl fluoride with a LOD of 1.99 ng/g and an RSD value of 39.7% (6.64 ng/g). The performance of the HS-SPME-GCMS method was more sensitive than the gas syringe method for all fumigants, except sulfuryl fluoride.

Due to residue issues, the world is increasingly demanding residue-free treatments. The main components of insects' cuticular lipids are hydrocarbon compounds. SAS powders may change the hydrocarbons on the cuticle, impacting an insect’s self-protection mechanism(s) against toxic gas chemicals, possibly by acting as a barrier between the insect and the surrounding phosphine environment. X-ray micro-computed tomography (Micro-CT) scanning

of SAS treated and untreated *T. castaneum* indicated that the SAS powder penetrates the tracheal system of *T. castaneum* and potentially blocks it, leading to asphyxiation. Micro-CT 3D reconstruction model of *R. dominica* showed the internal body fluid was completely depleted and the internal organs shrank. Based on metabolomics, several energy metabolites and derivatives were found to alter after applying food-grade SAS powders to adult *T. castaneum* and *R. dominica.* Phosphine-resistant adults are known to downregulate or slow the consumption of energy substances to survive phosphine fumigation. Fortunately, the food-grade SAS powders accelerated the carbohydrate metabolism leading to the depletion of monosaccharides, and the blocking of the β-oxidation pathway causing the accumulation of free fatty acids (FFAs). The excess FFAs, including saturated and unsaturated FFAs, possibly induce the lethal toxicity of the fatty acids. The associated bioassay results show that hydrophilic (HL) SAS and hydrophobic (HB) SAS controlled the larvae and adults of *T. castaneum* and *R. dominica*; however, HB-SAS was more efficient than HL-SAS when the moisture content and relative humidity were high.

HB-SAS stimulated *T. castaneum* to increase respiration and produce benzoquinones and derivatives, leading to its death within two hours of treatment. The respiration rate of the insects was monitored by Mass Spectrometry (MS), and varied with HL-SAS and hydrophobic HB-SAS treatments. Volatile organic chemicals were identified and quantitated from adult *T. castaneum* by headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GCMS). Three benzoquinone derivatives, ethyl p-Benzoquinone, methyl p-Benzoquinone, and ethyl 1, 3-Benzenediol, were increased significantly by 133.1, 43.1 and 41.9 folds, respectively. Importantly, these benzoquinone derivatives can be used as biomarkers to identify phosphine-resistant strains of *T. castaneum* two hours after SAS treatment by HS-SPME-GCMS.

The smaller particle size allowed the two SAS dusts to pass through open spiracles during air exchange. Due to their light weight, SAS particles are carried along with airflow into the tracheole tubes, which lie within the haemolymph and internal tissues. Small amounts of biofluid in the tracheole tubes evaporated due to the SAS treatments leading to the overwhelming loss of oxygen and water near the muscle cells. Therefore, the irritation of the SAS powder particles provides high insecticidal efficacy, even against phosphine-resistant individuals.

In conclusion, food-grade SAS powders kill phosphine-resistant insect adults, *T. castaneum* and *R. dominica,* by depleting sugar energy and inhibiting the β-oxidation of FFAs

to energy substances. Consequently, SAS powders offer a viable, pesticide residue free alternative to phosphine for managing and eradicating stored product insects.

Contents

List of Figures and Tables

List of Abbreviations

µl	Microliter
2D	2-Dimensional
3D	3-Dimensional
ANN	Artificial Neural Network
ANOVA	Analysis of Variance
BA	Benzyladenine
BET	Brunauer-Emmett-Teller
BJH	Barrett-Joyner-Halenda
BPNN	Back Propagation Artificial Neural Network
CA	Controlled Atmosphere
CAR	Carboxen
CAS	Chemical Abstracts Service
CAW	Carbowax
CL	Confidence Interval
Cm	Centimetre
DCM	Dichloromethane
DE	Diatomaceous Earth
df	Degrees of Freedom
DHS	Dynamic Headspace
DVB	Divinylbenzene
EI	Electronic Impact
EPA	Environmental Protection Agency
ESEM	Environmental Scanning Electron Microscope
EV	Electronic Energy
F	F Value
FID	Flame Ionization Detection
FPD	Flame Photometric Detector
GC	Gas Chromatography
GRAS	Generally Recognized as Safe
H	Hour
HS	Headspace
Hz	Hertz
IARC	International Agency for Research on Cancer
IE	Ionization Energy
IPM	Integrated Pest Management
IR	Infrared Radiation
ISO	International Organization for Standardization

KD	Knockdown Down
KD50	Knockdown 50% of the Population
KD95	Knockdown 95% of the Population
Kg	Kilogram
kW	Kilowatt
LD50	Lethal Dose for Control 50% of Population
LD95	Lethal Dose for Control 95% of Population
LED	Light-Emitting Diode
m.c.	Moisture Content
mg/L	Milligram Per Litre
Min	Minute
mL	Millilitre
MS	Mass Spectrometry
MU	Murdoch University
NIR	Near Infrared Light
NIST	National Institute of Standards and Technology
OP	Organophosphorus
PC	Principal Component
PCA	Principal Component Analysis
PCs	Principal Components
PDMS	Polydimethylsiloxane
ppm	Parts Per Million
R.H.	Relative Humidity
RI	Rendition Index
ROI	Region of Interest
RT	Retention Time
SAS	Synthetic Amorphous Silica
SE	Standard Error
SEM	Scanning Electron Microscope
Sig	Significance Level
SNK	Student-Newman-Keuls
SNP	Silica Nanoparticles
SPME	Solid Phase Microextraction
SPSS	Statistical Package for The Social Sciences
TIC	Total Ion Chromatogram
UV	Ultraviolet Light
Vis	Visible Light
VOCs	Volatile Organic Compounds

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: _____

Date: 20th November 2021

Publications and Presentations

Patents

Ren, Y, Agarwal, M, Du, X, McKirdy, H (2021) Method for the Control of Insects in Stored Products and Structures. *International PCT Patent Plants*, PCT/Australia2021/050915.

Agarwal, M, Ren, Y, Du, X, McKirdy, H (2021) A Method for Controlling Field Insects. *International PCT Patent Plants*, PCT/Australia2021/050917.

Journal Publications

Du, X, Zhang, W, Liu, B, Liu, T, Xiao, Y, Taniguchi, M, Ren, Y (2019) Optimization and Validation of HS-SPME-GCMS Method for Determination of Multifumigant Residues in Grain, Oilseeds, Nuts, and Dry Fruit. *Journal of AOAC International*, 102 (6).

Poster Presentations

Du, X., Lu, Y.J., Hardy, G., Emery, R., Zhang, W.J. and Ren, Y.L., Can the DI-SPME gas chromatography mass spectrometer be a tool for identification of stored grain insects based on fatty acids and sterols profiling. *12th International Working Conference on Stored Product Protection (IWCSPP)* in Berlin, Germany on October 7th-11th, 2018.

Acknowledgements

Firstly, I would like to express my deep gratitude to Professor Yonglin Ren, Head of Postharvest Biosecurity and Food Safety Laboratory, for his help during my research and his patience for many questions, valuable training, providing corrections and critical suggestions on experimental work and during my thesis writing stage. He has been a colossal source of inspiration for me during my PhD studies. I am also grateful to my co-supervisor Professor Giles Hardy, Director of the Centre for Phytophthora Science and Management, Murdoch University. He was very kind and generous in his auspicious help, scholarly guidance, extensive reviews of thesis, great encouragement and affectionate support.

I take great pleasure to express my sincere and intense sense of gratitude to my external co-supervisor Mr Robert N Emery, Principal Entomologist, Department of Primary Industry and Regional Development, Western Australia. For imparting all his experience, knowledge and skills in me, and his continuous efforts to accommodate a supportive environment throughout my research. My sincere thanks passed to advisory committee chair Dr Stephen Milroy, who keep update my research progress and helped me engage with the research milestones.

With a special mention to Dr Jeremy Shaw and Associate Professor Peta Clode from Centre for Microscopy, Characterisation and Analysis (CMCA), The University of Western Australia (UWA). It was fantastic to have the opportunity to conduct part of my research in your facilities.

I am also grateful to the team/university staff: Dr Manjree Agarwal, Dr Junxi Li, Shutting Pan, Nuccia Eyres and David Cousins for their unfailing support and assistance.

Very special gratitude goes out to all down at the Department of Primary Industry and Regional Development's 'Boosting Grains Research and Development' PhD program, supported by Royalties for Regions for helping and providing the funding for the work.

And finally, last but by no means least, I take this opportunity to express my thanks to all my family members who have supported me along the journey of my PhD study.

Thanks for all your encouragement!

1 General Introduction and Literature Review

This chapter outlines the general introduction (section 1.1), the physiological and biochemical effects of Synthetic Amorphous Silica (SAS) on stored grain insects (section 1.2), and the thesis aims (section 1.3). Section 1.4 includes an outline of the remaining chapters of the thesis.

1.1 GENERAL INTRODUCTION

1.1.1 Global and Australian Grain Industry

Grains are small, hard, dry seeds, harvested for human and animal consumption, to provide daily dietary energy. Today, more than 41% of human and 45% of animal calories are provided through grains (FAO 2002). As they are harvested dry, grains are more durable than other food sources, and are well recognized as industrial agriculture worldwide. FAO's forecast for 2020 world grain production is 2,750 million tonnes (Figure 1.1) (FAO 2020), valued at over $500 billion. The Australian grain industry plants about 22.3 million hectares annually, and their production contributes a substantial share of Australia's economy (AEGIC 2016). As domestic consumption of wheat is low, wheat is one of Australia's key exports after the mining industry. Wheat exports were valued at Australia $3,675 billion and were one of the top 25 exports of goods and services, representing 7.3% of the total agricultural & fisheries exports in 2018-2019 (DFAT 2019).

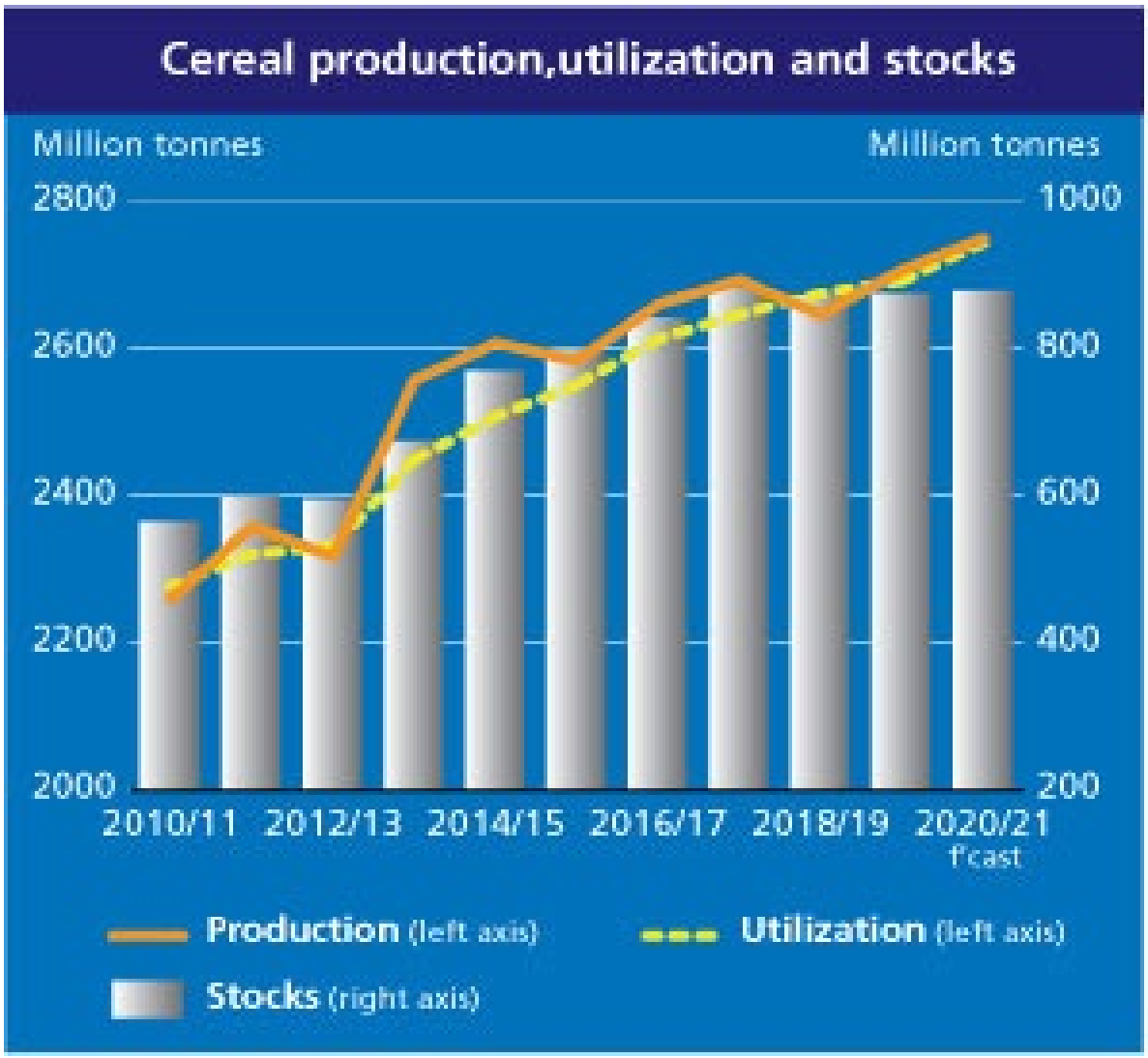


Figure 1.1. Trends of cereal production, utilization and stocks (FAO 2020)

1.1.2 Grain Storage Systems

As grain is harvested seasonally, excess grain must be stored for long-term consumption. Robust grain storage systems are necessary and important to reduce risks associated with food security. To feed the world's population, raw and processed agricultural products have to be secured during transportation and stored for several years. Grain storage technologies are applied with considerations to climatic conditions, capacity, financial affordability, and construction material availability. In general, grains are stored in bags or in bulk in a variety of storage structures such as in brick wall bins, underground storage bunkers, or in metal and concrete silos (FAO 1994; CBH 2003; Singh *et al.* 2017). In Australia, grain is stored in silos, grain bags or bunkers (Figure 1.2) (GRDC 2013). Individual on-farm silos normally store between 80 to 1,000 tonnes of grain. Sealed and unsealed silos constitute 79% of Australian on-farm grain storage facilities (Figure 1.2). In contrast, bulk handlers worldwide tend to use large-scale air-tight horizontal or vertical silos with aeration systems, and this is particularly true in Western Australia (CBH 2003). The capacity of the single silo constructed by Australian bulk handlers ranges from 1,000 to 34,000 tonnes (CBH 2003).

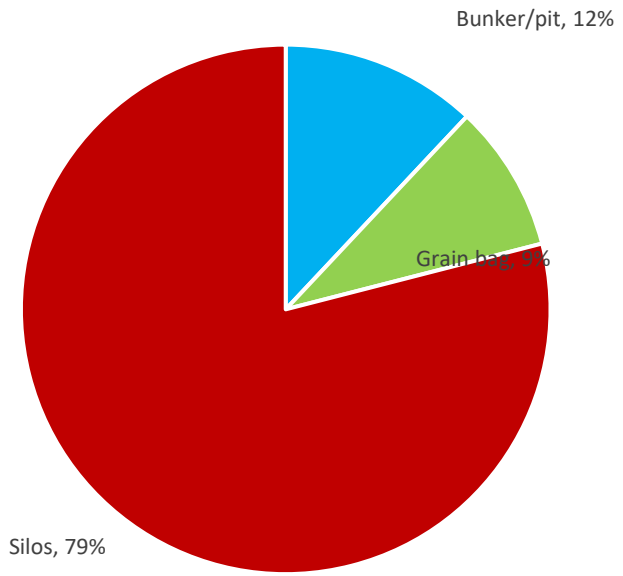
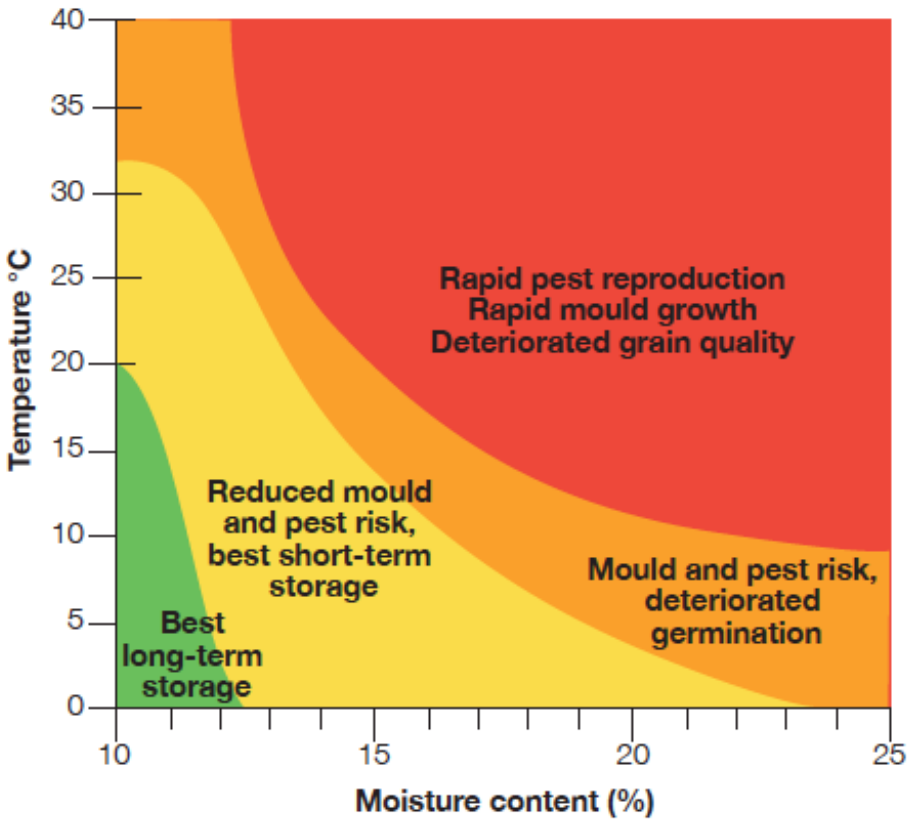


Figure 1.2. Pie chart of Australian On-farm Grain Storage System from Kondinin Group NAS 2011 (GRDC 2013).

1.1.3 Abiotic and Biotic Constraints to the Long-Term Viability of Stored Grain

The quantity and quality of stored grain can be threatened by abiotic and biotic components, such as temperature and humidity and pests and fungi. As a general rule of thumb, high temperatures and high moisture contents of stored produce significantly influence the metabolic rates of the stored products and biological organisms, which release more heat and moisture (Figure 1.3). Furthermore, the abiotic and biotic cycle provides the favourable living conditions leading to a high population density of biological organisms, such as pests, fungi, and bacteria (Figure 1.3). In consequence, the quantity and quality of stored grains are expected to be reduced significantly, including reduced germination rates and losses in market value.



Source: Csiro Ecosystems Sciences

Figure 1.3. Effects of temperature and moisture on stored grain

When the temperature and moisture content are over 25°C and 14.5%, respectively, fungi may grow and produce mycotoxins, potentially hazardous to mammals, in grain. Grains can be contaminated by fungi spores through the whole

supply chain from harvest to people. Thus, mycotoxins are one of the biggest food security challenges worldwide (Sinha and Sinha 1992; Fleurat-Lessard 2004).

The average mass loss of stored grains caused by insects was 5% of worldwide total storage weight, and 2% for rodents (Rajendran 2002). If an importing country finds grains contaminated with live insects, the whole shipping consignment can be rejected. A single ship carries typically between 30,000 to 90,000 tons of grain, so rejection results in significant food wastage and economic losses (Emery *et al.* 2003). Stored grain insects feed on these grain resources and contaminate the quality of attacked commodities with body fragments, faeces, smell, and a range of metabolic products (Snelson 1987). In association with increased insect metabolism, the increased moisture content can assist mould growth to constitute further sanitation and quality problems.

1.1.4 Stored Grain Insects

1.1.4.1 History and Australian Stored Grain Insects

The earliest record of stored grain insects was a flour beetle found in an Egyptian tomb dating back to 1345 B.C. (Buckland 1981). The species of postharvest insects vary according to climate conditions and local agricultural practices. Over 1,025 known insect species, including beetles, moths and mites, are responsible for damage to stored grain products, and over 20 of these pests have major economic impacts worldwide (Rajendran 2002). In Australia, there are six significant stored grain insects, including lesser grain borer (*Rhyzopertha dominica*), red flour beetle (*Tribolium castaneum*), rice weevil (Curculionidae: *Sitophilus oryzae* (Linnaeus, 1763), saw-toothed grain beetle (Silvanidae: *Oryzaephilus surinamensis* (Linnaeus, 1758)), flat grain beetle (Laemophloeidae: *Cryptolestes ferrugineus* (Stephens, 1831)), and psocids (*Order Psocoptera*) (Table 1.1).

Table 1.1. Cereal grains - resistance and efficacy guide for stored grain insects 2014 from storedgrain.com.au

TREATMENTS	WHP (DAYS)	 LESSER GRAIN BORER (*Rhyzopertha dominica*)	 RUST-RED FLOUR BEETLE (*Tribolium castaneum*)	 RICE WEEVIL (*Sitophilus oryzae*)	 SAW-TOOTHED GRAIN BEETLE (*Oryzaephilus surinamensis*)	 FLAT GRAIN BEETLE (*Cryptolestes ferrugineus*)	 PSOCIDS (booklice) (Order *Psocoptera*)	 STRUCTURAL TREATMENTS
Grain disinfectants – used on infested grain to control full life cycle (adults, eggs, larvae, pupae).								
Phosphine (eg Fumitoxin®)[1,3] when used in gas-tight, sealable stores	2							
Sulfuryl fluoride (eg ProFume®)[10]	1							
Grain protectants – applied post harvest. Poor adult control if applied to infested grain.								
Pirimiphos-methyl (eg Actellic 900®)[7]	nil[2]							
Fenitrothion (eg Fenitrothion 1000®)[4 & 7]	1–90							
Chlorpyrifos-methyl, (eg Reldan Grain Protector®)[5]	nil[2]							
'Combined products' (eg Reldan Plus IGR Grain Protector)[9]	nil[2]							
Deltamethrin (eg K-Obiol®)[10]	nil[2]							
Spinosad and Chlorpyrifos-methyl (eg Conserve On-Farm™)[9]	nil[6]							
Diatomaceous earth, amorphous silica – effective internal structural treatment for storages and equipment. Specific use grain treatments.								
Diatomaceous earth, amorphous silica (eg Dryacide®)[8]	nil[2]							

KEY

Effective control. Resistance widespread (unlikely to be effective). Strong phosphine-resistant strains of rusty grain beetle (*Cryptolestes ferrugineus*, also known as flat grain beetles) have been identified in some locations. Resistant species likely to survive this structural treatment. Not registered for this pest. **WHP** – withholding period.

1 Not effective in unsealed storages and selects for resistance. **2** When used as directed on label. **3** Total of (exposure + ventilation + withholding) = 10 to 27 days. **4** Nufarm label only. **5** Stored grains except malting barley and rice/ stored lupins registration for Victoria only/ not on stored maize destined for export. **6** When applied as directed, do not move treated grain for 24 hours. **7** Registered for use on cereal grain. **8** Do not use on stored maize destined for export, or on grain delivered to bulk-handling authorities. **9** Registered for use on cereal grains except maize, malting barley and rice. **10** Restricted to licensed fumigators or approved users.

Source: Registration information courtesy of Pestgenie, APVMA and InfoPest (DAFF) websites

1.1.4.2 Stored Grain Insects Biology

Most stored grain insects belong to the largest order of the Coleoptera. Stored grain insects belonging to the Coleoptera vary in length from about 1 mm to 9mm (Mason and McDonough 2012). Most stored grain insects undergo complete metamorphosis with four stages: the egg, larvae, pupae, and adult. Except for the psocids belonging to the Psocoptera which do not have a larval stage (Mason and McDonough 2012; CGC 2019). They are also characterized by their two pairs of wings, the elytra and hind wings. Elytra wings are hardened and leathery down the middle of the back. The membranous hind wings are folded under the elytra and are designed for flying, and are how these insects easily immigrate to and between grain storage structures. They have multiple pairs of legs, and their bodies can be divided into three segments: head with eyes and antennae, thorax with wings, and abdomen with tail-like or fork-like tip (Mason and McDonough 2012).

Based on diet sources, grain insect pests can be categorized into primary and secondary stored grain pests. The chewing mouthparts of primary grain insects have well-developed mandibles to attack the whole kernel of cereal grains and cereal products. Secondary insects only feed on flour or damaged grain (DPIRD 2019). For example, lesser grain borer and rice weevil are primary stored grain insects, while the rust-red flour beetle, saw-toothed grain beetle, and flat grain beetle belong to secondary grain storage insects (DPIRD 2019). The secondary insects normally lay eggs in the crevices of kernels or in flour, whilst the primary insects prefer to lay their eggs inside grain kernels. The larval stage is largely responsible for growth as they consume several times their body weight. In contrast, insects do not feed and move during the pupal stage. After undergoing dramatic internal and external changes described as holometabolous development, the adult insects migrate and reproduce for wide distribution. The average minimum life cycle of stored grain insects ranges from 15 to 38 days at 27-35°C and 65% to 80% R.H. (Mason and McDonough 2012). Stored grain insects not only feed on commodities directly but can also generate heat and moisture in the bulk grain due to their metabolic activities (feeding, excretion and respiration). High temperatures and moisture provide favourable living conditions and lead to outbreaks of stored products insects (Table 1.2).

Table 1.2. Biological table of two major stored grain insects, *Tribolium castaneum* and *Rhyzopertha dominica* (Mason and McDonough 2012).

	Tribolium castaneum	*Rhyzopertha dominica*
Adult length	3– 4.5mm	3mm
Pest type	Secondary pest	Primary pest
Commodities attacked	Dried material of animal and plant origin, especially cereal grain and products, oilseeds	Cereal grains, especially wheat, barley, rice, and sorghum
Economic importance	High, especially in mills and processing plants and in grain stored in warm to hot climates	High, even in modern bulk storage systems
Distribution	Worldwide	Worldwide, especially in warm temperate to tropical regions
Life cycle	Optimum: 20 days at 35– 38°C >70% R.H. Range: 22– 40°C, R.H. >1%.	Optimum: 25 days at 34°C, 70% R.H. Range: 20– 38°C, >30% R.H.
Eggs	Laid outside the grain (in flour)	Laid on the grain or inside tunnels bored by adults into the grain
Larvae	Mobile, external feeder	Internal feeders producing lots of flour, immobile when mature
Adults	Long-lived, feed on commodity, fly	Long-lived, feed on commodity

1.1.5 Fumigation and Residue Measurement

Harvested grain is commonly stored for the long term in grain storage facilities. In Australia, 35 million tonnes worth $8.7 billion were produced in 2016-2017 (DAWR 2018). Fumigants are widely used from pre-harvest for soil fumigation to post-harvest for grain, oilseeds, fruit and nuts. Hence, the residues of fumigants are a crucial index for determining food, occupational and environmental health and safety. Therefore, it is critical to eradicate insect infestations present in grain (Hwaidi *et al.* 2016). In reality, it is often tough to track the fumigation history of food, particularly in the grains industries, because fumigants are so widely used, especially for post-harvest treatments. A restrictive Codex Limit of Maximum Residue Levels (MRLs) lead to food safety concerns associated with these fumigants, and some purchasers set lower limits, and challenges on the use of fumigants for the treatment of grain have been issued by several government authorities and international organizations (Commission 2008; APVMA 2012). Individual and combined analytical methods to determine residue levels of phosphine, methyl bromide, cyanogen, sulfuryl fluoride, ethylene oxide, propylene oxide, and ethyl formate have been reported (Stenger *et al.* 1939; Romano and Renner 1975; Scudamore and Goodship 1986; Tschickardt and Lauterwald 2002; Ren and Mahon 2007; Park *et al.* 2014; Jimenez *et al.* 2015; Kang and Shin 2015). Some multi-fumigant residue detection methods have been developed for grain using different sample preparation processes and analytical instruments, for example, microwave irradiation (Ren and Mahon 2007) and charcoal adsorption (Dimitriou and Tsoukali 1998). However, most currently developed fumigant residue methods use a serial dilution of authentic standards without considering the effect of time on the equilibrium of fumigant distribution between different phases when a food matrix is a part of the calibration system, although, Amrein *et al.* (2014) indicated that complex matrices can significantly affect the accuracy of measurement. In heterogeneous matrices, for example, with unground grain, the analytical procedure is time-dependent so that an analysis of incurred residues depends on knowing the levels of chemical present. The solution is to convert the matrices from heterogeneous to homogeneous by grinding milling (Desmarchelier and Ren 1999).

1.1.6 Phosphine Resistance of Stored Grain Insects

Only phosphine has been identified and recognized worldwide for disinfestation of stored grain insects for the international grain trade, after restrictions to the use of

methyl bromide were imposed by Montreal Protocol (UNEP 1987; Chaudhry 2000). Consequently, due to the over-reliance on one particular chemical, the levels of insect resistance to chemicals are increasing worldwide. This chemical resistance has been detected in many on-farm and bulk handler sites in western and eastern Australia (Jagadeesan and Nayak 2017) (Figure 1.4).

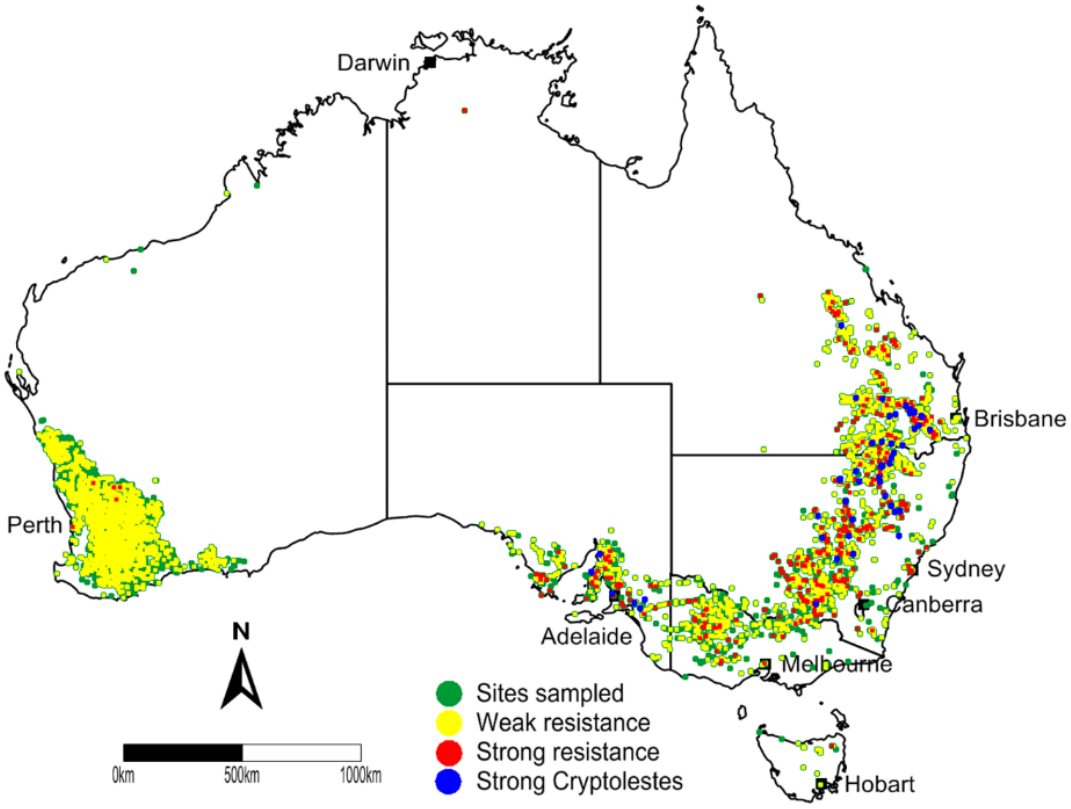


Figure 1.4. Phosphine resistance map 1986-2014 from stored grain insect resistance monitoring and management (https://www.agric.wa.gov.au/exporting-western-australia/stored-grain-insect-resistance-monitoring-and-management).

1.2 INSECT ECOLOGY AND PHYSIOLOGY

SAS treatment doesn't request gastight storage facilities and expensive annual maintenance costs, so this technology provides a promising future. However, there is little published evidence on the mode of action of SAS. Therefore, it is important to understand how SAS affects insect ecology and physiology, which plays an important role in protecting insect body and metabolism.

1.2.1 Insect Respiration

To produce essential energy for the insect body cells, insects need to exchange carbon dioxide (CO_2) gas with oxygen (O_2) from the environment through their respiratory system. Carbon dioxide, a toxic by-product of insect cellular respiration, must be expelled the insect body. An insect's respiratory system, including spiracles and tracheae, introduces air through pairs of muscular openings to internally perform the gas exchange. These external openings are called spiracles, which control gas flow into the internal densely networked trachea respiratory tubes. The trachea network connects interior organs immersed in haemolymph liquid to the environment in series.

1.2.1.1 Structure of the Spiracle

Insect spiracles are normally in pairs located along the side of the thorax and abdomen on exoskeletons to regulate the respiration gas flow and water loss rate. The structure of the spiracle plays a critical role in self-protection from toxic gases, such as phosphine and methyl bromide, and adaptation to the environment. The spiracle arrangement varies with different species. Muscles adjacent to the spiracles can be contracted to open and close the gas gate. The central nervous system mainly controls these muscles (Klowden 2007).

1.2.1.2 Structure of the Tracheae

Behind the spiracles, the tracheal network lies transverse and longitudinal. The tracheal trunk branches into the tracheole, a complex network of respiratory tubes of the trachea. The branch is normally 1 micrometer in diameter. The benefit of the fine tracheole system is that the phase surface is increased for more efficient gas exchange (Klowden 2007). At the end of each tracheal branch, the tracheole tubes exchange the gases and water content by dissolving gases in the tracheole liquid and diffusing them to the internal body cells eventually (Figure 1.4). At the same time, the end products of energy metabolism, named water and carbon dioxide, also can diffuse out and release through the tracheae (Harrison 2012). Tracheoles accumulate in digestion and flight segmentations, which conduct intensive metabolic activities. Limited by lack of sensitive analytical methods, there is no published research that report direct evidence of the influence of inert dust on small insect’s respiration patterns and metabolic rate.

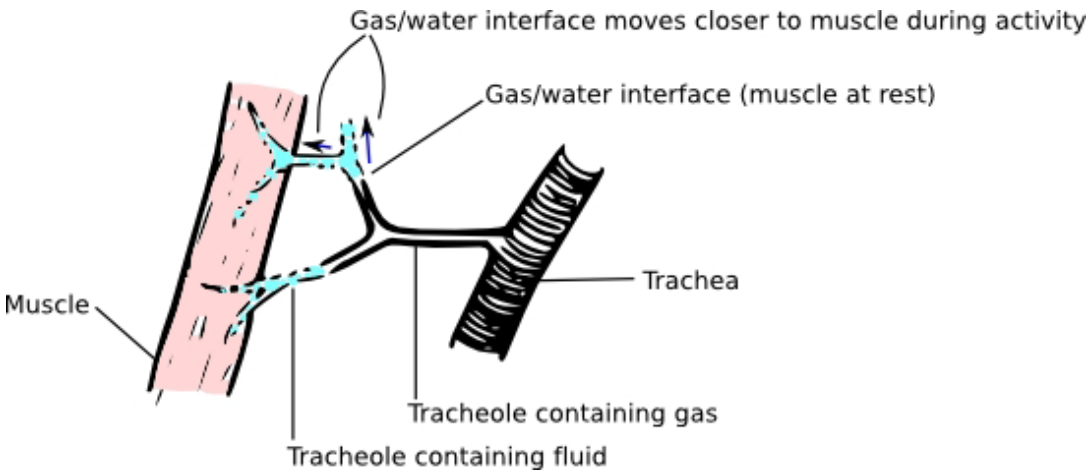


Figure 1.5. Gas/Water interface in the tracheole from Amateur Entomologists' Society (https://www.amentsoc.org/insects/fact-files/respiration.html).

1.2.2 Insect Cuticle Protection Layer

The arthropod (insect) cuticle is the first layer of protection from environmental damage (Wigglesworth 1957; Zacharuk 1972). Insect cuticle is secreted from the epidermis based on a non-cellular membrane. A hydrophilic procuticle layer sits above the epidermis and can be divided into the endocuticle and exocuticle layers. The epicuticle layer composed of four layers, lies above the procuticle layer. From top to bottom, the components of the four layers are the cement layer, wax layer, cuticulin layer, and innermost protein layer. These disparate layers have various components and functions. The innermost protein layer is very important for regulating morphological changes during insect growth. The cuticulin layer, containing lipids and sclerotin, gives the insect both the strength and hardness of sclerotin and waterproofing properties of wax (Wigglesworth 1990). The wax layer is a monolayer of wax molecules followed by a cement layer overlaid for protection. The physiological structure of the insect cuticle layer has been described and reviewed in detail (Wigglesworth 1957; Zacharuk 1972; Filshie 1982). The main function of the epicuticular layer is to prevent water evaporating from the insect. Subramanyam and Roesli (2000) state that the epicuticle is the most important cuticle layer and is related to insect survival. Gibbs (1998a) stated that it is difficult to link the differences of cuticle lipid composition to water loss or cuticle permeability. The mechanism of the

water proofing ability of insect cuticle lipids remains unclear. A clear understanding will help explain the mode of action of inert dusts, and other insecticides.

1.2.3 Lipids on the Insect Cuticle Layer

Lipids are small molecules generated and metabolized by enzymes rather than genetically encoded. There are between 10,000 - 100,000 distinct chemical entities under the lipid scope (Wenk 2010). Lipids are defined and divided into eight categories; fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Fahy *et al.* 2005; Fahy *et al.* 2009). Glycerophospholipids, sterols, and sphingolipids dominate a large lipid portion of biological membranes, which belong to amphiphilic lipids (both hydrophilic and hydrophobic). The signalling lipids are highly bioactive secondary metabolites of membrane lipids, which are normally soluble and diffusible, and control insect physiology. For example, arachidonic acid can be metabolized to prostaglandin H2 and lipoxin B4, which are pro-inflammatory and anti-inflammatory bio-agents, respectively (Wenk 2010).

1.2.4 Fat Body

The fat body is an essential metabolically functional component of insects. The main fat body cell stores energy of glycogen and triglycerides and releases energy for the demands of the insect. The lipids are stored in the form of cytoplasmic lipid droplets. Lipidomics play a key role in growth, reproduction, and fitness during long-term non-feeding periods (Arrese and Soulages 2010). The major components of lipid droplets are non-polar lipids, instead of amphiphilic lipids, glycerolipids (triacylglycerol), and sterol-esters (Wenk 2010). The insect fat body is loose tissue and covered with thin lobes in the haemolymph. Wenk (2010) found that, during homogenizing insect bodies, free fatty acids and sterol lipids were produced from the catabolic reaction of the lipid components. Metabolic changes in flying insects indicate that metabolism increases 50 to 100-fold during flight which resulted from converting amino acids, sugars, and lipids stored in the fat body (Storey 1985). However, there is little published information on stored grain insect metabolism when under stress.

1.2.5 Current Research Status of Mode of Actions (MoAs)

Attempts to find inert dusts to replace toxic chemicals for insect pest control date back to the 1920's. The use of conventional inert dusts, such as diatomaceous earth

(DE), in stored product pest management is restricted by several industrial concerns on low efficacy and human health (Bodroža-Solarov *et al.* 2012). Due to the high dosage required, and the impact on the loading angle of bulk grain, there has been limited application of inert dust in large scale bulk handlers' facilities (Golob 1997). In response to these concerns, food-grade synthetic amorphous silica (SAS) powders have been developed and introduced at a large commercial scale to bulk handling systems in China and Australia (Ren and Agarwal 2014; Perišić *et al.* 2018) and they have the potential to be used as phosphine resistance breakers in bulk handling systems.

Food grade SAS causes exposed insects to dehydrate more efficiently than conventional DE dusts, but death by desiccation is difficult to explain. There are five proposed modes of action (MoA) of inert dusts, including SAS and DE, to control stored grain insects, three of which are related to water loss and cuticle impairment (Wigglesworth 1944; Subramanyam and Roesli 2000). The most widely accepted insecticidal hypothesis is that the inert dust adheres to the insects' body and absorbs the epicuticular lipid layer, which in turn impairs the water balance through the cuticle (Chiu 1939; Wigglesworth 1944, 1945, 1947; Tarshis 1960; Tarshis 1961; Cook *et al.* 2008; Van Den Noortgate *et al.* 2018). There is a lack of solid scientific evidence to validate these MoAs thoroughly, and conflicting results that indicate insect death cannot be explained by desiccation alone (Chiu 1939). Previous researchers have observed a strong correlation between the loss of wax lipids and mortality. However, it was not always straightforward. Van Den Noortgate *et al.* (2018) reported that a zeolite material (NH4-MOR-38) absorbed the most waxes out of 24 porous materials. However, its insecticidal activity was lower than another zeolite material (H-BEA-300) that only adsorbed 18.2% of what NH4-MOR-38 adsorbed (Van Den Noortgate *et al.* 2018). Stronger absorption of wax lipids did not lead to higher insecticidal efficacy.

1.3 ADVANCED ANALYTICAL TECHNOLOGIES

1.3.1 Measurement of Lipid Absorption Capability by Using Solid-Phase Microextraction Technology

Based on understanding the important role of lipid components in function and metabolome, the damage or removal of lipid components from an insect will lead to disorder and death. In 1961, Ebeling reported that the lipid absorption capability of the

inert dust affects the effectiveness of the treatment (Ebeling 1961). Hence, the lipid absorption capability of inert dust becomes a critical factor affecting treatment efficacy. Subramanyam and Rennie (2000) described a test method for calculating the oil absorption capability using linseed or similar oils. The linseed oil was absorbed and mixed with inert dust. Typical linseed oil contains α-linolenic acid (51.9-55.2%), palmitic acid (about 7%), stearic acid (3.4-4.6%), oleic acid (18.5-22.6%), and linoleic acid (14.2-17%) (Vereshchagin and Novitskaya 1965). The composition of linseed oil covers the range of fatty acids in the fat body of most stored grain insects. The range of the lipid oil absorption capability of DE (Diatomaceous Earth) dust ranges from 1.4 to 1.75 grams of linseed oil per gram of inert dust; the range of oil absorption capability of pure synthetic amorphous silica (SAS), which is less harmful to human health and more effective against insects, is 1.9-3 grams of linseed oil per gram of inert dust (Subramanyam and Roesli 2000). Recently, Korunic (2016) found an inert silica dust (Sipernat 50S), with the capacity to absorb 3.23 mL oil per g dust. However, the oil absorption data of modified synthetic amorphous silica is lacking. In this study, experiments will be designed to compare wax absorption capabilities between hydrophobic and hydrophilic synthetic amorphous silica, purchased from the same manufacturer.

The solid-phase microextraction (SPME) method is a solvent-free sampling technique that extracts gas, liquid, and solid samples using fibers, tubes, vessel walls, suspended particles, stirrers, or disks coated with the extraction phases (Figure 1.6). The extraction phases commonly contain liquid or solid surfaces, small and large pores, where the adsorptive and absorptive equilibrium extraction of targeted analytes happens. It has been developed to replace the conventional methods for extracting wax lipids from the insect's body. SPME has been reported to save on sample preparation time, reduce solvent purchase and disposal costs, and improve detection limits (Kataoka *et al.* 2000). Since Pawliszyn *et al.* (1999) developed the SPME technology, solid-phase microextraction has been applied to analyse pesticide residues in food, drinks, environmental water and soil samples with Direct Immersion-SPME and Headspace SPME (Boyd-Boland *et al.* 1994; Kataoka *et al.* 2000; Pawliszyn 2000; Doong and Liao 2001; Bonansea *et al.* 2013). More recently, the technique has been used with strawberries and cherries (Lambropoulou and Albanis 2003). Also, direct-

immersion solid-phase microextraction technology has been employed to monitor the metabolome of a living plant, e.g., apple (Risticevic *et al.* 2016).

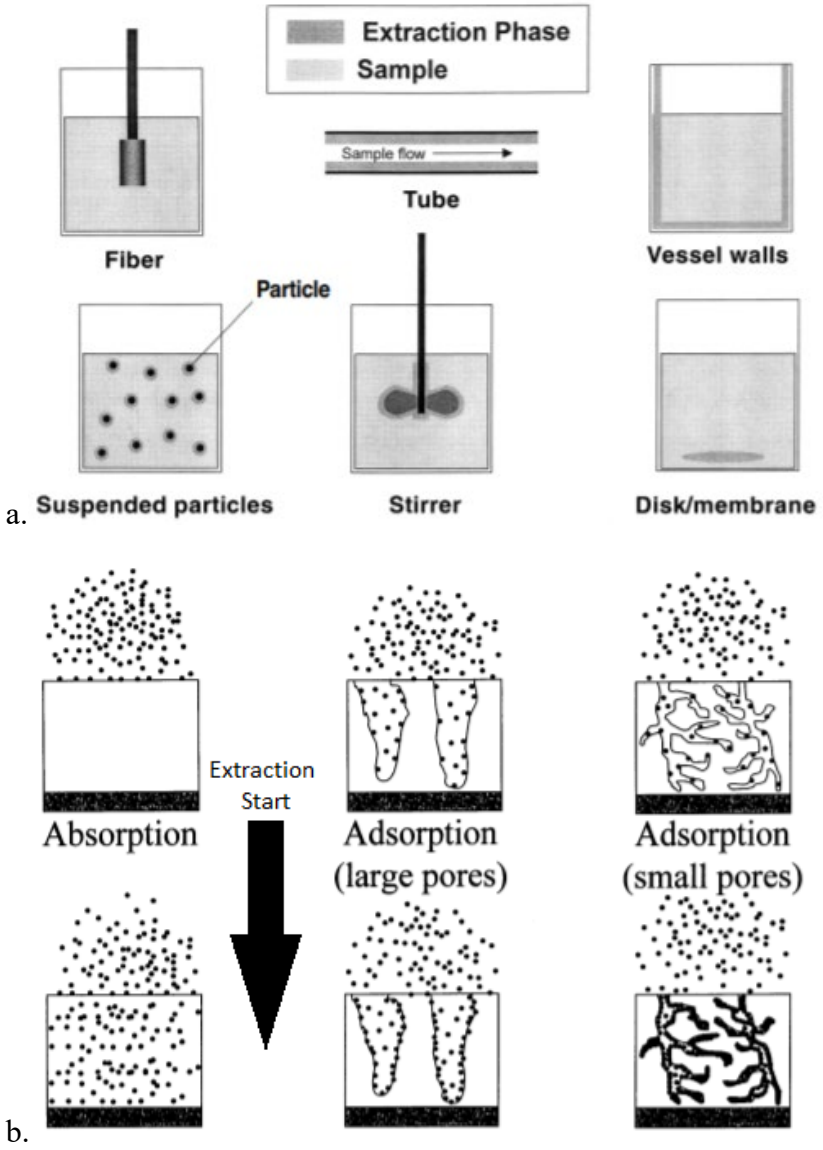


Figure 1.6. (a) Solid-phase microextraction (SPME) configurations and (b) schematic representation of absorptive versus adsorptive extraction and adsorption in small versus large pores (Pawliszyn 2000); Black arrow: SPME extraction time period.

1.3.2 Metabolomics

Metabolomics is defined as "the comprehensive quantitative analysis of all the metabolites of an organism or specified biological sample" (Raamsdonk *et al.* 2001). Metabolites are commonly referred to as "primary" and "secondary" metabolites. The differences between primary and secondary metabolites are directly involved in the normal metabolic pathway of growth, development, and reproduction. Although secondary metabolites indirectly contribute to these processes, some of them have important biological and ecological functions. Examples include pheromones, antibiotics, and pigments. Metabolites of foreign substances such as drugs are termed xenometabolites (Crockford *et al.* 2008). Analytical technologies GCMS, LCMS, and NMR are three popular approaches to metabolomics analysis (Nicholson *et al.* 1999) (Figure 1.7). However, due to the requirement of lower detection limits, the literature related to NMR will not be further reviewed. The complex mixture of a sample matrix can be minimized by applying separation technologies, e.g., Gas Chromatography (GC) and Liquid Chromatography (LC). Furthermore, the separation process can distinguish analytes based on the retention time, which can be used to calculate the retention index for metabolite identification purposes, even their isomers.

Figure 1.7. Gas chromatography-mass spectrometry (GCMS) (a) and liquid chromatography mass spectrometry (LCMS) (b) from Agilent; and nuclear magnetic resonance spectrometry (NMR) (c) from Bruker.

It is also important to improve sensitivity and employ a simple and sensitive trace analysis method to measure and identify these metabolites at the ppb (parts per

billion) level. Gas chromatography interfaced with mass spectrometry (GCMS) is one of the most popular methods for global metabolites analysis in the plant kingdom (Ogbaga *et al.* 2016). Since only volatile and semi-volatile compounds can be analysed by GCMS, chemical derivatization (untargeted profiling) is required for profiling global metabolites by reducing the boiling point and polarity of metabolites during GCMS analysis (Fiehn 2001). Fiehn (2001) suggested a combined targeted and untargeted Gas Chromatography-Mass Spectrometry metabolomics protocol, which includes sample preparation to data processing. As derivatization is involved, there are several drawbacks: bias and limited coverage of different derivatization agents; unstable trimethylsilyl (TMS) derivatives; and various waiting times (Villas-Bôas *et al.* 2011). To solve the last two issues, automated sample preparation approaches and automated derivatisation were evaluated and assessed by Gu *et al.* (2011) and Abbiss *et al.* (2015).

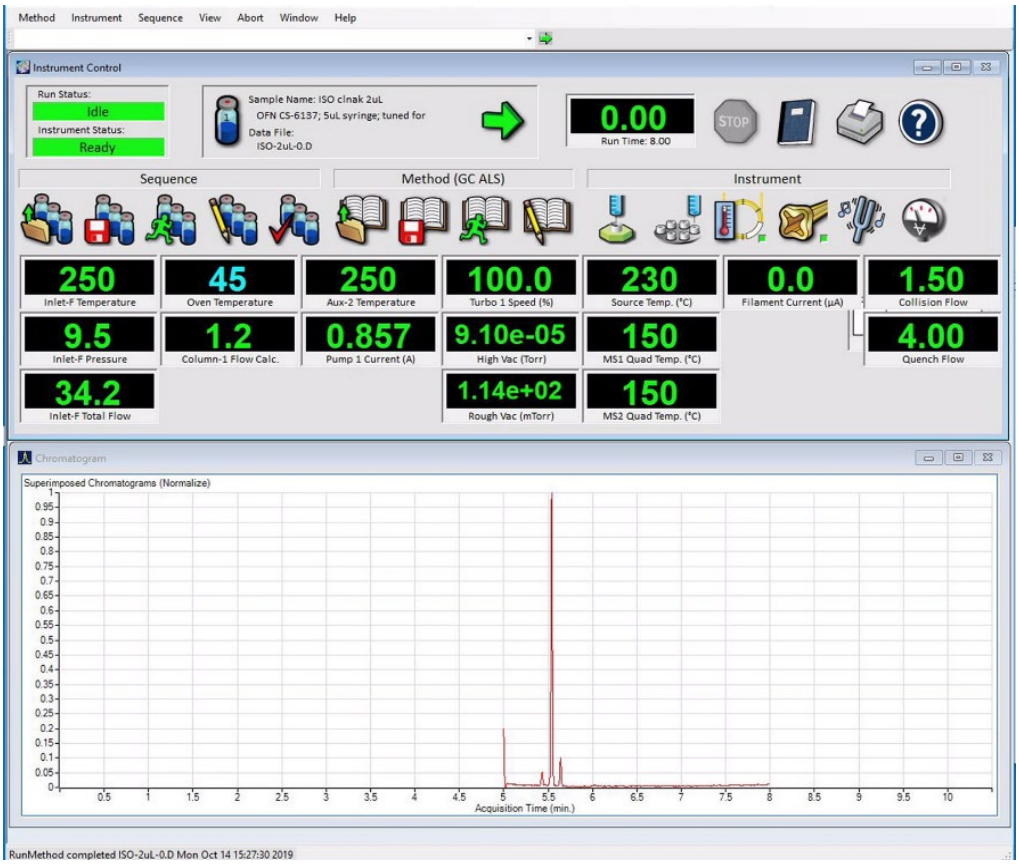
Additionally, these methods were designed for mammalian and plant tissues and are unsuitable for insect lipidomics. For example, the fatty acids are derivatized to the O-methyl oximes of fatty acids, which are fatty acid methyl esters (FAME). In this case, the error of qualitative and quantitative analysis of fatty acids occurs; hence this method fails to separate fatty acids from fatty acid methyl esters. Therefore, there is a need to develop a suitable GCMS metabolomics approach for insect lipid chemistry.

1.3.3 Multivariate Data Statistical Analysis (MVA)

Multivariate data analysis is a substantial statistical technique used for analysing big data with multiple variables simultaneously to identify patterns and relationships, especially for genomics, metabolomics, and lipidomics. The relative standard deviation (RSD, expressed as %) is used to present data reproducibility, and the maximum acceptance tolerances (MAT) of 20% and 30% are reported for liquid chromatography and gas chromatography coupled to mass spectrometry for large-scale metabolic profiling, respectively (Dunn *et al.* 2011).

In this study, GCMS data will be collected by data acquisition software, e.g., Agilent MassHunter, and identified by NIST 2014 database. Peak alignment and statistical analysis are then processed by Agilent MS Quantitative Analysis and MetaboAnalyst 3.0 (http://www.metaboanalyst.ca). The data can be used to compare the differences of metabolites between infested and healthy grain samples and monitor changes in insect metabolites affected by their food source.

a.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Data File:
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Method (GC ALS)
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250
Inlet-F Temperature
45
Oven Temperature
250
Aux-2 Temperature
100.0
Turbo 1 Speed (%)
230
Source Temp. (°C)
0.0
Filament Current (µA)
1.50
Collision Flow
9.5
Inlet-F Pressure
1.2
Column-1 Flow Calc.
0.857
Pump 1 Current (A)
9.10e-05
High Vac (Torr)
150
MS1 Quad Temp. (°C)
4.00
Quench Flow
34.2
Inlet-F Total Flow
1.14e+02
Rough Vac (mTorr)
150
MS2 Quad Temp. (°C)
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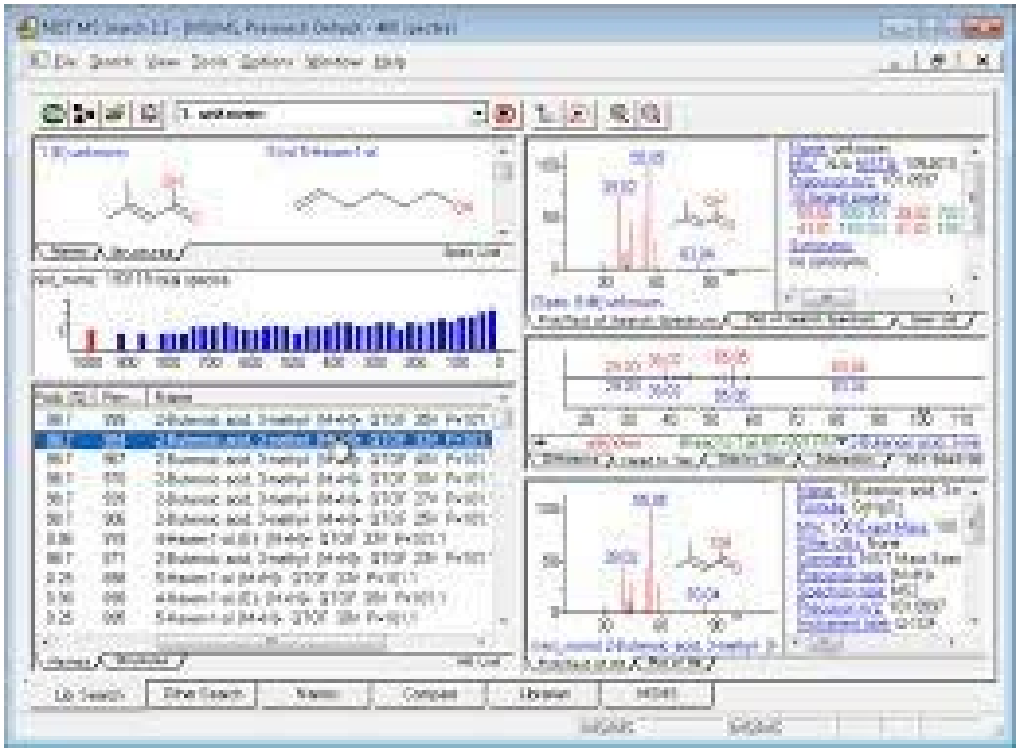
b.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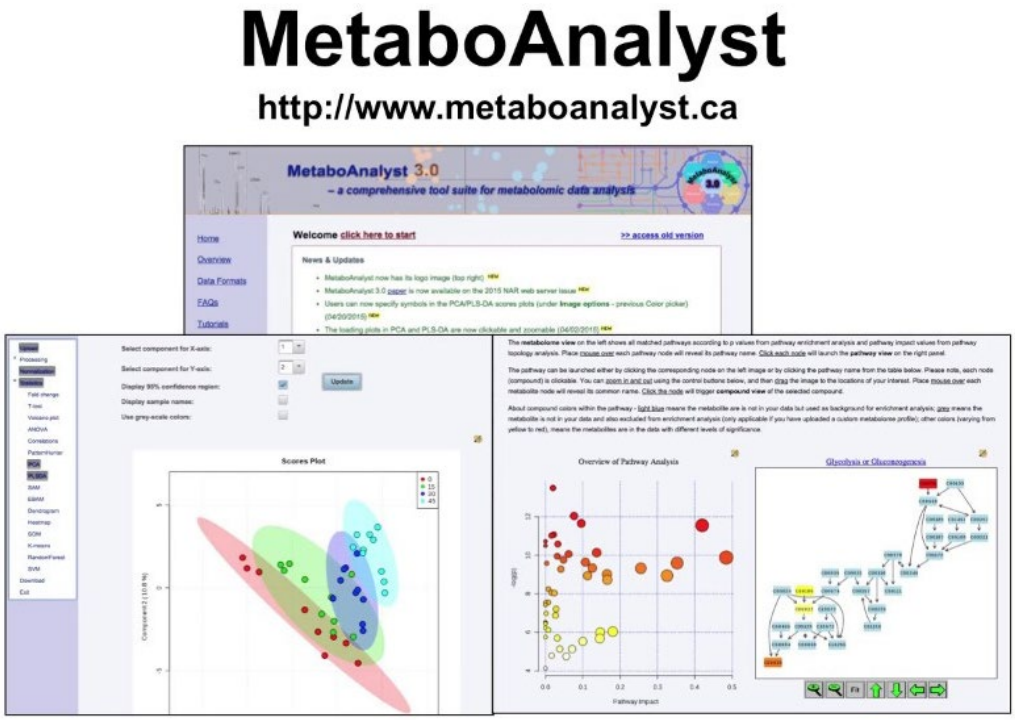
c. 

Figure 1.8. GCMS data acquisition software (a), NIST MS database (b), and web-based data process and statistical analysis platform, MetaboAnalyst (c).

1.4 RESEARCH AIMS

This research aims to discover and explain the insecticidal mechanism(s) of SAS on phosphine-resistant stored grain insects. Phosphine and methyl bromide are the only fumigants allowed to disinfest stored grain for international trade. However, the significant build-up of chemical resistance in key stored grain pests, has raised the urgent need to find a reliable tool for phosphine resistance management. Because of non-toxicity and high efficiency, food-grade synthetic amorphous silica powders (SAS) have been successfully introduced as a chemical resistance breaker in stored grain systems. There are no reports to data on the mode(s) of action of SAS treatment on phosphine-resistant stored grain insects.

One of the most popular modes of action (MoAs) by diatomaceous earth (DE) dusts on insects is that DE removes or absorbs the wax layer, resulting in high water loss leading to death. As the dosage of SAS is 10 times less than DE dusts, the total lipid absorption capacity of SAS powders is lower, but the insecticidal efficacy is higher than DE dusts (Ren and Agarwal, 2020). The hypothesis derived from laboratory observations is that the internal energy metabolism plays a more important

role than the loss in the wax layer on the mechanism(s) of insecticidal actions. Based on the integration of physiology and modern biochemical analysis, this research can help provide fundamental knowledge and a deeper understanding of how to control phosphine-resistant stored grain insects by SAS powders.

1.5 THESIS OUTLINE

Chapter 1 covers the general introduction, insect ecology and physiology and the advanced analytical technologies used in this study. Four experimental chapters (2, 3, 4, & 5) discuss the work involved in this research. Chapter 2 develops and optimizes the solid phase microextraction coupled gas chromatography mass spectrometry method for characterisation of fumigant behaviour and non-volatile metabolites excreted from stored grain insects. In Chapter 3, the innovative analytical method developed in Chapter 2 was validated on stored grain and other model insects. Chapter 4 examined and correlated the body weight loss during SAS treatment with the insects' energy metabolism. Chapter 5 focused on the respiratory and excretory responses of phosphine-resistant *T. castaneum* and *R. dominica* to SAS powders. Finally, Chapter 6 ties the experimental chapters together to give an overview of SAS modes of action and the implications for the effect of SAS on the overall energy metabolic pathway.

2 Optimization and Validation of HS-SPME-GCMS Method for Determination of Multi-fumigant Residues in Grain, Oilseeds, Nuts and Dry Fruit

Statement of Contribution

Title of Manuscript	Optimization and Validation of HS-SPME-GCMS Method for Determination of Multifumigant Residues in Grain, Oilseeds, Nuts, and Dry Fruit
Publication Status	◉ Published ○ Accepted for Publication ○ Submitted for Publication ○ Unpublished and Unsubmitted work written in a manuscript style
Publication Detail	*Journal of AOAC INTERNATIONAL, Volume 102, Issue 6,* ***2019****, Pages 1877-1883, doi.org/10.1093/jaoac/102.6.1877*

Principal Author

Name of Principal Author	Xin (Bob) Du
Contribution to the Paper	Data Collection & Analyses, Manuscript Development
Overall percentage (%)	62.5%
Signature	**Date:** 08 Aug 2019

Co-Author Contributions

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

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

Name of Co-Author	Bo Liu
Contribution to the Paper	Manuscript Development
Overall percentage (%)	7.5%
Signature	**Date:** 08 Oct 2019

Name of Co-Author	Tao Liu
Contribution to the Paper	Manuscript Development
Overall percentage (%)	5%

Signature	**Date:** 08 Oct 2019

Name of Co-Author	Wenjuan Zhang
Contribution to the Paper	Data Analysis
Overall percentage (%)	10%
Signature	**Date:** 08 Oct 2019

Name of Co-Author	Yu Xiao
Contribution to the Paper	Data Collection
Overall percentage (%)	5%
Signature	**Date:** 08 Oct 2019

Name of Co-Author	Miyuki Taniguchi
Contribution to the Paper	Data Collection
Overall percentage (%)	5%
Signature	**Date:** 01 Dec 2020

Name of Co-Author	Prof. Yonglin Ren
Contribution to the Paper	Design, Manuscript Development
Overall percentage (%)	5%
Signature	**Date:** 08 Oct 2019

ABSTRACT

Fumigants are approved in many countries and used to treat food, feed and seed. The optimization and validation of a high sensitivity headspace solid-phase micro-extraction gas chromatograph mass spectrometer (HS-SPME-GCMS) method for determination of eight fumigant residues; phosphine, methyl bromide, cyanogen, sulfuryl fluoride, ethylene oxide, propylene oxide, ethyl bromide, and ethyl formate, in a range of food matrices were developed. The food matrices included grain, oilseed, dried fruit and nuts. The new method was used to determine residue levels from a fumigant complex in food matrices by monitoring the change of the absorption of spiked standards and desorption of fumigant from fumigated samples. Based on the observation, the process of physical sorption and chemisorption was defined. The equilibrium time of the sample analysis was chosen at five hours. The Limits of Detection (LODs) of the fumigants were in the range of 0.03 to 1.99 ng/g. The response to a range of diluted authentic standards gave significant linear regressions ($r^2 >$ 0.9983). The relative standard deviations (RSDs) were ≤ 8.7% at the 3 ng/g level of aged spiking standard, except for sulfuryl fluoride which LOD was 1.99 ng/g and RSD value was 39.7% (6.64 ng/g). The performance of the HS-SPME-GCMS method was more sensitive than the use of a gas syringe, except for sulfuryl fluoride.

2.1 INTRODUCTION

Fumigants are widely used from pre-harvest for soil fumigation to post-harvest for grain, oilseeds, fruit and nuts. Hence, the residues of fumigants are a crucial index for fumigation determining food, occupational and environmental health and safety. In reality, it is often very hard to track the fumigation history of food, particularly in the grains industries, because fumigation occurs along the value chain, from farm through storage and manufacturing, especially for post-harvest treatments. A restrictive Codex Limit of Maximum Residue Levels (MRLs) led to food safety concerns associated with these fumigants, and some countries set lower limits, several government authorities and international organizations have issued challenges on the use of fumigants for the treatment of grain (Commission 2008; APVMA 2012).

Individual and combined analytical methods to determine residue levels of phosphine, methyl bromide, cyanogen, sulfuryl fluoride, ethylene oxide, propylene

oxide, ethyl bromide and ethyl formate have been reported (Stenger *et al.* 1939; Romano and Renner 1975; Scudamore and Goodship 1986; Tschickardt and Lauterwald 2002; Ren and Mahon 2007; Park *et al.* 2014; Jimenez *et al.* 2015; Kang and Shin 2015). Some multi-fumigant residue detection methods have been developed for grain samples using different sample preparation processes and analytical instruments, for example, microwave irradiation (Ren and Mahon 2007) and charcoal adsorption (Dimitriou and Tsoukali 1998). However, most current fumigant residue methods use a serial dilution of authentic standards without considering the effect of time on the equilibrium of fumigant distribution between different phases when a food matrix is a part of the calibration system. Although, Amrein *et al.* (2014) has indicated that the complex matrix can significantly affect the accuracy of measurement. In heterogeneous matrices, for example, for unground grain, the analytical procedure is time-dependent so that an analysis of incurred residues depends on knowing the levels of the chemical present. The solution is to convert the matrices from heterogeneous to homogeneous by, for example, grinding milling (Desmarchelier and Ren 1999).

Solid-phase micro-extraction (SPME) is a solvent-free technique for extracting whole matrices, which can alter the current conventional extraction methods for on-site routine analysis of residues. The concept of SPME started in the late 1980s (Pawliszyn and Liu 1987). This technology has since matured and been applied to trace chemical compounds or analyse pesticide residues (Arthur and Pawliszyn 1990; Du *et al.* 2016). However, few reports have focused on Headspace-Solid Phase Micro-extraction (HS-SPME) applications to the grain industry for analysis of multi-fumigant residues. Ren and Padovan (2012), and Amrein *et al.* (2014) reported that HS-SPME can analyse phosphine residues in agriculture commodity samples.

Analysis of sorption of a fumigant at trace levels in matrices is key to avoid underestimating fumigant residues. Therefore, the purpose of this study was to develop and validate a simple and non-destructive practical HS-SPME-GCMS trace analysis method using spiking standards while considering the time factor, to identify and quantify each of the eight representative fumigants, phosphine, methyl bromide, cyanogen, sulfuryl fluoride, ethylene oxide, propylene oxide, ethyl bromide, and ethyl formate, in different food matrices. The results of a headspace syringe injection method were used as a comparison. The method reported here has great potential to

monitor absorption, desorption, and residue of fumigants in comparison with low level of spiking standards.

2.2 MATERIALS AND METHODS

2.2.1 Commodities

Wheat used was Australia Standard White (ASW) wheat with 11.1% (w/w, wet basis) moisture content (m.c.) harvested in 2014-2015; canola and oats were harvested in 2012-2013 with 8.1% and 9.1% m.c. Almonds with 8% m.c. (Select Harvests Food Products Pty Ltd, Thomastown, VIC) and sultanas with 28% m.c. (Sunbeam Foods Pty Ltd. Irymple, VIC) were purchased from a local supermarket in 2014. The moisture contents were determined using a Graintec HE50 electronic moisture meter, Graintec Pty Ltd (Toowoomba, Australia). The moisture contents obtained were calculated from four replicate measurements.

2.2.2 Reagents and Apparatus

The eight compounds used, their purities and sources, are as follows: Phosphine (PH_3) (Fumitoxin®, 330 g/kg phosphine present as aluminium phosphide, Nufarm, Victoria, Australia), methyl bromide (MeBr) (1000 g/kg, Nufarm, Victoria, Australia), cyanogen (C_2N_2) (1000 g/kg cyanogen, Linde, Australia), sulfuryl fluoride (SF_2O_2) (Dow AgroSciences LLC, US), ethylene oxide (C_2H_4O) (2000 µg/mL in methanol), propylene oxide (C_3H_6O) (+/-)-propylene oxide), ethyl bromide (C_2H_5Br) (reagent grade, ⩾99% balanced with ethanol and water) and ethyl formate (EF) (reagent grade, ⩾97% balanced with ethanol) from Sigma-Aldrich, NSW, Australia. Handling, transferring, and airing fumigants and conducting fumigation must be conducted in a fume hood.

Eight different types of SPME fibers were used for the eight fumigants listed above. These were 100µm polydimethylsiloxane (PDMS), 30µm polydimethylsiloxane (PDMS), 7µm polydimethylsiloxane (PDMS), 85µm carboxen/polydimethylsiloxane (CAR/PDMS), 65µm PDMS/divinylbenzene (DVB), 60µm carboWAX/polyethylene glycol (PEG), 50/30µm DVB/CAR/PDMS and 85µm polyacrylate (PA). A manual holder was used and purchased from Supelco (Bellefonte, PA, USA). The fibers were conditioned following the manufacturer's

recommendations before use and cleaned between different extractions by exposing the fibers to 270ºC for 5 min. in a GC injection port.

Erlenmeyer flasks of 100 mL (Bibby Sterilin, Staffordshire, Cat. No. FE 100/3), each equipped with a cone/screw-thread adapter (Quickfit, STS; Bibby Sterilin) were used for the preparation of standards and extraction of fumigant residues from samples. A 100 μL gastight syringe with a Teflon-tipped plunger (SGE, Austin, Texas, USA) was used to withdraw headspace samples for comparisons with the SPME fiber injection method.

An Agilent 5977E GCMSD system (Agilent Technologies, Melbourne, VIC, Australia) was used to identify and quantify fumigant residues. The Agilent 5977E GCMSD was equipped with a split/splitless injector and an SPME inlet (Supelco, Bellefonte, PA, USA), which operated under splitless mode during the operation. The injection inlet was set at 160°C, and the GC purge valve was set to be switched on 0.5 min after injection. The eight fumigant gases were separated on an Agilent PoraPLOT Q 50 m × 0.32mm × 10μm column. Ultra-High Purity (UHP) helium was used as carrier gas at a constant pressure of 10psi. The initial oven temperature was held at 70°C for 10 min, then ramped up to 150°C at 10°C/min and held for 10 min. The MSD transfer line, the ion source, and the quad-pole temperatures were 200, 230 and 150°C, respectively. The selective ion monitor (SIM) mode was used for monitoring 2-4 MS fragment peaks of each fumigant gas (Table 2.1) with electron energy of 70eV. The solvent delay was set at 7 min. and the dwell time was varied from 50-200 ms depending on the different levels of fumigation concentration.

Table 2.1. The physical properties, mass spectrometers, retention times, and treatment dosage of eight common fumigants

Fumigant (Molecular Formula)	Boiling Point (°C) at 1 atm	Molecular Weight	Mass Spectrometer Fragments (m/z)	Retention Time (min)	Dosage of Fumigant (mg/L)
Sulfuryl fluoride (SF_2O_2)	-55.4	102	102, 83	7.801	25
Phosphine (PH_3)	-87.7	34	34, 33	8.478	1
Cyanogen (C_2N_2)	-21.1	52	52, 26	12.197	50
Ethylene oxide (C_2H_4O)	10.7	44	44, 29, 15	18.112	50
Methyl bromide (CH_3Br)	3.7	95	94, 96, 79	19.563	50
Propylene oxide (C_3H_6O)	34	58	58, 43, 39	24.770	50
Ethyl bromide (C_2H_5Br)	38	109	108, 110, 81	28.408	50
Ethyl formate ($C_3H_6O_2$)	54	74	31, 45, 74	28.797	80

2.2.3 HS-SPME Extraction Procedures

To obtain equilibrium, a 100 mL sealed flask containing 35 g of sample was placed in a water batch with a shaker for 5 min at 250 rpm. Each type of SPME fiber was exposed in the gastight flask at 35°C for 25 min. At the end of the defined extraction time, the fiber was withdrawn from the headspace into the needle. The fiber holder was removed from the extraction flask and inserted into the injection port. The fiber was extended into a GC inlet, where sample components were desorbed and injected into the GCMSD to determine the residue concentrations.

2.2.4 Preparation of Spiking Samples

Spiked wheat and canola samples were chosen as presentive and prepared by adding appropriate volumes of each of the eight fumigants into separate sealed flasks (100 mL) containing the samples to achieve concentrations of 0 (untreated blank), 0.3, 1, 3, and 10 ng of fumigant/g of grain.

2.2.5 Aged Fumigation Samples Preparation and Analysis

The fumigation dosages of sulfuryl fluoride (SF), phosphine (PH_3), cyanogen (EDN), ethylene oxide (EO), methyl bromide (MB), propylene oxide (PO), ethyl bromide (EB) and ethyl formate (EF) were 25, 1, 50, 50, 50, 50, 50 and 80 mg/L, respectively. In order to obtain samples containing aged multi-fumigant residues in samples, wheat, canola, oats, almond, and sultanas were fumigated in 100 mL flasks with phosphine and sulfuryl fluoride for 5 days then aerated for 7 days; and with cyanogen, methyl bromide, ethylene oxide, propylene oxide, ethyl bromide and ethyl formate for 1 day then aerated for 7 days. All aerations were conducted in a fume hood.

For monitoring fumigation concentration, 60 µL of head space was redrawn from 100 mL flask and transferred to GC injection port by a 100 µL airtight syringe. The HS-SPME extraction procedure of eight fumigation residues in aged samples was the same as described in 2.3.

2.2.6 Statistical Analysis

Measurement of GCMS peak areas was replicated 3 times and averaged. The variation of the peak areas, concentration levels, and standard deviations were calculated by Microsoft Excel 2010 Software and Statistic software OriginPro 7.5 SR1 (v7.5776).

2.3 RESULTS AND DISCUSSION

2.3.1 Optimisation of the HS-SPME-GCMS Trace Analysis Method

The eight SPME fiber coatings, and a range of extraction temperatures (23-45°C), times (5-90 min), desorption temperatures (150-250°C) and times (1-5 min) were investigated to determine the optimum operation procedures. The eight differently coated fibers were exposed in the diluted authentic standard at 23°C for a 30 min extraction. The 75µm carboxen/polydimethylsiloxane (CAR/PDMS) and 50/30µm DVB/CAR/PDMS fibers were more efficient at extracting the fumigant residues than the other SPME coatings (Figure 2.1). However, carboxen/ polydimethylsiloxane (CAR/PDMS) was the most effective fiber for extraction of high boiling point fumigant residues, e.g., propylene oxide, ethyl bromide and ethyl formate, and the 50/30µm DVB/CAR/PDMS fiber was the most effective for extraction of an expanded range of analytes with low boiling points, including, sulfuryl fluoride and phosphine. In general, divinylbenzene (DVB) and polydimethylsiloxane (PDMS) fibers were useful to capture phosphine and sulfuryl fluoride gas molecules. The length of the fiber can significantly affect the extraction of the total amount of fumigant, such as in this experiment, 50/30µm DVB/CAR/PDMS fiber (2 cm) was twice as long as the other fibers (1 cm), and in some cases, it can double the amount of fumigant captured from the headspace (Figure 2.1).

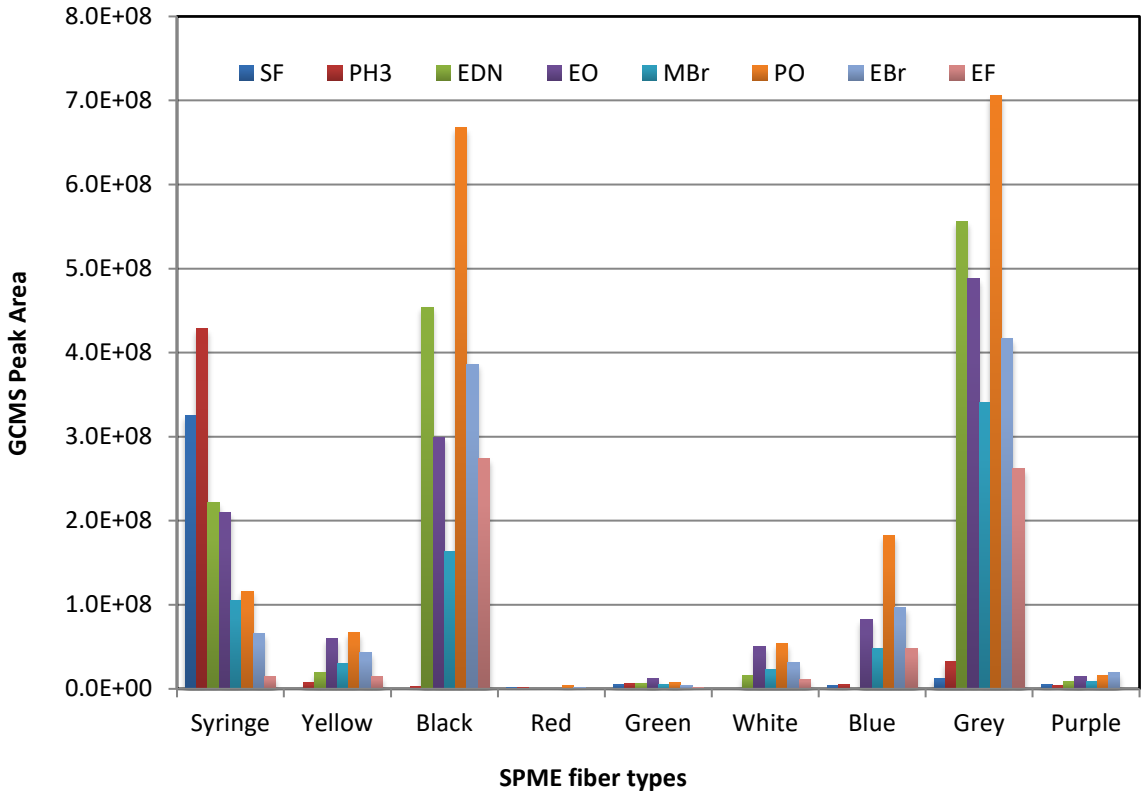
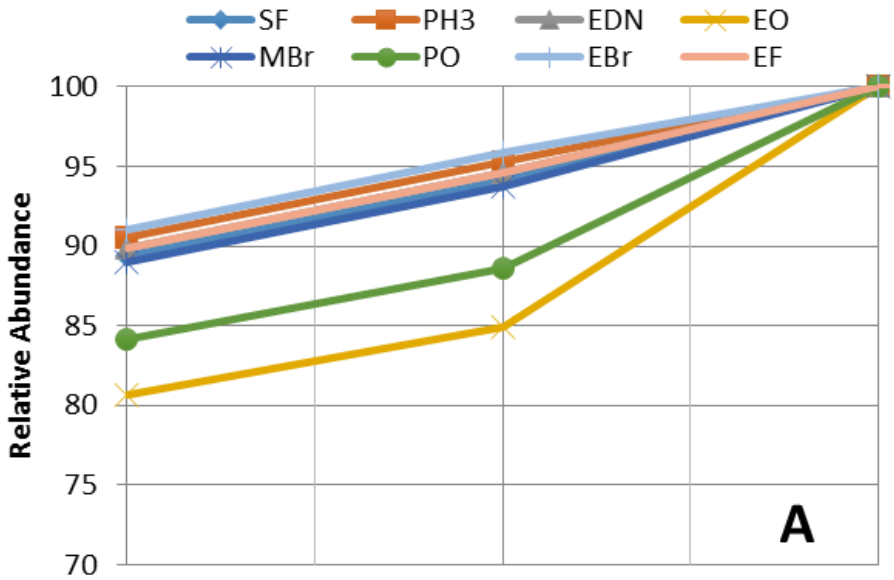


Figure 2.1. GC readings from a combination of eight fumigants (SF; PH_3; EDN; EO; MBr; PO; EBr; EF) extracted by eight different fibers after a 30 min extraction at 23°C. Where: Red fiber is 100µm Polydimethylsiloxane (PDMS), Yellow fiber is 30µm Polydimethylsiloxane (PDMS), Green fiber is 7µm Polydimethylsiloxane (PDMS), Black fiber is 75µm Carboxen/Polydimethylsiloxane (CAR/PDMS), Blue fiber is 65µm PDMS/Divinylbenzene (DVB), Purple fiber is 60µm CarboWAX/Polyethylene Glycol (PEG), Gray fiber is 50/30µm DVB/CAR/PDMS and White fiber is 85µm Polyacrylate (PA).

Compared to the performance between optimised SPME fibers and conventional syringe injection, the conventional method was 10 times more efficient for two lower boiling point fumigants of sulfuryl fluoride (-55.4°C) and phosphine (-87.7°C). This is because the SPME fibers extraction of fumigants is a physical equilibrium absorption process. The efficacy of extraction or physisorption highly depend on the boiling point of fumigants, e.g., the lower the extraction temperature, the more fumigant will be absorbed on the SPME fiber. This can be explained by the fact that the 75µm CAR/PDMS and the 2 cm 50/30µm DVB/CAR/PDMS absorbed the compounds with the higher boiling points at the higher partition coefficients in solid phase (e.g., methyl bromide, cyanogen, ethylene oxide, propylene oxide, ethyl

bromide and ethyl formate) and were more efficient than that conventional syringe injection. However, the HS-Syringe method was more sensitive than the HS-SPME method for analysis of phosphine in normal spiked standards. However, the opposite occurred when the aged samples were analysed, consistent with Ren and Padovan (2012). Therefore, considering the above factors and results, the 2 cm 50/30µm DVB/CAR/PDMS fiber was selected for further investigation in this report.

Since the highest boiling point of fumigant used in this investigation was ethyl formate with 54°C, the effect of temperature on the extraction of fumigant residues was evaluated at 23, 35, and 45°C. The chromatograph peak areas of the high boiling point fumigant that were extracted decreased by half from 23 to 45°C, but the extraction efficiencies for low boiling point fumigant were increased above 35°C, especially for phosphine. The syringe injection method was less sensitive to the extraction temperature than the SPME fiber method (Figure 2.2). This phenomenon is probably partly due to the equilibrium partition between the SPME fiber absorption property and the individual gas partial pressures. The fumigant gas partial pressure in the headspace decreased with increasing temperature of fumigated samples; therefore, the SPME fiber can extract more fumigant molecules during the extraction period. However, the efficacy of the SPME fiber absorption ability was reduced with increasing extraction temperature, except phosphine and cyanogen (Figure 2.2). Therefore, an extraction temperature of 35°C was chosen as optimal for the HS-SPME extraction of multi-fumigant residues.



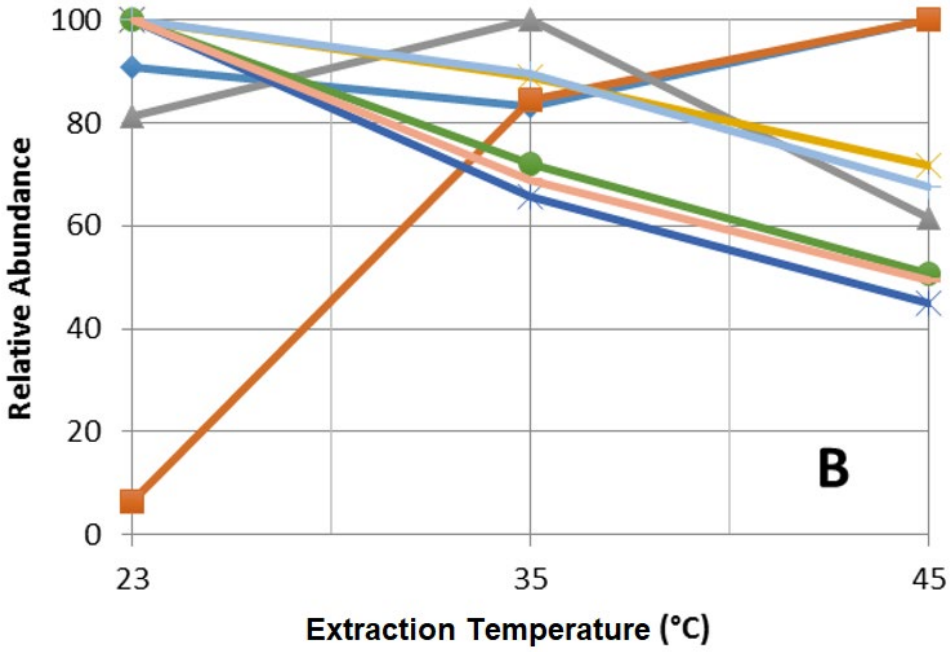


Figure 2.2. Comparison of the effect of temperature on the efficacy of the two different methods of residue extraction from a combination of 8 fumigants (SF; PH_3; EDN; EO; MBr; PO; EBr; EF). A: HS-Syringe injection method, B: HS-SPME extraction with a 50/30µm DVB/CAR/PDMS fiber after 30 min extraction.

The longer the extraction time of the SPME fiber in the headspace, the more fumigants were absorbed on the surface of the fiber (Figure 2.3). However, the rates of increase were varied depending on the fumigant boiling points. For example, the GCMSD readings of propene oxide and ethyl formate with boiling points of 34 and 54°C (relevant high boiling point fumigant) were increased more than 30% although extended for only 15 min of extraction time. However, absorption rates of other tested lower boiling point fumigants were increased less than 15% over the range of increasing extraction times. This was especially the case for phosphine, and there was no change in absorption on the SPME fiber during the time extension for 30 min. However, it was necessary to have a longer extraction time for fumigant compounds with higher boiling points, such as ethylene oxide, propylene oxide, methyl bromide, and ethyl formate. Therefore, a 30 min extraction time would be the most efficient to be considered for practical operation purposes.

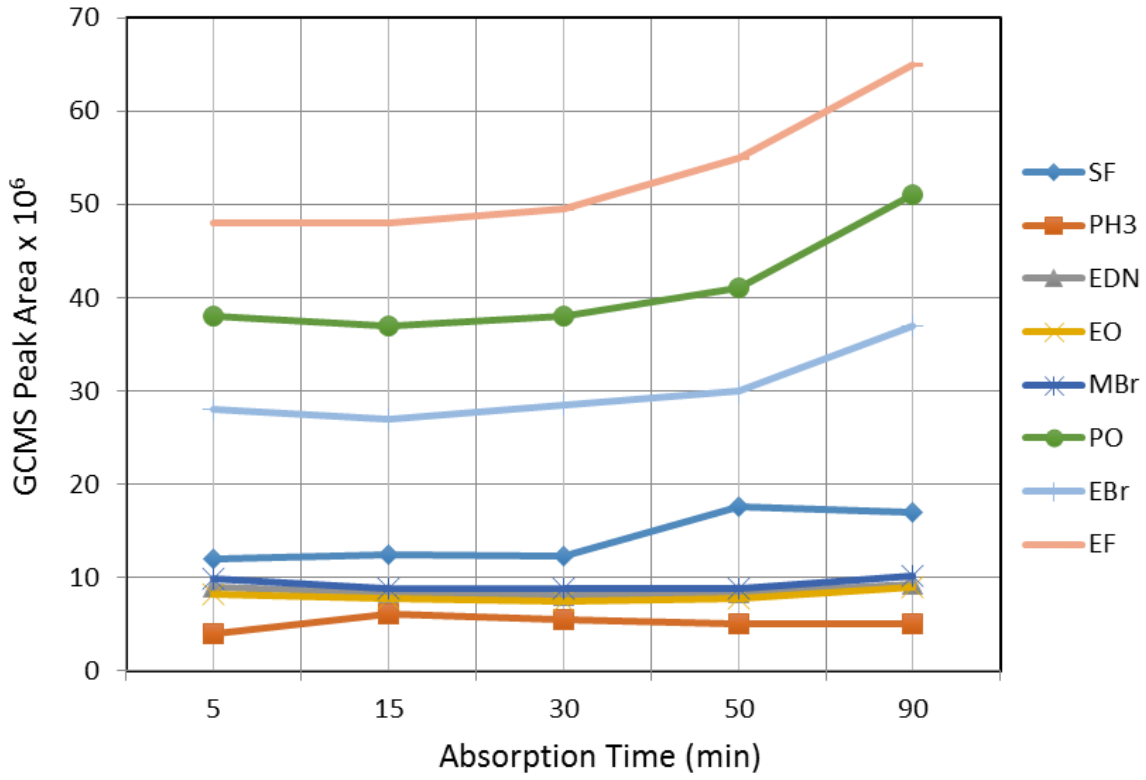


Figure 2.3. Effect of time on the HS-SPME extraction for 8 fumigants (SF; PH_3; EDN; EO; MBr; PO; EBr; EF) residues with 50/30µm DVB/CAR/PDMS fiber at 23°C.

Peak areas of detected fumigant decreased with increasing injection port temperature from 160°C to 250°C (Figure 2.4) (250°C was the medium operational temperature recommended by the manufacturer for the 50/30µm DVB/CAR/PDMS fiber). Higher injection port temperatures lead to fumigants being quickly and completely desorbed from the SPME fibers. However, higher sensitivity of the GC at higher injection port temperature did not occur in the case of phosphine, where sensitivity is reduced to 60% at 250°C. The average decline rate of most fumigants was 22% at 250°C (Figure 2.4). Also, the peaks of methanol (retention time 25.56 min) and ethanol (retention time 17.86 min) increased when the temperature increased from 200°C to 250°C. The methanol and ethanol fragments were degraded from analytes, such as propene oxide, ethylene oxide and ethyl formate at high temperature. Therefore, the GC injector temperature was set at 160ºC for optimal desorption.

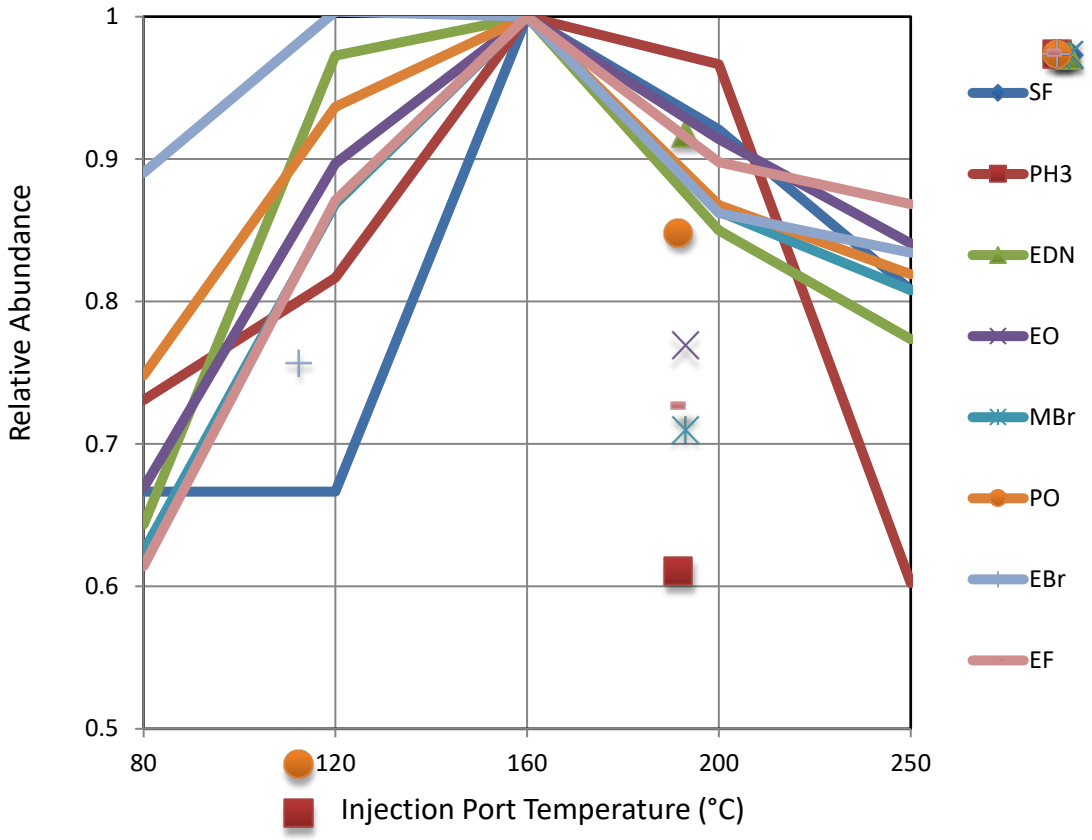


Figure 2.4. Effect of gas chromatograph injector temperature on the desorption of 8 fumigant residues (SF; PH_3; EDN; EO; MBr; PO; EBr; EF) from the 50/30µm DVB/CAR/PDMS fiber after a 30 min at 23°C.

The time of desorption of the fumigant from the HS-SPME fiber was decided by re-injecting the 50/30µm DVB/CAR/PDMS fiber into the GC injection port at 160°C for 1, 3, 5 and 30 min, and measuring the remaining relative abundances. Most fumigant absorbed on the fiber was desorbed completely in 3 min. Less than 0.5% of the relative abundance of seven absorbed residues were left after 1 min of desorption, except for sulfuryl fluoride with a relative abundance of 11.15% (Figure 2.5). After 5 min, the average relative abundance dropped to below 0.03%, with sulfuryl fluoride at 1.99%, and after 30 min, there were no residue peaks detected. It appears that the mechanism of absorption and desorption of fumigant was not related to the boiling point, but rather to the polarity and size of the fumigant molecules. Therefore, the fiber was desorbed for 5 min at 160°C to ensure complete desorption of fumigants from the fibers.

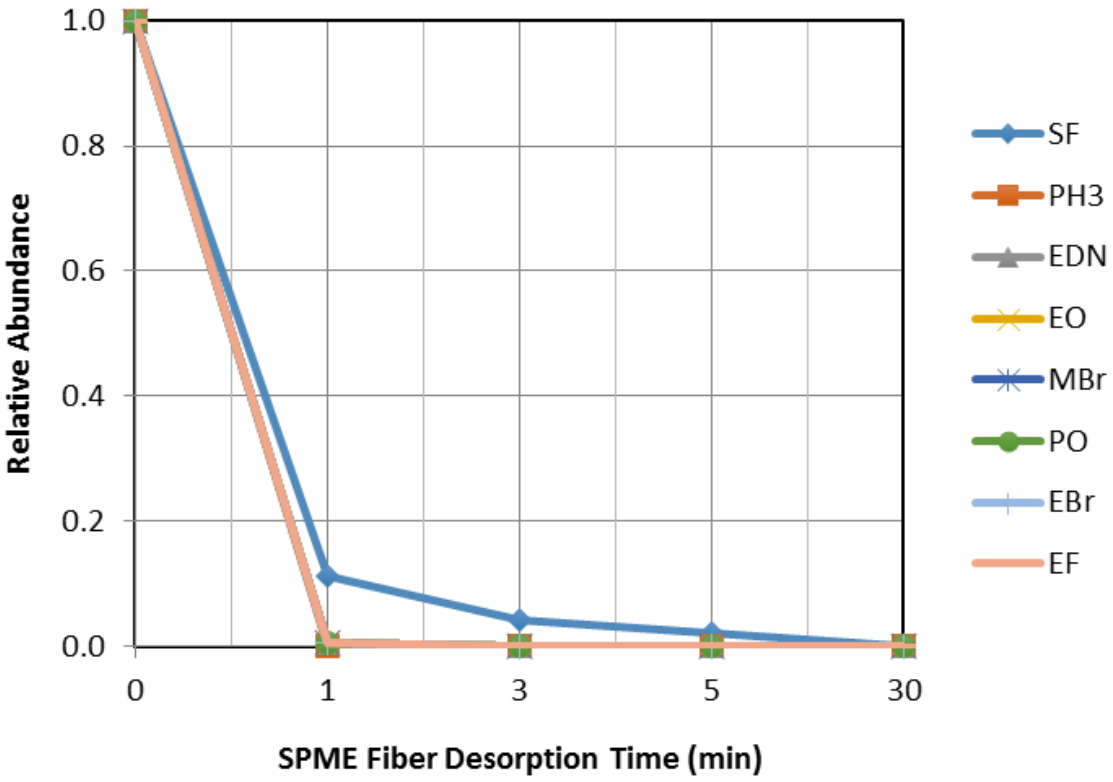


Figure 2.5. Effect of gas chromatograph injector dwell time on the desorption of 8 fumigant residues (SF; PH_3; EDN; EO; MBr; PO; EBr; EF) from the 50/30µm DVB/CAR/PDMS fiber after extraction at 23°C.

2.3.2 Calibration Curve, Repeatability, Limit of Detection (LOD) and Limit of Quantification (LOQ)

The GC response to the eight fumigants tested showed a wide range (0.005-13.75 ng/kg) >103 of linear relationships (r^2=0.9983-0.9999) to the diluted authentic standards (Table 2.2). The regression equations and correlation coefficients for each fumigant were calculated along with Limits of Detection (LOD) and Limits of Quantification (LOQ) (Table 2.2). The LODs and LOQs were determined experimentally by injecting the serial dilutions of authentic standards into sealed flasks (100 mL) containing wheat samples (35 g) and were defined as 3 and 10 times the standard deviation of measurements of the lowest standards close to the LOD estimated for an individual fumigant. The LODs of the multi-fumigants were in the range of 0.03 to 1.99 ng/g. The significance of the linear regressions (r^2) was greater than 0.9983. Repeatability of the 3 ng/g aged wheat spiking standard (n=4) is shown in Table 2.3. The relative standard deviations (RSDs) were less than 8.7%, except for sulfuryl fluoride which LOD was 1.99 ng/g and RSD value was 39.7% (6.64 ng/g).

Table 2.2. Parameters of linear regression analysis, and Limits of Detection (LOD, n=4) and Quantification (LOQ, n=4) of eight fumigant residues extracted by HS-SPME using a 2 cm 50/30µm DVB/CAR/PDMS fiber at 25°C for 30 min and detected by GCMSD

Fumigants	Calibration Curve	r^2	Range of Test (ng/g)	LOD (ng/g)	LOQ (ng/g)
Sulfuryl fluoride	y=8.7476χ-49.082	0.9999	0.129-12.87	1.99	6.64
Phosphine	y=6.2876χ-191.28	0.9983	0.043-4.29	0.47	1.55
Cyanogen	y=373.81χ+841.87	0.9999	0.006-6.56	1.09	3.65
Ethylene oxide	y=56.53χ+457.91	0.9994	0.005-5.55	0.05	0.16
Methyl bromide	y=976.71χ+17372	0.9992	0.120-11.98	0.47	1.57
Propylene oxide	y=1801.1χ+25322	0.9986	0.007-7.32	0.06	0.21
Ethyl bromide	y=3827.6χ+53545	0.9986	0.014-13.75	0.03	0.08
Ethyl formate	y=7109.1χ+59698	0.9996	0.009-9.33	0.08	0.27

Table 2.3. Relative standard deviations from analysis of samples treated at 3 ng/g of fumigant n=4

	GC Peak areas				Mean	STD	RSD
Fumigants	1st	2nd	3rd	4th			**(%)**
Sulfuryl fluoride	98.0	147.0	54.0	88.2	96.8	38.4	39.7
Phosphine	204.4	192.7	235.8	206.7	209.9	18.3	8.7
Cyanogen	7062.9	6749.1	7038.7	6897.3	6937.0	145.0	2.1
Ethylene oxide	9601.3	9520.4	9481.3	9695.0	9574.5	94.6	1.0
Methyl bromide	4291.3	4369.0	4518.7	4038.2	4304.3	200.9	4.7
Propylene oxide	31028.2	30194.3	29314.8	29105.2	29910.6	881.9	2.9
Ethyl bromide	57005.1	58068.3	55293.2	55825.2	56548.0	1240.6	2.2
Ethyl formate	268498.8	256481.3	253398.5	250684.4	257265.8	7854.2	3.1

2.3.3 Extraction Temperature of Aged Residue Samples

While the extraction temperature of fumigant was previously optimal at 35°C, this was re-evaluated to assess the temperature effect on the extraction of fumigant residues from aged samples. The repeat extraction temperature experiments were conducted using the fumigated wheat and canola samples that had been aired for 48 hr and then resealed. There were no differences in optimum temperature for the extraction of the fumigants between the freshly prepared spiking samples and fumigated aged residue samples, except for phosphine. Where there are ultra-low levels of residues, the SPME fiber can affect the partition coefficient between headspace and sample, which the use of a syringe cannot do. In this case, the SPME fiber trapped and accumulated phosphine gas molecules in the headspace. As the concentration of phosphine dropped, more phosphine gas molecules held in the food matrix dispersed into the headspace. The effect of high temperature of the fumigant extraction process decreased the SPME fiber performance, but it increased the amount of fumigant that escaped from the food matrix.

2.3.4 Sorption Study of Fumigant Residues in Different Food Matrix

The sorption study of residues in the range of different food matrices was carried out using the 3 ng/g spiking standard. All food matrices had similar absorption and desorption curves over time, but only the spiking standard for sultanas had a different absorption curve (Figure 2.6). This is because the commercial sultanas used in this study were covered by edible oil, and so formed a non-absorption substrate. It can be seen from Figure 6B that if the wheat, canola, oats, and almonds were considered as non-absorption substrates as well, the fumigant residues would be overestimated. Adamson (1990) reported that chemisorption might be slow and the rate behavior indicative of the presence of an activation energy; it may be possible for a gas to be physically adsorbed at first, and then, more slowly, to enter into some chemical reaction with the surface of the solid. Chemisorption is one kind of chemical reaction, so it should be reversible.

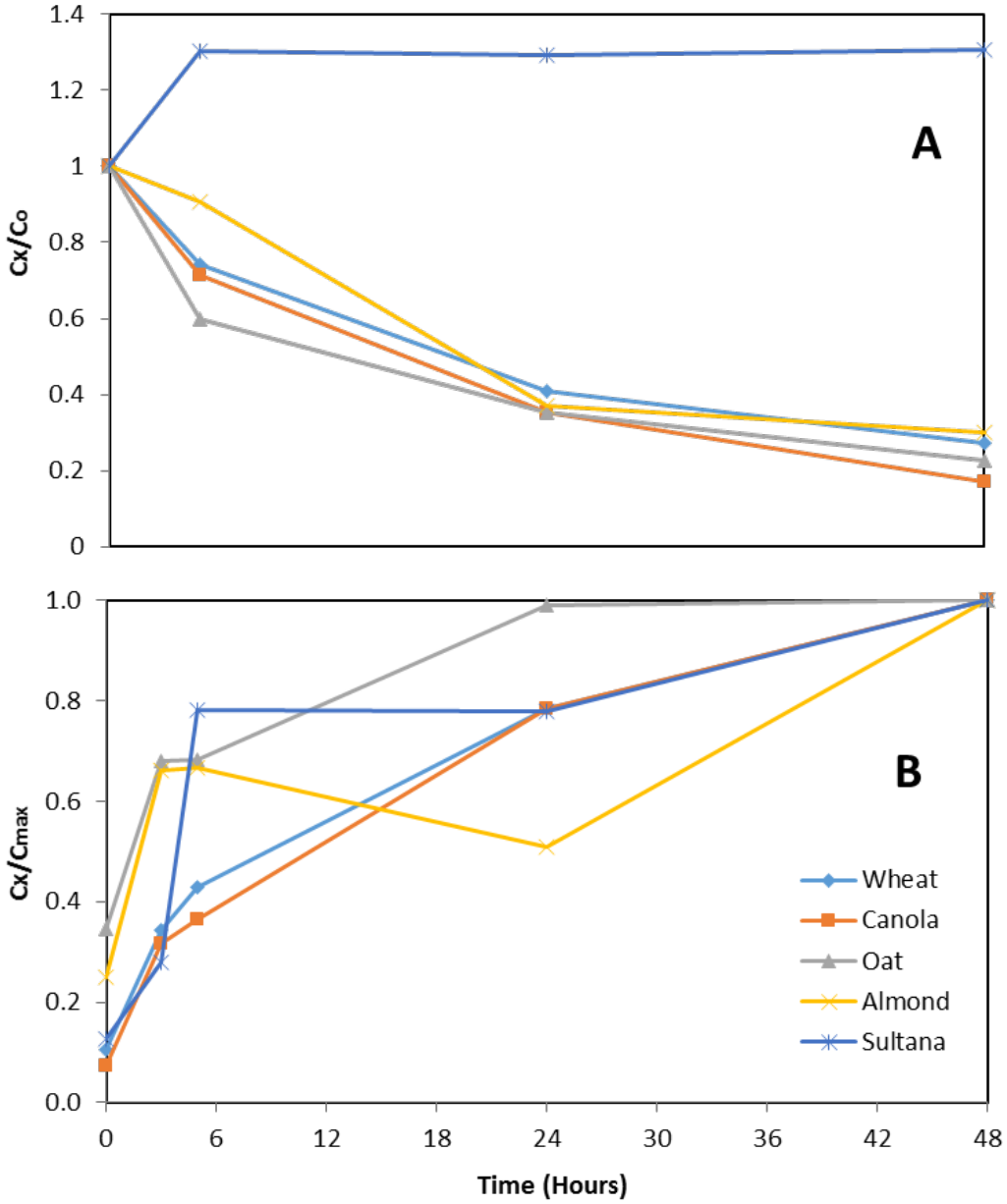


Figure 2.6. Absorption (A) and desorption (B) of fumigant over time using aged samples of 3 ng/g spiking fumigant standards on wheat, canola, oats, almonds, and sultanas. Where Cx/Co is the ratio of concentration in the headspace to total calculated applied concentration, and Cx/Cmax is the ratio of concentration in the headspace to the highest concentration.

Four processes were considered: physical desorption, chemical desorption, physical absorption, and fumigant degradation. During the aged sample desorption process, the fumigants were observed to quickly escape from the food matrix in the initial 3-5 h and increased steadily over 24 or 48 h. Significant degradation then

occurred in the hydrocarbon fumigants after 48 h, e.g., ethyl formate. Physical desorption transitioned to chemical desorption at around 5 h when desorption started with the sealing of the aged samples. In the absorption process of spiking standards, wheat, canola and oats had the same transition point at around 5 h. However, it was not obvious in almonds which have a much smaller surface area due to their larger individual size and the high commodity density. The long equilibrium time compared to other analytical methods avoided underestimating the fumigant residues. The total fumigant residue concentration after 5 h of equilibration was higher than after 0.5 h by 2.9-6.9 times (Table 2.4).

Table 2.4. The levels of total fumigant residues in a range of food matrix samples after 0.5 and 5 hours equilibrium

Matrixes	Levels of total fumigant residues (ng/g) at time of equilibrium		Ratios*
	0.5 (Hours)	5 (Hours)	
wheat	2.1	11.5	5.5
canola	1.4	9.7	6.9
oats	34.7	114.3	3.3
almond	0.0	0.1	2.9
sultanas	0.1	0.5	4.7

$$*Ratios = \frac{\text{The level at 5 hours equilibrium time}}{\text{The level at 0.5 hour equilibrium time}}$$

2.4 CONCLUSION

Initially, two methods were considered: Aged External Standard and Standard Addition. Due to the time delay in SPME extraction and the fast absorption rate, the standard addition method was considered not practical for this study. Hence, aged fumigation food samples were analysed by the method discussed above (Table 2.5). To summarise the explanations for the varied results:

I. The low fumigant residue levels of almonds were due to the high density to surface area ratio.

II. The low fumigant residue levels of sultanas were expected because of the substrate's homogenous character and high density to surface area ratio.

III. Finally, the high residue levels of cyanogen may be due to the conversion of hydrogen cyanide to cyanogen in the food matrix.

Table 2.5. Levels of fumigant residues in wheat, canola, oats, almond and sultanas treated with eight fumigants

Fumigant Residues	Concentration of fumigant residues in 3-week aged fumigation samples (ng/g)				
	Wheat	Canola	Oats	Almond	Sultanas
Sulfuryl fluoride*	0.29	1.16	0.00	0.00	0.00
Phosphine*	6.51	0.71	0.00	0.00	0.00
Cyanogen	852.33	91.03	1500.09	0.02	0.07
Ethylene oxide	3.28	4.54	0.00	0.00	0.00
Methyl bromide	3.39	1.14	4.45	0.02	0.03
Propylene oxide	7.32	2.80	5.46	0.00	0.00
Ethyl bromide	12.95	26.71	8.07	0.01	0.01
Ethyl formate	25.96	16.50	11.19	0.03	0.00

* According to industrial protocol, Sulfuryl fluoride and Phosphine are fumigated for 5 days. Other fumigants were used for 1 day.

An innovative multi-fumigant residue detection method based on HS-SPME sampling technology with GC-MSD analysis was established. The method was highly sensitive, was reproducible, and provided simultaneous confirmation of the identity of the individual fumigants and a quantification of the residues present in the different food matrices. The limits of quantitation (0.08-6.64 ng/g) were, in all cases, significantly lower than the MRLs established for wheat, canola, oats, almonds, and sultanas (APVMA 2012). The proposed method could be adapted to other fumigant residues with more accurate results and better performance than the currently used syringe injection method, except in the case of sulfuryl fluoride. It is also possible, in the future, to develop this method into a universal and simultaneous fumigant residue analysis method. This is the first report of a sorption study of residual fumigant levels in heterogeneous food matrices by a HS-SPME-GCMSD method.

3 The Physical Effect of Synthetic Amorphous Silica (SAS) Powders on *Tribolium castaneum* and *Rhyzopertha dominica*

ABSTRACT

Previous research has developed and identified a novel use for existing synthetic amorphous silica (SAS) powders. In this study, the effect of storage temperature, grain moisture content, and relative humidity on the efficacy of hydrophobic (HB) and hydrophilic (HL) synthetic amorphous silica (SAS) powders were evaluated. SAS powders proved highly efficacious against four key species of grain storage pests. However, it has been theorized that SAS powders may have a novel mode of action related to their relatively small particle size coupled with strong particle charge, another novel feature of SAS powders that promotes sorption. The physical effect of synthetic amorphous silica (SAS) powders was explored on *Tribolium castaneum* and *Rhyzopertha dominica.*

The loss in body weight of *T. castaneum* exposed to SAS powders showed that the body weight loss was more than the water loss by over 13%, indicating the loss includes body water and volatile metabolites produced during dehydration. Also, the weight loss rate of freeze-killed insects was higher than live controls between the second and seventh day, which implies the cuticle barrier can only protect insects from desiccation when energy metabolism is active. Scanning electron microscopy showed that the SAS powder attached at the opening of the spiracles at the apex of the abdomen. X-ray micro-computed tomography (Micro-CT) showed that the SAS powder could penetrate and block the tracheal system, leading to the collapse of the internal organs.

The bioassay examined how the environmental factors, grain moisture content (11.4% and 13% m.c.) and relative humidity (50% and 70%) affected the efficacy of SAS on *R. dominica* and *T. castaneum* negatively. Higher storage temperature at 35°C, higher effectiveness of SAS against both species were observed.

This study demonstrated that both types (HL and HB) of SAS powders are promising alternatives to chemical fumigants to control storage insect pests. A higher dose rate for *R. dominica* might be needed under high relative humidity in wheat grains with higher moisture content.

3.1 INTRODUCTION

Australia is at imminent risk of losing the grain disinfestation chemicals that currently underpin $7.8 billion worth of grain exports. Fumigation is widely used as an effective method to manage pests from small to large-scale grain silos. Many gaseous chemicals have been developed and registered as fumigants worldwide, such as phosphine (PH_3), methyl bromide (MB), sulfuryl fluoride, carbonyl sulfide, ethyl formate, and ethanedinitrile (EDN) (Bond 1984; Hooper *et al.* 2003; Bartholomaeus and Haritos 2005; Ren and Mahon 2006; Sriranjini and Rajendran 2008; Athanassiou *et al.* 2015). However, only phosphine has been identified and recognized worldwide for the disinfestation of stored grain insects for international grain trade after restrictions to the use of methyl bromide were imposed by the Montreal Protocol (UNEP 1987; Chaudhry 2000) because of its detrimental impact on the environment. The phenotyping of phosphine resistance in grain storage pests has been detected in many on-farm and bulk handler sites in Western and eastern Australia (Jagadeesan and Nayak 2017). Fumigants are cost-effective and easy to apply and have assisted the industry in achieving the 'nil tolerance' status for grain insect pests that have guaranteed Australia's international market access over many years.

However, the use of toxic fumigants is now under threat from a combination of increased global market sensitivity to residues, developing chemical resistance in key grain insect pests, and potential changes in OH&S and security legislation. Due to hurdles relating to toxicity, residues, the environment, and work safety issues, it is almost impossible to register new chemicals to control grain storage pests. Furthermore, fumigation is limited to sealed storage structures, accounting for about 30% of the Australian storage facilities. This change has placed increasing pressure on grain management during transport, exacerbated by farmers storing larger volumes of grain on-farm. Therefore, the industry urgently needs new technologies to protect grain during transport and respond to the pressures of deregulation.

As mentioned above, to safeguard market access, the grain industry needs to develop practical and cost-effective non-chemical technologies to manage grain in storage and transport. Diatomaceous earth (DE)-based insecticides are obtained from geological deposits of diatomite, which are fossilized sedimentary layers of microscopic algae called diatoms (USGS 2021). The second form of silica used in insect control is amorphous silica (AS), made by heating ordinary sand to a very high temperature. Attempts to seek inert dusts to replace toxic chemicals against insect pests date to the 1920s. The use of conventional inert dusts, such as diatomaceous earth (DE), in stored product pest management is restricted by several industrial concerns relating to its low efficacy and impacts on human health (Bodroža-Solarov *et al.* 2012). Due to the high dose rates needed, the small loading angle of bulk grain flow limits inert dust application in bulk handlers' facilities (Golob 1997). In response to these concerns, food-grade synthetic amorphous silica (SAS) powders have been developed and introduced to large commercial scale bulk handling systems in China and Australia (Ren and Agarwal 2014; Perišić *et al.* 2018). Therefore, SAS powders can potentially be used as a phosphine resistance breaker in central bulk handling systems.

Diatomaceous earth products have been considered as a technology for bulk storage use for many years, but this interest has not translated into widespread adoption and use. This is because the previous products were not cost-effective and had a negative effect on the grain storage industry's ability to store and move grain. Specifically, the products resulted in reduced bulk density and flowability of grain, very long insect kill times, low efficacy against some insect species, application difficulties to apply, and reduced efficacy at high moisture contents. In addition, several factors are known to affect the effectiveness of DE, including relative humidity, temperature, geological source, insect species, insect life stage, the strain of insect, and grain and insect density (USGS 2021; Waqas *et al.* 2010; Korunic *et al.* 1998, Kostyukovsky *et al.* 2010 and Athanassiou *et al.* 2008). For example, the moisture content of maize grain significantly affects the efficacy of inert dusts, which could achieve superior control of *Sitophilus zeamais* Motsch when it did not exceed 12% (Vayias and Stephou, 2009).

Synthetic amorphous silica (SAS) powders are recognized for their insecticidal activity. SAS powders, categorized as a type of inert powder are composed of nanometer-sized primary particles, of nano- or micrometer-sized aggregates and

agglomerates in the micrometer-size range, and may provide a promising alternative strategy to manage pests (Wang *et al.* 2011 and Cai *et al.* 2010). These powders have shown low toxicity towards mammals, little potential for insect resistance, and a good residual effect (Fields 2002). Food grade SAS causes exposed insects to dehydrate more efficiently than conventional DE dusts, but death by desiccation is difficult to explain. There are several proposed modes of action (MoA) for inert dusts, including SAS and DE, to control stored grain insects and these are related to water loss and cuticle impairment (Wigglesworth 1944; Subramanyam and Roesli 2000). Therefore, the insecticidal efficacy of the SAS powder-based insecticides might vary with temperature and relative humidity. Previous studies have reported that their performance was reduced as ambient humidity increases and temperature decreases (Cao *et al.* 2010; Wang *et al.* 2011 and Baliota *et al.* 2022).

The mechanism(s) of action of SAS powders has not yet been fully elucidated. However, they are thought to act differently to inert dusts like silica gel, nanostructured alumina, and diatomaceous earth (DE). This study aimed to systematically investigate the influence of temperature, moisture content, and relative humidity on the effectiveness of SAS powders to control storage insects in grain. In addition, the experiment results underpinned the significance of the loss of organic chemicals, which may be more crucial than water loss.

3.2 MATERIALS AND METHODS

3.2.1 Insects

Insects used in this study were the lesser grain borer, *Rhyzopertha dominica* (F.) and the red flour beetle, *Tribolium castaneum* (Herbst). The *T. castaneum* included phosphine susceptible (MUTC1) and resistant (MUTC-RS) strains, and the *R. dominica* was the phosphine susceptible (MURD2) strain, all held at the Post-harvest Plant Biosecurity and Food Safety Laboratory, Murdoch University, Western Australia, Australia. All test species were established by adding adults (400-500) to media (1 kg) at 25°C and 65% R.H. The *T. castaneum* culture was established on a medium comprising 1-part baker's yeast and 12 parts wholemeal flour milled from Australian soft wheat (var. Rosella). The *R. dominica* was established on a medium

containing 40 parts wheat and 1-part wholemeal flour. The new generations of mixed age (1-2 weeks) adults were used.

For preparing clean and undamaged insects, a 9 cm diameter filter paper was left in the 2 L glass jars for approximately 30 mins to allow the insects to cling onto the paper. The paper was then flipped over directly above moist tissue paper, and gently tapped twice to separate the insects from their respective food sources. The insects were then cleaned further by transferring them to wet Kleenex® tissue paper (PN: 4456, 305 × 240mm, Kimberly-Clark Worldwide, Inc., Australia), and then placed in a plastic food display tray (456 × 318 × 57mm, NALLY, Australia). Adults that climbed off the wet tissues were collected and transferred to a clean 500 mL glass bottle with a mesh lid for experiments. This procedure was repeated several times until 50 adult insects per sample were obtained.

3.2.2 Synthetic Amorphous Silica (SAS) and Diatomaceous Earth Powder

Two synthetic amorphous silica powders were used. These were a hydrophilic SAS (HL-SAS) and a hydrophobic SAS (HB-SAS). The HL-SAS and HB-SAS consist of extremely fine white solid powder silicon dioxide. HL-SAS (purity >99.9% w/w) with a Brunauer–Emmett–Teller (BET) theory surface area of 260-280 m^2/g, an oil-carrying capacity of 280-300 mL/100 g, and an average diameter of 5µm. The HB-SAS (purity >99.9% w/w) is surface modified with polysiloxane with a surface area >230 m^2/g, an oil-carrying capacity >350 mL/100 g, and an average diameter of 4.5-5.5µm.

Natural diatomaceous earth, Dryacide™ (DE) (Purity >90% w/w, Entosol, NSW, Australia), was used as the reference material. The supplier did not provide the surface area and the oil-carrying capacity. However, the typical surface area and oil-carrying capacity of similar diatomaceous earth products were reported in the range of 1-23.8 m^2/g (Tsai *et al.* 2006; Nattrass *et al.* 2015) and 150.5-188.2 mL/100 g (Subramanyam and Roesli 2000; Korunic *et al.* 1997), respectively.

3.2.3 Electron Imaging Scan of SAS Treated *Rhyzopertha* Spiracle and the Apex of the Abdomen

To determine the penetration of powder into the spiracles, which are covered by wings and sternal plates, treated *Rhyzopertha* specimens were coated with gold and observed under a scanning electron microscope Zeiss 55VP (SEM) located at the

Centre for Microscopy, Characterisation and Analysis (CMCA), The University of Western Australia (Figure 3.1). The SEM can perform X-ray microanalysis using Oxford Instruments X-Max 80 silicon drift EDS system with AZtec and INCA software, to analyze elements in the range Be to Pu.

Figure 3.1. High-resolution scanning electron microscope (SEM) Zeiss 55 VP-SEM used for scanning the SAS treated adult *R. dominica*.

3.2.4 X-Ray Micro-Computed Tomography (Micro-Ct) Scanning SAS of Treated and Untreated *T. castaneum* Tracheal Systems

A Zeiss Versa XRM-520 was used to scan SAS treated and untreated *T. castaneum* to create high-resolution 3D imaging at the Centre for Microscopy, Characterisation and Analysis, University of Western Australia. The Zeiss Versa XRM-520 system (Carl Zeiss Pty Ltd, North Ryde, Australia) (Figure 3.2) is a 3D X-ray microscopy optimised for non-destructive microtomography with a versatile combination of sub-micron resolution and contrast, sample flexibility, and the large working distance required to address emerging research challenges. The X-ray source was 20-90kV with a 9, 18 and 35µm pixel size resolution. Zeiss Avizo Fire 3D tomography image reconstruction software was installed on a high-speed workstation with graphics acceleration GPU for visualisation and data processing.

Figure 3.2. The Zeiss Versa XRM-520 system for scanning SAS treated *T. castaneum* and *R. dominica*.

3.2.5 Treatment of Insects with SAS and DE Powder

The dosages used were 0.5 g/m^2 for both SAS powders and 10 g/m^2 for DE. For the SAS treatments, 1.2 mg SAS powder was deflocculated and collected in 55mm diameter 50 mL glass jars and gently shaken for 5 seconds. For the DE treatment, 24 mg Dryacide DE was sieved through a 250-µm wire mesh into a 50 mL glass jar. Clean empty glass jars were used as controls. Clean and undamaged adult *T. castaneum* and *R. dominica* were added to the glass jars at 0.1 g per jar (approximately 60 and 100 adults for *T. castaneum* and *R. dominica*, respectively. Each experiment was duplicated and conducted at 23±1°C and 65±5% R.H.

3.2.6 Time-body Weight Loss Bioassays

Fifteen live *T. castaneum* adults were treated as above, then transferred into a 2 mL self-standing polypropylene tube (HS10060, Sigma-Aldrich, Castle Hill, NSW, Australia) with duplication for each treatment. *T. castaneum* adults from the same batch of insect culture killed by freezing were also prepared and stored in liquid nitrogen for metabolites analysis. Sample tubes were filled with SAS powders without insects as an interference control sample. The initial weights of tubes without lids,

insects and SAS particles were recorded individually. The mass changes of each sample tube were weighed at 2, 4, 7, 14 and 21 days. Before and after weighing samples, a 1 g certified calibration weight was also weighed to check the performance of the balance (0.01 mg, Masscal Recision Calibrations, Melbourne, VIC, Australia) to ensure consistency between all weight measurements. Based on the principle of mass balance, the weight loss rate was calculated as for equations 3.1a and b:

$$\Delta W_{time} = W_{vial} + W_{insect} + W_{SAS} - W_{time} \quad \text{Eq. 3.1a}$$

$$Weight\ loss\ rate_{time} = {}^{\Delta W_{time}}/_{W_{insect}} \quad \text{Eq. 3.1b}$$

Where: ΔW_{time} = Weight loss of total system weight;

W_{vial} = Weight of individual glass vial;

W_{insect} = Weight of initial insect bodies;

W_{SAS} = Weight of synthetic amorphous silica dioxides (SAS);

W_{time} = Total weight at different measurement times.

3.2.7 The Influence of Storage Temperature, Grain Moisture Content and Relative Humidity on Insecticide Efficacy of SAS Powders on Insects

3.2.7.1 Preparation of Grain Samples

Wheat harvested in 2015/16 was purchased from Cooperative Bulk Handling in Perth, Western Australia, and stored at 4°C. The wheat was sterilized at -20°C for four days before use to remove any insect infestation, and then kept at 4°C for two days to defrost. The thawed wheat was conditioned at room temperature for 24 h before treatment.

Two moisture contents were used. For the relatively low moisture content, wheat samples were purchased from a local farm with 11.4% m.c. For the high moisture content experiments, a calculated amount of distilled water was mixed with wheat kept in a sealed plastic bag to equilibrate at 25°C to achieve 13% m.c. The moisture content of the grain was determined using a Pfeuffer HE50 Grain Moisture Meter (Graintec, Australia) and expressed as a percentage calculated from three replicates.

3.2.7.2 Insect Preparation

Clean glass jars of 4 L capacity were used to mix 2 kg wheat with SAS at dose rates of 50, 100, and 150 mg/kg. The jars were shaken and rolled for 2-3 mins for even mixing and allowed to settle for 10 mins. The well-mixed wheat and SAS powders were placed into 120 mL glass jars, each containing 50 g fitted with a plastic screw cap with steel mesh in the centre.

3.2.7.3 Bioassays of Evaluation of Temperature, Moisture Content and Relative Humidity on the Efficacy of SAS Powders

One hundred *T. castaneum* and *R. dominica*, were released into each jar. Untreated wheat was used as the control sample. For each dose and temperature, there were 4-8 replicates. All treated and control samples were placed in a cultural cabinet at 27±1°C and 60±5% R.H with natural light periods for up to 3 weeks during the experiment. When the jars were opened, dead insects were discarded, and live insects were transferred back to each of the experiment conditions mentioned below.

Three temperatures, 15, 25 and 35°C, respectively, were used. Wheat grains with 11.4% m.c. were treated as described above, and then kept at each temperature under 60±5% R.H. Three SAS dose rates were applied: D1=50, D2=100 and D3=150 mg/kg (SAS/Wheat).

Two relative humidity levels, 50% and 75%, respectively, were set up. Wheat grains with 11.4% m.c. were treated as described above, and then kept at 50% and 75%, R.H. at 25°C. Three dose rates were applied: D1=50, D2=100, and D3=150 mg/kg (SAS/Wheat), respectively.

Wheat grains with two moisture content levels, 11.4% and 13% m.c. were treated as described above, and then kept at 35°C at 75±5% R.H. to achieve a balance of equivalent relative humidity. Three dose rates were applied: D1=50, D2=100, and D3=150 mg/kg (SAS/Wheat), respectively.

3.2.8 Imaging Reconstruction and Statistical Analysis

High-powered Micro-CT workstations with the Avizo Fire software for visualisation and data processing reconstructed and generated a 3D computed tomographic model with 3001 projections collected for an HB-SAS powder treated *R. dominica*. Data entry and basic statistical analyses were performed using Microsoft Excel Office 365 (Microsoft, NY, USA). Analyses of differences between groups were

conducted using unsupervised and supervised multivariate statistical analysis techniques and one-way analysis of variance (ANOVA) followed by the Student-t post hoc test to evaluate the bioassay data through Metaboanalyst 4 (Pluskal *et al.* 2010; Chong *et al.* 2018).

3.3 RESULTS

3.3.1 SEM Scan of *T. castaneum* Cuticle Layer with HB-SAS

At 10kV energy and 20µm aperture, the SEM microscope magnified the object 18,000 times to show the HB-SAS powder accumulates around the secretory spot and attaches to sensory hairs (Figure 3.3). The associated x-ray EDS spectra confirmed that the adherent particles were silica.

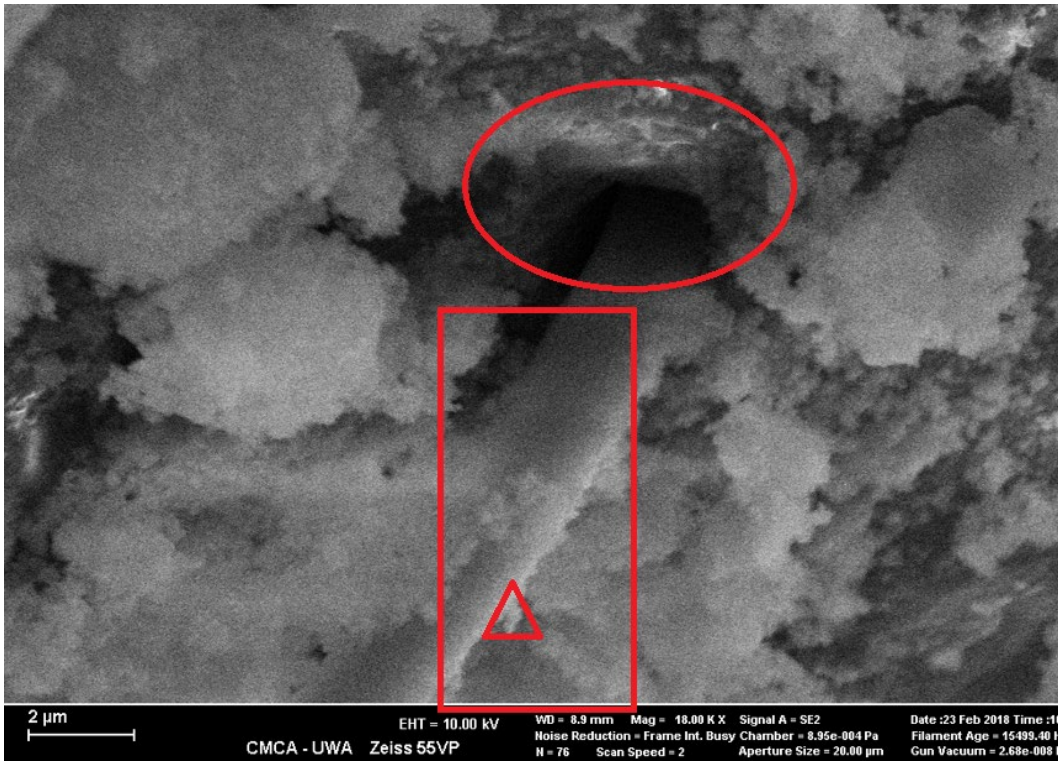


Figure 3.3. Scanning Electron Microscopy image of the secretory spot (red oval), sensory hair (red rectangle) and SAS powder (red triangle) of the treated *T. castaneum*

3.3.2 X-Ray Micro-Computed Tomography (Micro-CT) Scanning HB-SAS Treated and Untreated *T. castaneum* Tracheal System

An untreated control and the treated *T. castaneum* were scanned in phase-contrast mode to generate more contrast. Phase-contrast normally requires long scan times to achieve higher resolution. Binned imaging makes it faster but leads to a lower

resolution. The Versa 520 can obtain a maximum pixel resolution of 0.7 microns, however, such small size of the tracheal system is a challenge to resolve. There was unmistakable evidence that the tracheal system (highlighted in green) of *T. castaneum* was blocked with the HB-SAS (red arrows) powder (Figure 3.4). This indicates that the HB-SAS powder penetrates *T. castaneum* by inhalation into its tracheal system.

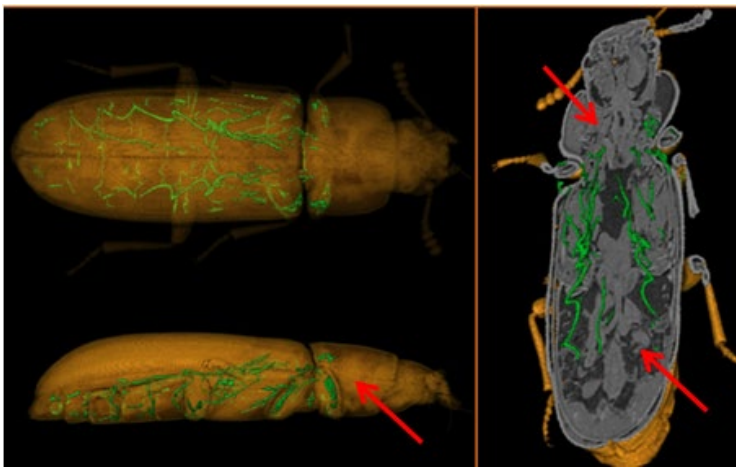
a.  b. 

Figure 3.4. X-ray micro-computed tomography (Micro-CT) scanning of an HB-SAS treated (a) and untreated (b) *T. castaneum*. The tracheal tubes of *T. castaneum* treated with HB-SAS (in green) where the trachea is blocked (red arrows)

After data pre-processing and background subtraction, the internal desiccated space of *R. dominica* treated with HB-SAS powders was extracted by Avizo Fire and is highlighted in blue (Figure 3.5). The blue 3D model indicates that the interior of the HB-SAS treated insect is desiccated throughout, including the legs, head, and antennae. As the area of desiccation expanded, the internal organs shrank and became non-functional.

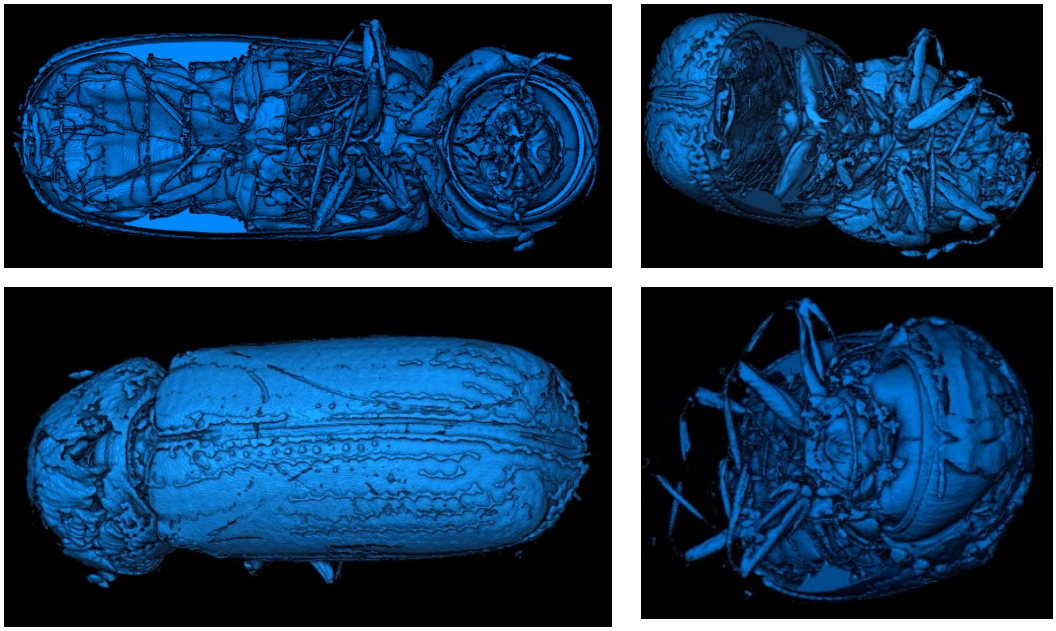


Figure 3.5. Four angle screenshots of an X-ray micro-computed tomography (Micro-CT) 3D reconstruction of the desiccated space in *R. dominica* two days after treatment with HB-SAS powder. The 3D reconstruction model subtracted the background of the insect's exoskeleton and its interior organs.

3.3.3 Insect Time-Body Weight Loss Bioassays

The bioassay examinations focused on distinguishing water loss and weight loss during treatment with the two SAS powders. The maximum body weight loss (BWL) was 62% of the initial weight of live *T. castaneum* adults at 28 days (Figure 3.6). Insects treated with the hydrophilic and hydrophobic SAS powders reached their maximum BWLs of 53% and 50%, respectively on day seven (Figure 3.6). The maximum BWL of pre-freeze killed *T. castaneum* adults approached 43% on day 16. The pre-killed insect without SAS and pre-killed insects treated with the hydrophilic and hydrophobic SAS powders lost 43%, 46% and 43%, respectively, on the same day (day 15) as those without SAS powders (Figure 3.6). The hydrophilic SAS powder particles absorbed unknown compounds to give a light pink colour, but hydrophobic ones did not (data not shown). The pink colour indicates that the unidentified metabolites have their hydrophilic functional groups attracted to the hydrophilic surface of the SAS powder.

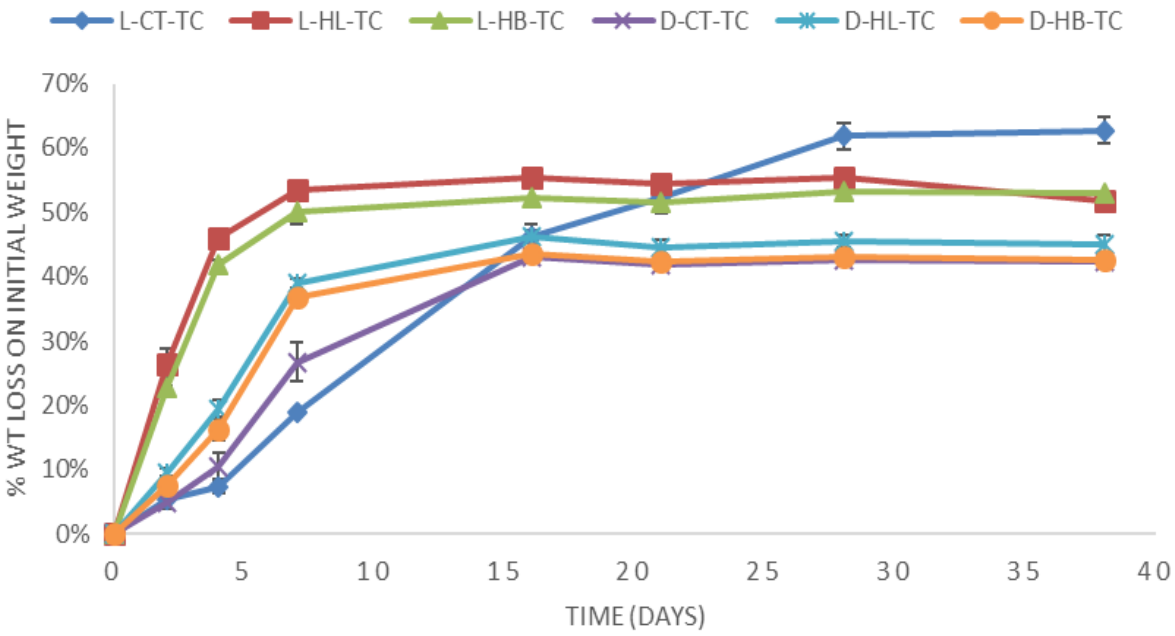


Figure 3.6. Time-body weight loss (%) curves of phosphine resistant *T. castaneum* comparing weight loss rate, based on initial weight, among control, hydrophilic and hydrophobic SAS powders. L-CT-TC: Alive control group of *T. castaneum*; L-HL-TC: Alive *T. castaneum* exposed to hydrophilic SAS powders; L-HB-TC: Live *T. castaneum* exposed to hydrophobic SAS powders; D-CT-TC: Freeze-killed control group of *T. castaneum*; D-HL-TC: Freeze-killed *T. castaneum* exposed to hydrophilic SAS powders; D-HB-TC: Freeze-killed *T. castaneum* exposed to hydrophobic SAS powders

3.3.4 Effect of Temperature on the Efficacy of SAS Powders

The effects of temperature on the efficacy of the SAS powders are presented in Tables 3.1 and 3.2. The mortality of *T. castaneum* and *R. dominica* was positively linked to the environment's temperature. The mortality of *T. castaneum* and *R. dominica* was significantly affected by dosage, temperature, time and the type of SAS from high to low F value ($P<0.01$) (Table 3.5a).

as the temperature increased for the different dosages of hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders from 50 to 150 mg/kg, the mortality was 100% faster than the DE dust in less than one week. HL-SAS and HB-SAS required at least four and three weeks, respectively, to reach 100% mortality at 15°C. With increasing temperature, *T. castaneum* was more sensitive to both SAS powders, except at the low

dosage (50 mg/kg). At 25°C, the SAS powders reduced the exposure time to three weeks and one week at 100 mg/kg and 150 mg/kg, respectively. When the temperature reached 35°C, no *T. castaneum* adults survived after one week at 100 mg/kg and 150 mg/kg of HL-SAS or HB-SAS, respectively (Table 3.1). *R. dominica* had similar patterns as TC, except for HL-SAS at 35°C. At 25°C, the SAS powders reduced the exposure time to three weeks and one week at 100 mg/kg and 150 mg/kg, respectively. When the temperature reached 35°C, *R. dominica* adults survived beyond four weeks at all dosage levels, except at 150 mg/kg of HB-SAS (Table 3.2). Fifty-three out of 100 control *R. dominica* adults were sensitive to 35°C beyond two weeks of exposure (Table 3.2). Overall, the SAS powders performed best on *T. castaneum* and *R. dominica* adults exposed to treated wheat grain at 11.4% m.c. and 35°C and 25°C at 60±5% R.H.

Table 3.1. Mean mortality (%) of *T. castaneum* adults exposed to wheat grain treated with hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders at 11.4% m.c. and 60±5% R.H. at different temperatures over 1-5 weeks.

Temp	Time (Wk)	HL-SAS (mg/kg)			HB-SAS (mg/kg)			Ctl
		50	100	150	50	100	150	
15°C	1	25b	77c	86eg	26b	81c	83g	0a
	2	79c	93e	92eg	65d	94e	97ef	0a
	3	96e	99ef	99f	87g	98ef	100f	0a
	4	99ef	100ef	100ef	97ef	100f		0a
	5	100ef			100g			0a
25°C	1	56j	91eg	100ef	10k	74c	100ef	1a
	2	90eg	99ef		92eg	99ef		4a
	3	99ef	100ef		98ef	100ef		5i
	4	100ef			98ef			15k
	5				99ef			25b
35°C	1	67d	100ef	100ef	52i	100ef	100ef	0a
	2	93e			93e			0a
	3	97ef			98ef			1a
	4	99ef			99ef			1a
	5	100f			100ef			13k

*Means followed by the same letter are not significantly different at P≤0.05. Each data is the mean of 4 or 8 replicates.

Table 3.2. Mean mortality (%) of *R. dominica* adults exposed to wheat grain treated with hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders at 11.4% m.c. and 60±5% R.H. at different temperatures over 1-5 weeks.

Temp	Time (Wk)	HL-SAS (mg/kg)			HB-SAS (mg/kg)			Control
		50	100	150	50	100	150	
	1	40c	77fg	85g	39c	63e	73f	4a
15°C	2	77fg	93gh	93gh	82g	93gh	94hi	12ab
	3	77fg	93gh	93gh	82g	93gh	94hi	12ab
	4	85g	96hi	98i	94hi	99i	95hi	20b
	5	86g	97i	100i	91gh	99i	100i	23b
25°C	1	97i	97i	100i	97i	99i	100i	2a
	2	99i	98i		99i	100i		6a
	3	100i	100i		99i			6a
	4				100i			8a
35°C	1	82g	82g	96hi	87g	94hi	100i	16a
	2	86g	86g	97i	91 hi	97i		53d
	3	91gh	89gh	99i	93 hi	98i		68ef
	4	91gh	-	99i	94hi	99i		69ef

*Means followed by the same letter are not significantly different at P≤0.05. Each data is the mean of 4 or 8 replicates.

3.3.5 Effect of Relative Humidity on Efficacy of SAS Powder

The effect of relative humidity on the HL-SAS and HB-SAS was evaluated at 25°C and 11.4% m.c. for three weeks (Table 3.3). The mortality of *T. castaneum* was significantly affected by dosage, relative humidity, type of SAS and time from high to low F value (P<0.01). However, *R. dominica* had lower F value of relative humidity (F=2.089) than *T. castaneum* (F=540.405) (Table 3.5b). For the *T. castaneum* adults, increasing relative humidity at three different dosages of the hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders from 50 to 70% R.H, the mortality was reduced by up to 3.4 and 1.2 times at 50 and 100 mg/kg, respectively. However, it remained highly efficient at 150 mg/kg and all adults were killed in less than a week. In contrast to *T. castaneum*, *R. dominica* had an opposite trend. The mortality of *R. dominica* increased by 1.2, 1.6 and 1.8 times in response to the 50, 100 and 150 mg/kg doses for both the HL-SAS and HB-SAS powders, respectively, in the first week. After two

weeks, compared with the first week, the mortality of *R. dominica* decreased by 0.77 and 0.88 times when the relative humidity was increased from 50% to 70%. There were no significant differences between the HL-SAS and HB-SAS powders (Table 3.3).

Table 3.3. Mean mortality (%) of *T. castaneum* and *R. dominica* adults exposed to wheat grain treated with hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders at 11.4% m.c. and 25°C under different relative humidity over three weeks.

Species	Relative humidity	Time (Week)	HL-SAS (mg/kg)			HB-SAS (mg/kg)			Control
			50	100	150	50	100	150	
T. castaneum	50±5%	1	78gh	97il	100lm	34cd	97il	100lm	1a
		2	91il	100lm		61ef	100lm		1a
		3	100lm			91il			1a
	70±5%	1	25c	79gh	100lm	10b	80gh	100lm	1a
		2	32cd	90il		10b	92il		0a
		3	34cd	93il		13b	95il		1a
R. dominica	50±5%	1	26c	45d	44d	35cd	46d	47d	1a
		2	51de	86hi	91il	61ef	91il	91il	4a
		3	61ef	91il	97il	68fg	98	98lm	6ab
	70±5%	1	25c	74g	78gh	41cd	68fg	86hi	3a
		2	31cd	78gh	83gh	47de	74g	87hi	6ab
		3	36cd	80gh	86hi	51de	76gh	90il	6ab

*Means followed by the same letter are not significantly different at P≤0.05. Each data is the mean of 4 or 8 replicates.

3.3.6 Effect of Grain Moisture Content on the Efficacy of SAS Powders

Given that the SAS powders lead to dehydration, insects need water from food to stay hydrated. The efficacy of the SAS powders was tested on grain with 11.4% and 13% m.c. at 35°C and 75±5% R.H. for two weeks (Table 3.4). The mortality of *T. castaneum* was significantly affected by dosage, moisture content, exposure time and SAS powder types from high to low F value ($P<0.01$). However, *R. dominica* had higher F value of SAS powder ($F=332.186$) than *T. castaneum* ($F=11.724$) (Table 3.5c).

As the moisture content increased at three different dosages of the hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders, the mortality of adult *T. castaneum* decreased significantly by 11, 4.2, and 1.06 times at 50, 100 and 150 mg/kg, respectively. However, it remained highly efficient at 150 mg/kg and killed all adults in week two.

Rhyzopertha dominica had a similar pattern to *T. castaneum*, where the mortality of *R. dominica* decreased from low to high levels of moisture content significantly to 10, 15, and 43% mortality after the first week by 5.5, 4.4 and 1.7 times at 50, 100 and 150 mg/kg of HL-SAS, respectively. Given the high moisture content reduced the efficacy of HB-SAS, the mortality of *R. dominica* decreased from 60, 92, and 98% at 11.4% m.c., to 11, 41, and 78% at 13% m.c. by 5.5, 2.2, and 1.3 times at 50, 100 and 150 mg/kg of HB-SAS after the first week (Table 3.4).

Table 3.4. Mean mortality (%) of *T. castaneum* and *R. dominica* adults exposed to wheat grain treated with hydrophilic (HL-SAS) and hydrophobic (HB-SAS) powders at different moisture contents at 35°C and 75±5% relative humidity over 2 weeks.

Species	Moisture content	Time (Week)	MU8 (mg/kg)			MU9 (mg/kg)			Control
			50	100	150	50	100	150	
T. castaneum	11.4%	1	23c	96hi	100i	22c	79g	100i	1a
		2	46d	100i		63f	99i		1a
	13%	1	4a	25c	94hi	2a	19c	95hi	0a
		2	30c	86gh	100i	7ab	78g	100i	1a
R. dominica	11.4%	1	22c	66f	75g	60f	92hi	98i	2a
		2	29cd	71fg	83g	61f	94hi	98i	7ab
	13%	1	10b	15bc	43d	11b	41d	78g	3a
		2	17bc	27c	50e	18bc	49e	84gh	8b

*Means followed by the same letter are not significantly different at P≤0.05. Each data is the mean of 4 or 8 replicates.

Table 3.5. Analysis of variance analysis (ANOVA) results of effect of temperature (a), relative humidity (b) and moisture content (c) on the efficacy of SAS powders against *T. castaneum* and *R. dominica* adults.

a.

Source	*T. castaneum*			*R. dominica*		
	df	*F*	*P*	df	*F*	*P*
Temperature	2	170.771	<0.01	2	146.357	<0.01
SAS powder	1	24.047	<0.01	1	16.520	<0.01
Dosage	3	2032.461	<0.01	3	646.354	<0.01
Time	4	440.601	<0.01	4	65.247	<0.01

b.

Source	*T. castaneum*			*R. dominica*		
	df	*F*	*P*	df	*F*	*P*
Relative humidity	1	540.405	<0.01	1	2.089	<0.01
SAS powder	1	103.970	<0.01	1	30.316	<0.01
Dosage	2	1953.862	<0.01	2	638.710	<0.01
Time	2	38.084	<0.01	2	173.810	<0.01

c.

Source	*T. castaneum*			*R. dominica*		
	df	*F*	*P*	df	*F*	*P*
Moisture content	1	286.055	<0.01	1	418.534	<0.01
SAS powder	1	11.724	<0.01	1	332.186	<0.01
Dosage	2	1964.373	<0.01	2	448.078	<0.01
Time	2	166.934	<0.01	2	17.873	<0.01

3.4 DISCUSSION

The lowest dose (50 mg/kg) of the SAS powders tested in this study efficiently controlled the stored grain insects at all temperatures, under 70±5% R.H. in the wheat grain at 11.4% m.c. The normal dose level (100 mg/kg) provided complete control of all insects within 1-4 weeks. The high dose (150 mg/kg) tested for HL-SAS and HB-SAS was much lower than any reported for DE-based inert dusts, typically applied at 1,000 mg/kg. Finally, it is also worth mentioning that the SAS does not affect the grain flow and angle of repose when treating exported grain, which DE dusts do (Ren and Agarwal 2014).

3.4.1 The Body Weight Loss

Live *T. castaneum* lost approximately 10% more of their initial weight than pre-freeze dead beetles when desiccated by the HL-SAS and HB-SAS powders. This indicates that weight loss includes body water and volatile metabolic products, such as carbon dioxide, metabolic water from the TCA cycle (Krebs cycle), and other volatile organic chemicals (VOCs). Furthermore, more body weight loss occurred in the HL-SAS powder than in the HB-SAS powder. The former powder absorbed unknown compounds to give a pink colour, but the hydrophobic powder did not. The pink colour in the HL-SAS powder indicates that the unidentified metabolites had their hydrophilic functional groups attracted to the hydrophilic surfaces of the powder. Hodges *et al.* (1996) reported *T. castaneum* infested flour or rice turned pink contributed by benzoquinones at high population densities. Therefore, the unidentified pink metabolites on hydrophilic SAS may be benzoquinones or their derivatives as well.

The Micro-CT 3D model indicated a complete desiccation occurred on the test insects by SAS powders (Figure 3.5). In contrast, DE dusts couldn't desiccate insects completely with even higher dosage, 34-37% water remaining at the death (Rigaux *et al.* 2001). So, the MoA of desiccation may contribute more on SAS treatment, rather than DE dusts.

Over seven days, the high weight loss rate of freeze-killed *T. castaneum* illustrates and strengthens the fact that the insect's cuticle layer protects it from water loss. In addition, due to the inactivity of dead insects, the SAS powders should remove less of the wax layer from the dead insects than living insects, and the wax layer loss was not directly correlated to the weight loss rate. Therefore, the metabolic products play an important role in maintaining water content in the *T. castaneum* body by triggering and sustaining the water retention capability of the cuticle layer. This result is consistent with Li (2018), who reported that synthetic amorphous silica invaded the intersegmental frictional joints of *T. castaneum* and absorbed the

vital body fluids. The lethal effect was not instantaneous, but structural damage was irreversible. The insects' locomotion and behaviour were severely affected, which made activities necessary for survival and reproduction difficult or impossible, including feeding and mating (Li 2019).

3.4.2 Effect of Storage Conditions and Environment on the Efficacy of SAS Powders

Well-documented research on diatomaceous earth (DE) dust have revealed that DE products are sensitive to the moisture content of grain and ambient humidity when used in structural treatment applications (Cao *et al.* 2010; Wang *et al.* 2011 and Baliota *et al.* 2022). An increase in moisture content of treated grain or air relative humidity will considerably reduce the efficacy of DE, especially if the moisture content is more than 14% or relative humidity exceeds 70%. In the present study, grain moisture content significantly affected the efficacy of the DE inert dust. In contrast, the results clearly showed that at 13% m.c. and 75±5% R.H., both HL-SAS and HB-SAS powders still achieved 100% mortality in *T. castaneum*.

The efficacy of the two synthetic amorphous silica powders (HL-SAS and HB-SAS) tested in this study, was affected differently by variations in temperature, grain moisture content, and relative humidity for *T. castaneum* and *R. dominica* adults (Sections 3.3.4 to 3.3.6). Increased temperature leads to intense metabolic activities associated with the proactive release and passive water loss from the two insects. In turn, high temperatures increased the pesticide efficacy of HL-SAS and HB-SAS within one week. While a low temperature of 15°C suppressed the insects' physiological activities, such as sexual reproduction, water evaporation and respiration, all essential for survival. This is one of the major reasons that both primary and secondary insects need at least three to five weeks to reach 100% mortality when treated with HL-SAS and HB-SAS.

The mode of action by desiccation is one of the leading causes of insect death. When the moisture content and relative humidity of the environment were high, sufficient water was available to replenish the moisture lost by desiccation caused by the SAS powders. Moisture content and relative humidity represented the internal and external water sources, respectively in the insects. Internal water intake is more direct and efficient than external water. Artificially high moisture content may worsen the agglomeration of HL-SAS on the surface of grain kernels, and in turn, reduce the amount of effective pesticidal HL-SAS particles. Therefore, the decreased mortality observed was expected in these water-related experiments.

3.5 CONCLUSION

The high efficacy of both SAS powders could not be explained by existing modes of action of DE dust. The desiccation process takes a relatively long time, typically weeks, to act. However, the SAS powders could kill the two grain insects in days, even hours. Therefore, it is expected that other modes of action are synergistic with each other. The SEM and micro-CT results were combined to prove that the SAS powders targeted the vulnerable cuticle parts of adult *T. castaneum*, such as the secretory spot with the sensory hair and the tracheal system. It explains the large empty desiccated space throughout the insect's body, including the legs, head, and antennae. In addition, the internal organs were shrunk and malfunctional (Figures 3.4 and 3.5).

This study is also the first to systematically investigate the influence of temperature, moisture content, and relative humidity on the effectiveness of SAS powders to control storage insects in grain. Exposure to high temperatures, low moisture contents, and low relative humidity ensures that HL-SAS and HB-SAS are highly efficient for controlling primary and secondary grain insects. Since HL-SAS is a food-grade powder, it can provide grain farmers and bulk handlers with a safe and environmentally friendly management tool. However, further toxicological and respiratory tests need to be conducted to investigate the feasibility of using SAS powders to treat grain for food and feed. It is also worth bearing in mind that the multiple MoAs contribute to insect mortality to varying degrees. The efficacy of the different pesticidal actions also depended on the insect species.

4 Biological and Behavioural Responses of Stored Grain Insects to Synthetic Amorphous Silica

ABSTRACT

Food-grade synthetic amorphous silica (SAS) powders have been used for pest control in grain storage globally. Importantly, they can kill phosphine-resistant stored product insect pests. However, SAS powders' mechanism(s) as an insecticide to kill stored product insect pests is unclear.

This study explored how food-grade SAS powders affect the biology and behaviour of stored grain insects. GCMS and LCMS were combined to acquire the profiles of 836 and 919 metabolite features from hydrophilic (HL) SAS and hydrophobic (HB) SAS treated *T. castaneum* and *R. dominica*, respectively. Principal components analysis and sparse Partial-Least Squares Discriminate analysis distinguished significant differences between HL-SAS, HB-SAS, and the DE dust. Lipid compounds were identified in extracts from HL-SAS and HB-SAS powders collected from *T. castaneum* bodies. The results indicated that both HL-SAS and HB-SAS absorb cuticle wax and lipids damaging the insect's cuticle. The results also showed that HB-SAS and HL-SAS powders significantly reduced monosaccharides and amino acids, such as β-alanine, asparagine, and serine. Free fatty acids accumulated in the treated insect bodies. Direct immersion solid-phase microextraction (DI-SPME) technology was also used to validate the accumulation of free fatty acids without chemical derivatisation.

Based on the model insect *T. castaneum,* HL-SAS and HB-SAS powders had no significant effect on egg hatching. The efficiency of SAS powder on the nascent larvae found HB-SAS to be more effective than HL-SAS on the newly emerged larvae, as 100% mortality was achieved in six and seven days with HB-SAS and HL-SAS, respectively. The freshly hatched larvae were killed within seven days. The efficiency of SAS powder on the 1st instar larvae indicated that both HL-SAS and HB-SAS powders killed larvae within three to four days.

The bioassay results revealed there might be other pesticidal mechanisms behind HB-SAS, enhancing the killing power of HB-SAS powders. The indicative modes of action were dehydration and asphyxiation associated with accumulated storage lipids and glycerol. In

contrast, the decline in the levels of most carbohydrates, amino acids, and the tricarboxylic acid (TCA) cycle metabolites indicated the physiological actions were due to a depletion in energy metabolism.

4.1 INTRODUCTION

Fumigation is an effective method widely used to manage pests with gaseous chemicals, called fumigants, in small to large-scale grain storages e.g., sheds, bunkers as well as silos. Many chemicals have been developed and registered as fumigants worldwide, such as phosphine (PH_3), methyl bromide (MB), sulfuryl fluoride (SF), carbonyl sulphide (COS), ethyl formate (EF), and ethanedinitrile (EDN) (Bond 1984; Hooper *et al.* 2003; Bartholomaeus and Haritos 2005; Ren and Mahon 2006; Sriranjini and Rajendran 2008; Athanassiou *et al.* 2015). However, phosphine is well recognised worldwide for the disinfestation of stored grain insects for the international grain trade, after restrictions to the use of methyl bromide were imposed by the Montreal Protocol (UNEP 1987; Chaudhry 2000). Phosphine resistance in grain storage pests has been detected in many farmer and bulk handler sites in western, southern and eastern Australia (Jagadeesan and Nayak 2017).

The use of conventional inert dust, such as diatomaceous earth (DE), in stored product pest management is restricted due to industrial concerns on their low efficacy and impacts on human health (Bodroža-Solarov *et al.* 2012). Also, due to the high dosage of DE required, the bulk grain flowability is changed as the small loading angle of bulk grain flow limits inert dust application in bulk handlers' facilities (Golob 1997). In response to these limitations, food-grade synthetic amorphous silica (SAS) powders have been developed and introduced to large commercial scale bulk handling systems (Ren and Agarwal 2014). Furthermore, SAS powders can be used as a phosphine resistance breaker in central bulk handling systems. There are five proposed modes of action (MoA) for inert dusts, including SAS and DE, to control stored grain insects. However, the death caused by the SAS powders is difficult to explain based on these five MoAs. Two MoAs are related to asphyxiation due to the dusts blocking spiracles and their oral ingestion (DeCrosta 1979; Cook and Armitage 1999) and three are related to water loss and cuticle impairment (Wigglesworth 1944; Subramanyam and Roesli 2000). The most widely accepted insecticidal mechanism against stored grain insects is that the inert dust absorbs the epicuticular lipids and impairs the water balance through the cuticle (Chiu 1939; Wigglesworth 1944, 1945, 1947; Tarshis 1960; Tarshis 1961; Cook *et al.* 2008; Van Den Noortgate *et al.* 2018). There is a lack of solid scientific evidence to validate these MoAs thoroughly, and conflicting results indicate that insect death cannot be explained by desiccation alone (Chiu 1939). Researchers have strongly correlated the loss of wax lipids and mortality (Chiu 1939; Wigglesworth 1947; Tarshis 1961; Cook *et al.* 2008; Van Den Noortgate *et al.* 2018). However, these findings were not always straightforward. For example, Van Den

Noortgate *et al.* (2018) reported that a zeolite material (NH4-MOR-38) absorbed the most waxes out of 24 porous materials, but its insecticidal activity was lower than another zeolite material (H-BEA-300), which only adsorbed 18.2% of what NH4-MOR-38 adsorbed. Stronger absorption of wax lipids did not lead to higher insecticidal efficacy. Food grade SAS powders cause exposed insects to dehydrate more effectively than conventional DE dust (Li *et al.* 2019), but the death by desiccation is difficult to explain.

Synthetic amorphous silica (SAS) powders are generally recognised for their insecticidal activity because they have low toxicity towards mammals, little potential for insect resistance developing, and no residue issues. The mechanism(s) of action of SAS powders is still to be determined, but they are thought to act similarly to inert dusts like silica gel, nanostructured alumina and diatomaceous earth (DE) (Chiu 1939; Wigglesworth 1947; Tarshis 1961; Cook *et al.* 2008; Van Den Noortgate *et al.* 2018). The most widely accepted explanation for the mode of action of inert dusts is that they act by adsorbing the lipids from the insect cuticle, causing death by desiccation (Korunić 1997; Korunić *et al*. 1988). Therefore, the insecticidal efficacy of the SAS powder-based insecticide might vary with temperature, moisture content and relative humidity. Previous studies have reported that their performance declines as ambient humidity increases and temperatures decrease (Subramanyam and Roesli 2000). The toxicity of a precipitated silica powder Sipernat® 22S (formerly Wessalon S) in whole wheat to adult *Sitophilus granarius*, *T. castaneum*, *Cryptolestes pusillus,* and *Oryzaephilus surinamensis* decreased with increasing relative humidity. The moisture content of maize grains significantly affected the efficacy of inert dust, which can achieve superior control of *Sitophilus zeamais* if the grain does not exceed 12% moisture content.

Although there have been many studies on amorphous silica and silicate-based mineral dusts to protect grain against pest insects, little has been published on the variations in efficacy of synthetic amorphous silica powders with variations in temperature and grain moisture content. In addition, few researchers have addressed the performance of hydrophilic and hydrophobic powders under various temperatures and moisture content.

A previous study identified a novel use for an existing synthetic amorphous silica (SAS) product (Ren and Agarwal 2014; Li *et al.* 2019). These results showed that SAS powder is highly efficacious, providing a solution to the issues that have prevented the widespread use and adoption of similar products by the grains industry and offering the industry a non-chemical alternative for the control of insects in stored grain. Currently, the accepted mode of action for silica-based products is via adherence to the insect's body with subsequent damage

to the protective waxy layer of the insect's cuticle by sorption and abrasion to a lesser degree. The result is water loss from the insect's body and ultimately death. SAS powder particles are relatively small and coupled with a high ratio of surface area to volume, so SAS provides a higher capacity to absorb oils and promote sorption than traditional DE dust (CRC3003 report 2018).

Metabolomics has great promise as a method for investigating and comparing metabolic changes under different environmental stresses in stored products, pests, and other insects (Christiansen *et al.* 2012, Wang *et al.* 2017). In the present study, *T. castaneum* is used as a biological model, as it has spread worldwide through international trade in the food industry and developed significant phosphine resistance (Weston and Rattlingourd 2000; Campbell *et al.* 2010). Understanding how SAS powders affect insect metabolism is fundamental to predicting its effects on energy-demanding activities, including insect movement and survival.

Compared with susceptible populations, a lower respiration rate and reduced reproduction are the physiological basis of phosphine resistance (Pimentel *et al.* 2007). The molecular biological mechanisms of phosphine resistance of stored grain insects have been well studied (Schlipalius *et al.* 2012; Oppert *et al.* 2015; Kim *et al.* 2019). Phosphine-resistant insects can display many differential expressions of cytochrome P450, cuticle, carbohydrates, transporters, and many mitochondrial genes (Oppert *et al.* 2015). The metabolic pathway of β-oxidation from free fatty acids to acetyl-CoA, which eventually forms energy substances (several equivalents of ATP), is downregulated in the strongly phosphine-resistant rice weevil, *S. oryzae* (Kim *et al.* 2019). This observation was also found in desiccated insects (Sawabe and Mogi 1999; Robert *et al.* 2008). Significant and rapid weight loss induced by SAS powders elicited a general inhibition of aerobic metabolism correlated to mortality of *T. castaneum*. In addition, the overloaded free fatty acids can stress and damage the membranes of organism cells to induce chemical toxicity (Hoekstra *et al.* 2001).

This study seeks to clarify the mode of action of SAS powders and evaluate the effect of SAS powders on the life cycle and biological behaviour of *T. castaneum* and *R. dominica.* To evaluate the effects of SAS powder on insect biochemical changes, the insects' body waxes and lipids were also analysed using DI-SPME coupled with GC-MS. A combination of phenotyping and metabolomics analysis was used to verify the hypothesis that the metabolic energy changes caused by SAS contribute to insect mortality. The results will guide the development and production of more effective SAS formulations against stored product insect pests, particularly phosphine-resistant insects.

4.2 MATERIALS AND METHODS

4.2.1 Insect Cultures

A phosphine-susceptible strain (MUWTC-CH-S) and a phosphine-resistant strain (MUWTC-SR-500) of *T. castaneum* were obtained from the Department of Primary Industries and Regional Development, Western Australia, Australia (DPIRD) and cultured over ten generations in the Post-harvest Biosecurity and Food Safety Laboratory, Murdoch University. *T. castaneum* were fed with wheat flour supplemented with 7.7% yeast in 2 L clear glass jars covered with a metal mesh lid at 29±1°C, and 65±5% R.H. The adults of the phosphine-susceptible strain (never exposed to phosphine) (SS_Uxp) and a strongly resistant strain (SR_Uxp) of *T. castaneum* were used for free fatty acids profiling (Section 4.2.6). A phosphine-susceptible strain (MUWRD-S-6000) and a resistant strain (MUWRD-SR-500) of *R. dominica* were also obtained from DPIRD, and fed with wheat kernels in 2 L glass jars at 29±1°C, and 65±5% R.H. The evaluation of phosphine resistance was conducted and determined by the FAO 1975 method (FAO 1975).

4.2.2 Synthetic Amorphous Silica (SAS) and Diatomaceous Earth

Two synthetic amorphous silica powders MU8 (HL-SAS) and MU9 (HB-SAS) were used. Both are extremely fine white solid silicon dioxide powders with an average particle size of 118 and 117 nm, respectively (Ren and Agarwal 2014). MU8 is hydrophilic (purity >99.9% w/w) with a Brunauer–Emmett–Teller (BET) surface area of 260-280 m^2/g, an oil-carrying capacity of 280-300 mL/100g, and an average diameter of 5µm. MU9 is hydrophobic (purity >99.9% w/w), and the surface is modified with polysiloxane with a surface area >230m^2/g, an oil-carrying capacity >350 mL/100g, and an average diameter of 4.5-5.5µm.

Diatomaceous earth, Dryacide® (DE) - >90% w/w natural diatomaceous earth; <10% w/w silica gel, was obtained from Entosol (Australia) Pty Ltd, NSW, Australia. The supplier did not provide the surface area and oil-carrying capacity. However, the typical surface area and oil-carrying capacity of similar diatomaceous earth products were reported in the range of 1-23.8m^2/g (Tsai *et al.* 2006; Nattrass *et al.* 2015) and 150.5-188.2 mL/100 g (Subramanyam and Roesli 2000), respectively.

4.2.3 Treatment of Insects with SAS Powders and DE Dust for Bioassays

Before treating the insects with the SAS powders and DE dust, all insects were cleaned to remove surface contaminants. To prepare clean and undamaged insects, a 9 cm diameter filter paper (Whatman®, Grade 113) was left in the 2 L glass jars for approximately 30 mins to

allow the insects to cling to the paper. The paper was then flipped over above moist tissue paper and gently tapped twice to separate the insects from their respective food sources. The insects were then cleaned further by transferring them to wet Kleenex® tissue paper (305 × 240mm, Kimberly-Clark Worldwide, Inc., Australia) and placed in a plastic food display tray (456 × 318 × 57mm, NALLY LTD, Australia). The adults that climbed off the wet tissues were collected and transferred to a clean 500 mL glass bottle with a mesh lid for the experiments. This procedure was repeated several times to obtain 50 adult insects per sample.

Ten grams of the HL-SAS, HB-SAS were added into the funnel of the powder applicator (Ryobi 20L Stainless Steel Wet Dry Workshop Vacuum), and then blown into a modified fume hood within 20 sec. Based on DE (Dryacide) usage label, the DE dusts were poured and stirred in the Petri dishes for further use. Five seconds after blowing all the powder, ten uncovered Petri dishes, half of which were empty and the other half contained 1 g of insects (approx. 600 *T. castaneum* or 900 *R. dominica* adults), were placed into the fume hood for 2 minutes. This process resulted in the deposition of only deflocculated powder onto the insects' bodies and Petri dishes. The amount of powder deposited was calculated by comparing the weight of the Petri dishes before and after collecting the powder in the fume hood. The powder was collected in the five empty Petri dishes, and five Petri dishes each containing 1 g of insects were used as control. This deflocculation process was repeated three times (3 replicates) for two strains each of *T. castaneum* and *R. dominica* treated with HL/HB-SAS powders. Each replicate had five Petri dishes. The pure deflocculated powder was collected in the Petri dishes, the insects were kept in Petri dishes with powder, and the clean insects were kept as a control for 30 hours at 25±1°C and 60±5% R.H. The mortality of adult insects was checked at 18, 24 and 42 hours and the dead insects were frozen immediately at -20°C for MS-based global metabolites profiling.

4.2.4 Chemicals, Reagents and Solid-phase Microextraction (SPME) Fibers

PHOSTOXIN® pellets containing aluminium phosphide (4Farmers Australia Pty Ltd, Welshpool, WA, Australia) were mixed with 10% sulfuric acid (Merck Australia, NSW, Australia) to generate phosphine gas. Acetonitrile and n-Alkanes (C7-40) retention index (RI) standards were purchased from Sigma-Aldrich (Sigma-Aldrich, Castle Hill, NSW, Australia). The three-coating phase 50/30µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) fiber was purchased from Supelco (Bellefonte, PA, USA) and conditioned following the manufacturer's recommendations before use. The fiber was operated with a manual holder (Supelco, Bellefonte, PA, USA).

All solvents were HPLC grade. Acetonitrile, methanol, 2-propanol and chloroform were purchased from Fisher (NSW, Australia). Ammonium acetate, formic acid, leucine enkephalin, and phosphoric acid were purchased from Sigma (NSW, Australia). Liquid nitrogen was purchased from BOC (WA, Australia) to freeze the insect samples. Triglyceride Mix standards (17811-1AMP, 09/2018, TG 27:0, TG 33:0, TG 39:0, TG 45:0, TG 51:0) were supplied by Sigma (NSW, Australia).

4.2.5 Extraction of Insect Metabolites for GC-MS and LC-MS Analysis

All insect samples collected for the metabolites profiling were quenched in the liquid nitrogen for 5 seconds and stored in freezer (-20°C) until the analysis.

For lipids profiling analysis of insect extractions by the HL-SAS and HB SAS powders and the DE dust, adult *T. castaneum* and *R. dominica* were extracted in 10 mL n-hexane for further analysis. There were approximately 15 mg of insects (approx. 10 *T. castaneum* /15 *R. dominica* adults). The insects included the clean insects (control), the insects treated with the HL-SAS and HB-SAS powders, and the DE dust. These were prepared for lipids analysis using the following steps: (i) brush as much as possible of the SAS powders and DE dust off the insects with a fine round craft paintbrush (Renoir Nylon Craft Paint Brush, Size 0, Bunnings, Australia), and keep the particle materials in glass Petri dishes, (ii) blow the SAS powders and DE dust off the insects' bodies into glass Petri dishes using a laboratory rubber suction ball fitted to a glass pipette, and (iii) wash the insect bodies by rinsing with n-hexane for 5 seconds to remove any residual powder and dust adhered to insects' cuticle layer, these residues were discarded. Then, the powders, dust and insects were separately transferred to glass vials (20 mL) and immersed with 10 mL HPLC-grade hexane for 24 hours to extract the lipids. Untreated SAS powders, DE dust and insects were extracted with hexane as a control. The powder/dust/insect-hexane mixtures were centrifuged (5000 g) for about 30 minutes at 4°C to separate the solids from the solution. The supernatant hexane layer was withdrawn, concentrated under a stream of nitrogen, and then the extracts were reconstituted in 1 mL of acetonitrile ($C_2H_3N_2$).

To evaluate changes in energy metabolites, 15 mg of each phosphine-resistant *T. castaneum* and *R. dominica* adult insects were homogenised in 2 mL BeadBug™ microtubes containing 1 mL pre-frozen solvent mixture to extract for 5 min with four replicates. This method was adequate for extracting the various chemical groups, such as amino and carboxylic acids, carbohydrates, sugar alcohols and fatty acids. After 5 min centrifuging at 10,000 g, 450

µL of the supernatant of the extraction mixture was filtered through 0.45µm filters (Millex-FG, Millipore, MA, USA), and transferred to two separate 2 mL amber GC vials. The two vials were consecutively dried using a 1.5-litre shelf freeze dryer (SJIA-5FE, CN). Derivatisation of dried samples was performed in two steps; first, 20 µL of a fresh methoxylamine hydrochloride solution (20 mg/mL in pyridine) was added, followed by 91 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) with 1% Trimethylchlorosilane (TMCS) and Retention Index (RI) determined. The sample preparation was the same as above, except ten biological replicates were used to compare the metabolite changes between phosphine-susceptible (SS) and strongly resistant (SR) *T. castaneum*.

4.2.6 Solid-phase Microextraction Fiber (SPME) for Lipids Extraction

All insect samples collected for the metabolites profiling were quenched in the liquid nitrogen for 5 seconds and stored in freezer (-20°C) until the analysis. Treated and control insect samples (25 mg) were homogenised in a 2 mL BeadBug™ microtube containing 1.5 mL HPLC grade acetonitrile and extracted for 5 minutes. One millilitre of supernatant was transferred into a 2 mL amber GC vial with a septum. The three-phase SPME fiber was pierced through the septum and placed into the solution to extract and enrich fatty acids and alkanes. After two hours of extraction, the fiber was retracted into a protective sleeve and exposed to a GC injection port. The compounds were desorbed from the SPME fiber into the GCMS injection port.

4.2.7 Gas Chromatography and Mass Spectrometry (GC-MS) Based Global Metabolites Profiling

The derivatised samples were then evaporated in an injection inlet for an Agilent 7890B+5977E GCMSD (Agilent Technologies, VIC, Australia analysis. The GC was equipped with a split/splitless injector during the analysis, which operated under the splitless mode. The injection inlet was set at 300°C, and the GC purge valve was switched on 1 min after injection. An Agilent HP-5MS 30m × 0.25mm × 0.25 µm was used for separation. Ultra-high purity helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The initial oven temperature was held at 60°C for 2 min, then increased to 200°C at 7°C/min and then to 300°C at 5°C/min the held for 4 min. The Mass Selective Detector (MSD) transfer line, ion source, and quad-pole temperatures were 200, 230 and 150°C, respectively.

Agilent GCMS Mass Hunter Software was used for data acquisition and analysis, in which the extracts were randomly analysed in two independent iterations. The mass spectra of the unknown compounds were deconvoluted and identified by AMDIS_32 with the NIST MS

database 2014. The retention index was also used to assist in the identification of compounds. Peak detection, deconvolution, filtering, scaling, integration, and quantitation were conducted in the Mass Hunter Quantitative Analysis for GCMS Software (Ver. 7.045.7).

4.2.8 Liquid Chromatography and Mass Spectrometry (HPLC-MS) Analyses

All samples were analysed using a Waters Acquity UPLC-ESI-Q-Tof (WATERS Crop., USA). Data acquisition and processing were performed using Masslynx software (version 4.1). Chromatographic separation was performed on a Waters Acquity® BEH C18 column (2.1 × 100mm, 1.7 μm). The binary gradient was run with consisted eluents A (100% Water) and B (100% Acetonitrile) with 0.1% formic acid at room temperature with a flow rate of 0.2 mL/min and a 10 mL injection volume of sample extracts. Optimal separation was achieved using the following solvent gradient elution: mobile B started at 45%, increased to 75%, 85%, and 100% within time ranges of 0−20, 21−39, and 39−41 min, respectively, held for 2 min, then decreased to 45% for 30 s, followed by 90 s of re-equilibration resulting in a total run time of 45 min.

All samples were analysed twice in positive and negative ionisation mode with 50 to 1,000 Dalton full scan mode. The optimum MS parameters were operated at capillary voltage 3.1 kV; Sample cone 45V, Extraction 5.0V, ion guide voltage 3.0 V, and desolvation gas temperature 350°C with 350 L/min of desolvation gas, collision cell 0.6 mL/min of UHP Argon, Detector voltage 1820 V.

4.2.9 Evaluate the Effect of the SAS on the Hatching of *T. castaneum* Eggs

To evaluate the effect of SAS powder on the hatching of *T. castaneum,* one gram of mixed age and gender *T. castaneum* adults were transferred into a 2L glass jar with 20 black paper strips (15 cm (L) x 3 cm (W)) for 24-48 hours. Newly laid eggs were collected with the black strips and then divided into glass Petri dishes, each with 50 eggs and 6.4 mg HL-SAS or HB-SAS (equivalent 1 g/m^2) evenly distributed on the bottom. For the controls, the eggs were kept constant at 28±1°C and 65±5% R.H. eggs in clean Petri dishes. The number of newly emerged larvae was recorded daily until no new hatching occurred. There were five replicate Petri dishes.

4.2.10 Evaluate the Efficiency of the SAS on Nascent *T. castaneum* Larvae

To evaluate the efficiency of the two SAS powders on the nascent larvae of *T. castaneum*, an experiment was conducted to evaluate the efficiency of the powders on newly hatched larvae. The freshly hatched larvae were continually kept in the initial glass Petri dishes with 5 g wholemeal wheat flour mixed with 6.4 mg powder (equivalent dosage 1 g/m^2). Five replicate

Petri dishes were prepared. The number of dead larvae was recorded daily until all nascent larvae died. Petri dishes without powder but with food and nascent larvae were prepared as a control.

4.2.11 Efficiency of the SAS on the 1st instar *T. castaneum* Larvae

To evaluate the effect of SAS powder on the first instar larvae of *T. castaneum*, 1 g of mixed age and gender *T. castaneum* were transferred into a 2 L glass jar with 20 black paper strips (15 cm (L) x 3 cm (W)). After 48 hours, *T. castaneum* adults were removed from the 2L glass jar. These eggs developed to larvae at 28±1°C and 65±5% R.H. The first instar larvae (two-days-old) from the culture were released into glass Petri dishes. To each Petri dish, 5 g wholemeal wheat flour with either 6.4 mg HL-SAS or HB-SAS (equivalent dosage of 1 g/m^2) and 50 larvae of *T. castaneum* were added. Larvae in clean Petri dishes were prepared as controls. There were five replicate Petri dishes per treatment. The larvae in the Petri dishes with powder were kept for 24 hours and then moved to clean Petri dishes. The number of dead larvae was recorded daily until 100% mortality, or no more moulting was observed.

4.2.12 Statistical Analysis

Agilent Mass Hunter Software was used to acquire and analyse extracts, these were randomised and analysed in two independent iterations. The mass spectra of unknown compounds were deconvoluted and identified by AMDIS_32 with the NIST MS database 2014. The retention index was also used to help identify the compounds.

For quantitation of metabolite features, peak detection, deconvolution, filtering, scaling, and integration were processed by Mass Hunter Quantitative Analysis for GCMS (Ver. 7.045.7). Each lipid extract was analysed in duplicate, resulting in six technical replicates per biological replicate (e.g., each strain). Peak detection, deconvolution, filtering, scaling and integration were detected, extracted, and aligned using MZmine 2. Chromatographic peaks were extracted from 1-36 min with a retention time error window of 0.1 min and mass spectral peaks detected from 50 to 2000 m/z with a mass error window of 7ppm, generating a data matrix consisting of retention time, m/z, and peak intensity based on peak area for all features. Data entry and primary statistical analyses were performed using Microsoft Excel Office 365 (Microsoft, NY, USA). Analyses of differences between groups were conducted using unsupervised and supervised multivariate statistical analysis techniques, including principal component analysis (PCA), partial least squares regression - discriminant analysis (PLS-DA), and one-way analysis of variance (ANOVA) followed by the Student Newman-Keuls post hoc

test to evaluate and visualise the metabolomics data through MZmine2 and Metaboanalyst 4 (Pluskal *et al.* 2010; Chong *et al.* 2018).

4.3 RESULTS

4.3.1 Performance of UPLC-Q-TOF Lipids Analytical Method

The UPLC-Q-TOF lipids analytical method was able to qualify and quantify mono-, di, and triacylglycerols (MGs/DGs/TGs) extracted from *T. castaneum* treated with HB-SAS and HL-SAS (Figures 4.1 and 4.3). This method also detected the lipids adsorbed on the HB-SAS powders, which had been removed from treated insects (Figure 4.2).

Comparing the untreated control live insects, frozen dead insects, and the HB-SAS treated insect extractions showed that they had lost energy lipids, such as the triacylglycerols (TGs) group (Figures 4.1 and 4.2). Due to the complexity of lipid sub-class identification, for example, PE (Phosphatidylethanolamine) and PC (Phosphatidylcholine) could not be separated using this analytical method. As direct evidence, the lipid compounds absorbed onto the HL-SAS powder were observed directly from the HL-SAS powder collected from the treated insect bodies (Figure 4.3). The HB-SAS did not absorb cuticle wax and lipids or damage the insect's cuticle.

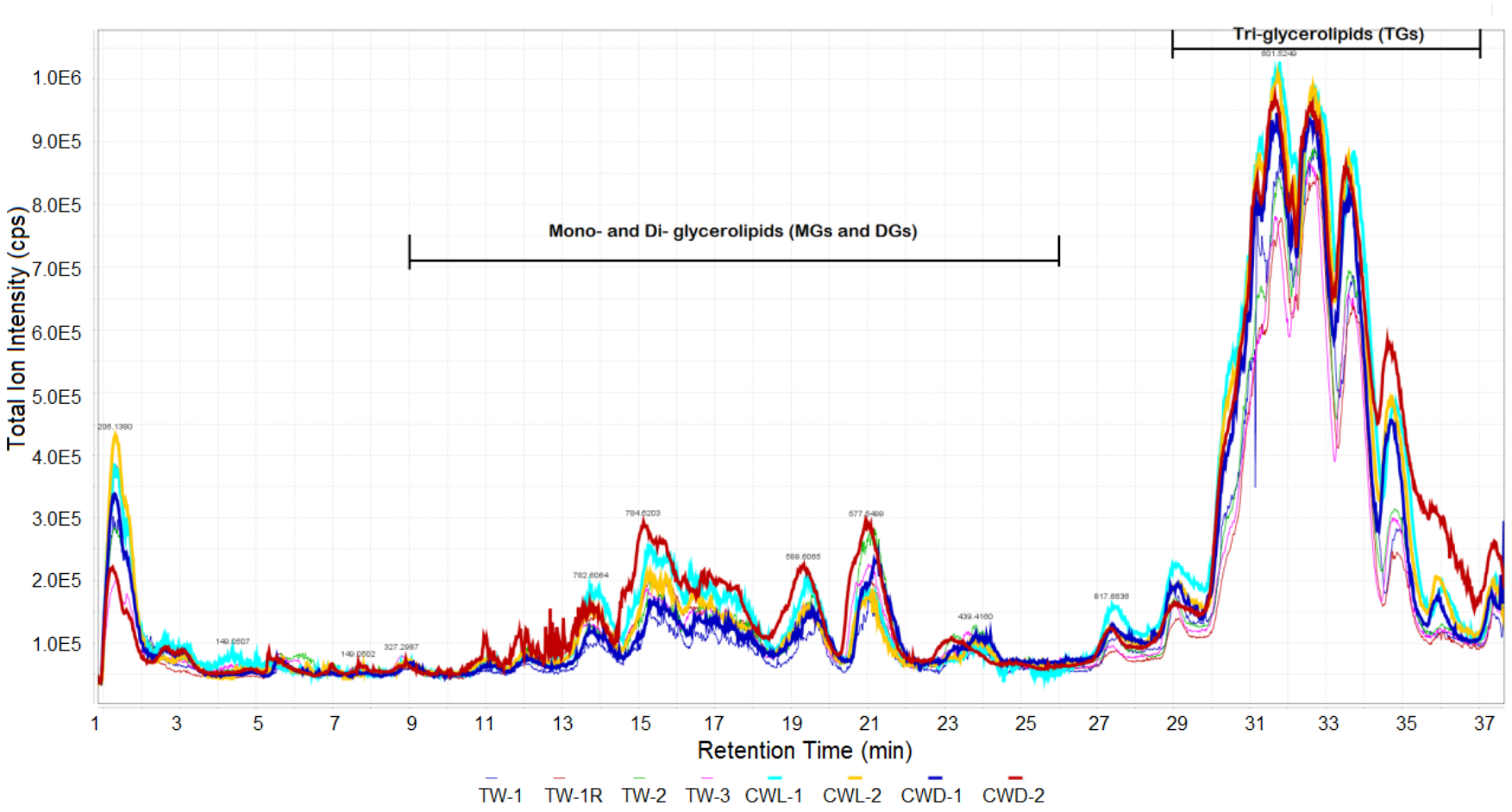


Figure 4.1. The total ion chromatograph (TIC) of UPLC-Q-TOF reflects the effects of HB-SAS powder on the lipid compounds obtained from the bodies of adult *T. castaneum* after HB-SAS treatment for 48 hours. CWL=Control live insects, CWD=Frozen dead insects, TW=HB-SAS treated insects.

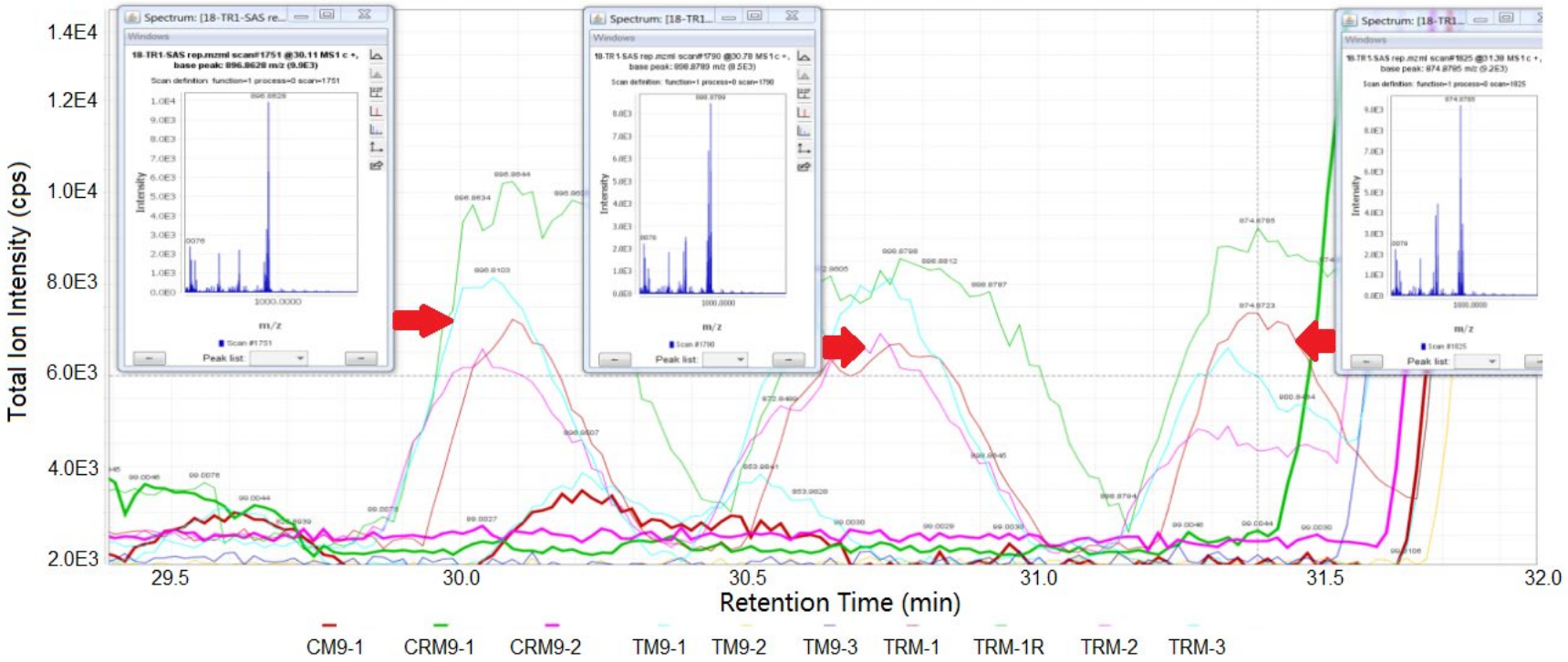


Figure 4.2. The UPLC-Q-TOF graph of lipid compounds absorbed by HB-SAS powder removed from adult *T. castaneum* after 48 hours of exposure to HB-SAS. Three lipids with a similar mass to charge ratio (m/z), 896.8628, 898.8789, and 874.8785, were well separated at the retention times of 30.11, 30.78 and 31.38 min. CM9=Control HB-SAS powder, CRM9=Control HB-SAS powder residue, TM9=Treated HB-SAS powder, and TRM=SAS powder collected from treated insects.

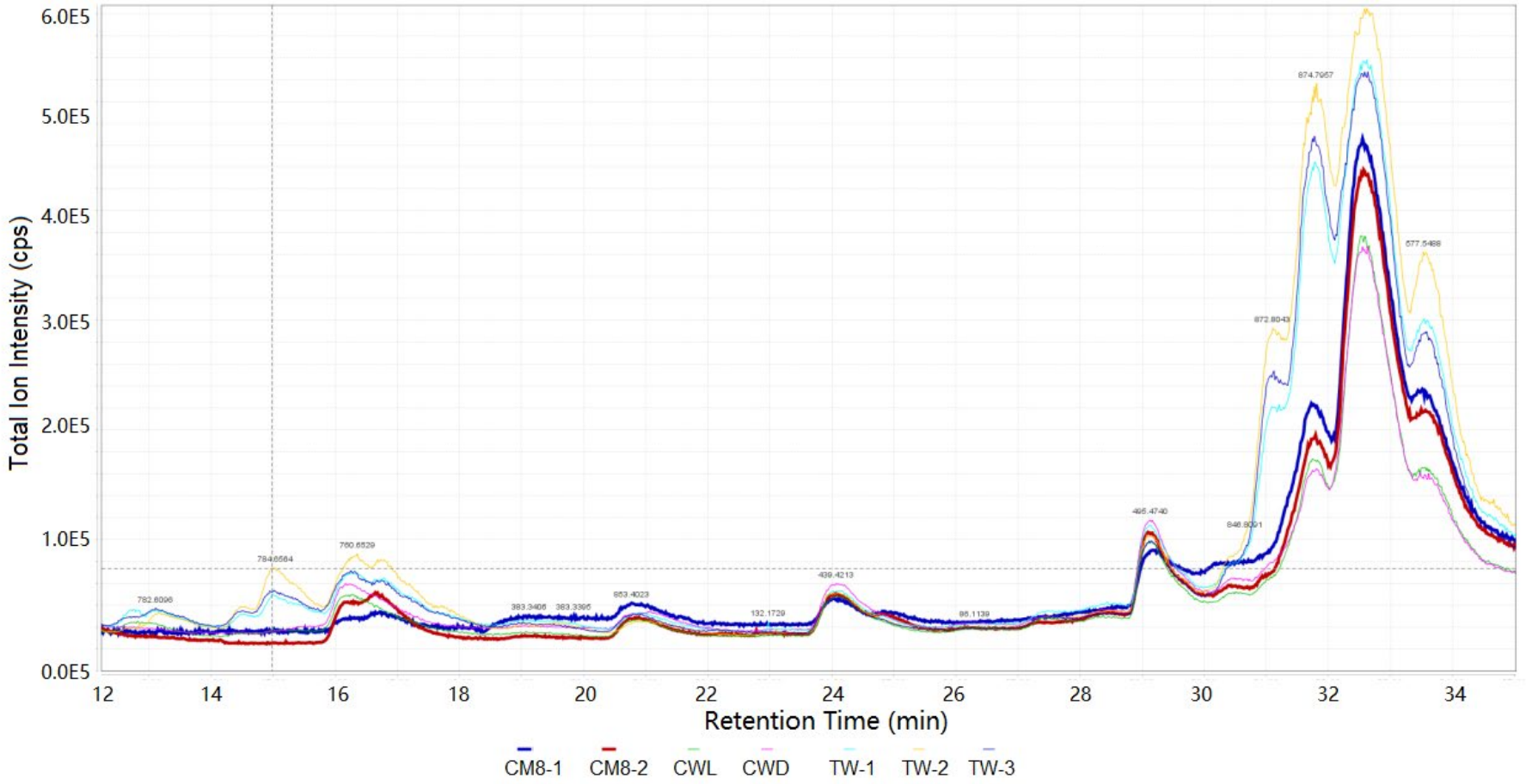
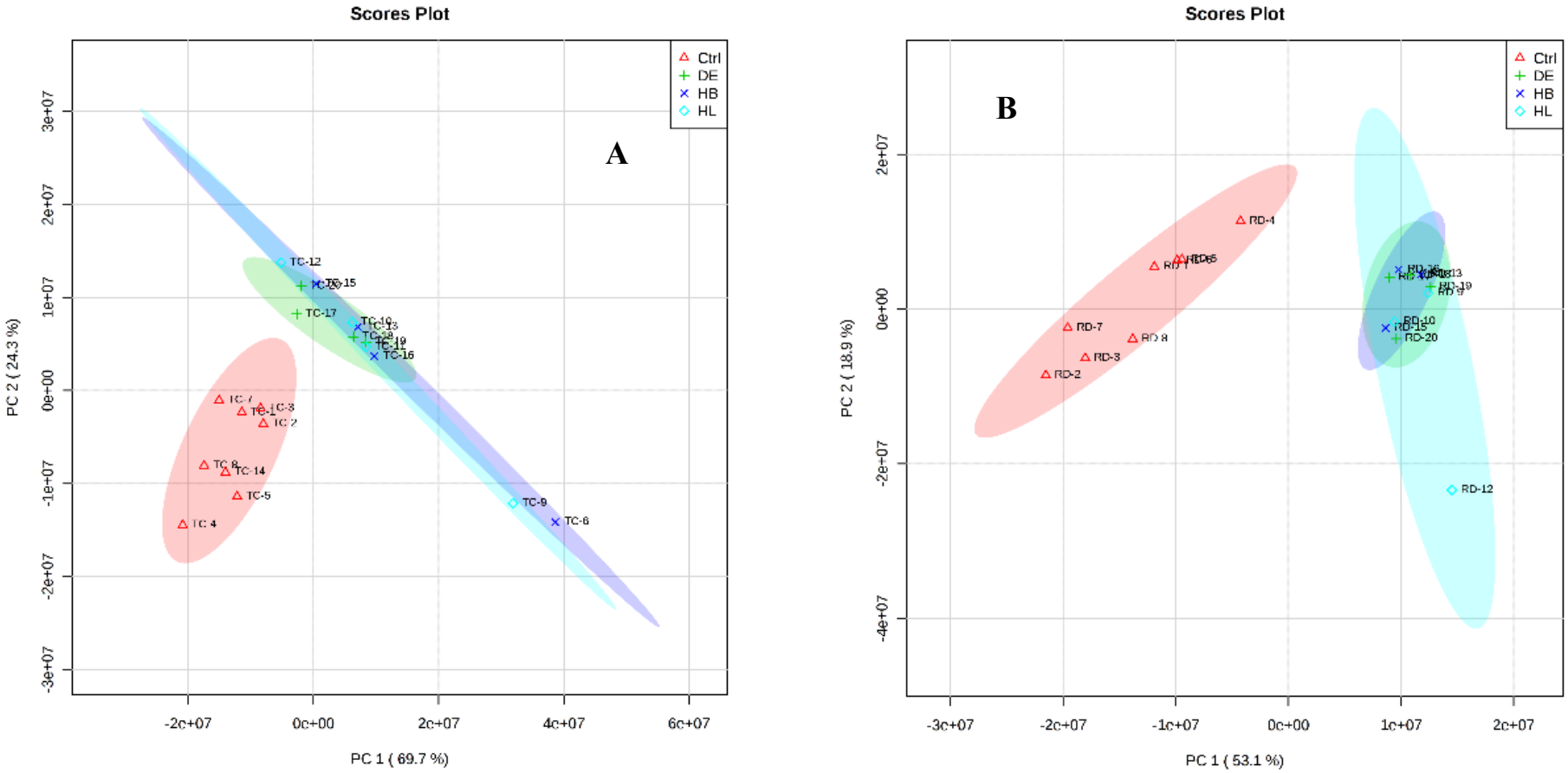


Figure 4.3. The UPLC-Q-TOF graph of lipid compounds absorbed by HL-SAS powder from *T. castaneum* after 48 hours of exposure to HL-SAS. CM8=Control HL-SAS powder, CWL/CWD=SAS powder collected from control live/dead insects, TW=SAS powder collected from treated insects.

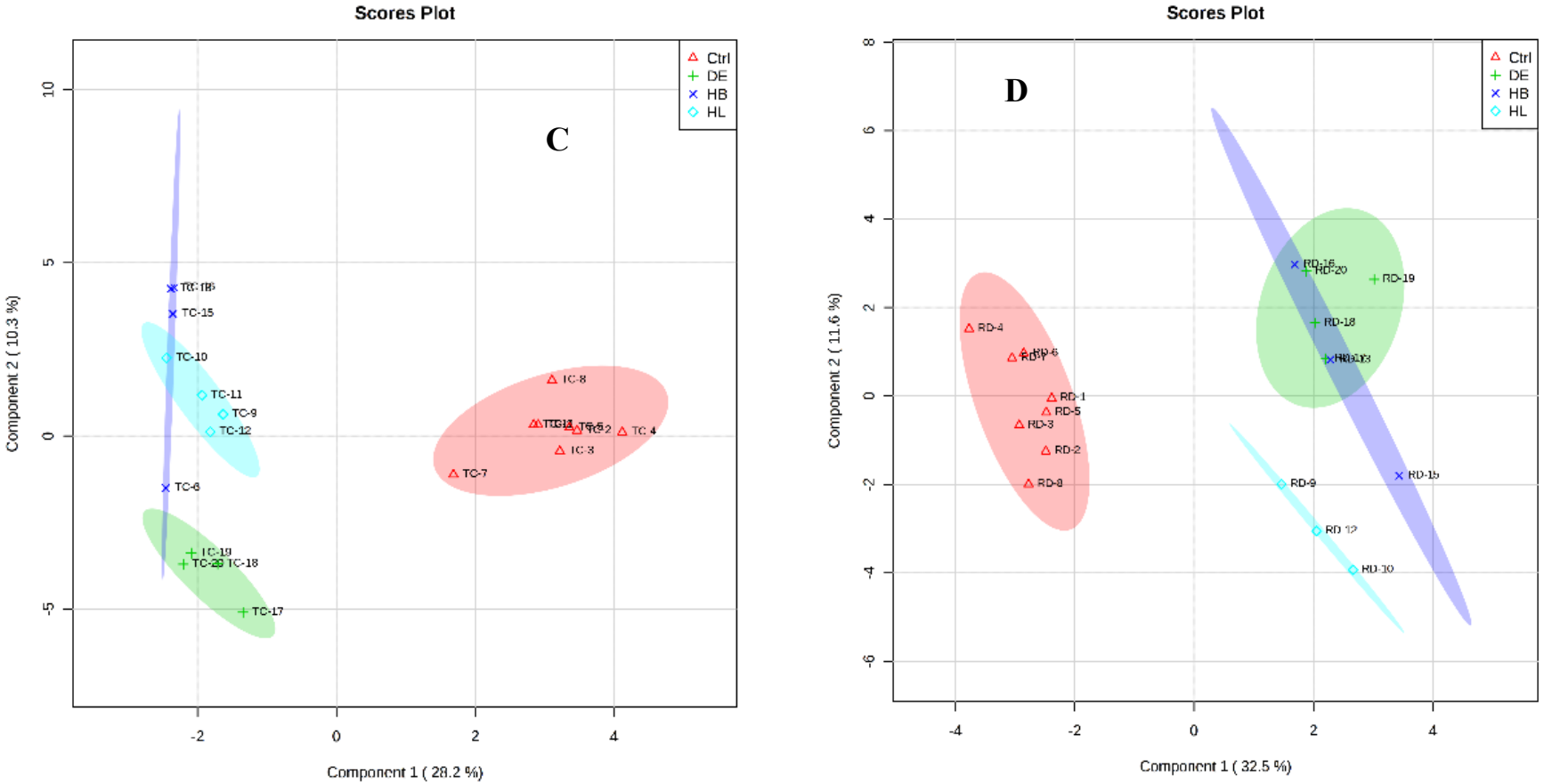
4.3.2 Mass Spectrometry-based Global Metabolites Profiling

By combining GC-MS, positive LC-MS, and negative LC-MS, 123, 584 and 126 features from a total of 836 features were detected from *T. castaneum,* and 112, 562, and 245 with 919 features in total were detected from *R. dominica.* The metabolic datasets were analysed without filtering and scaling in MetaboAnalyst 3 (Figures 4.4 and 4.5). Unsupervised multivariate analysis by principal components analysis (PCA) of all features illustrated clear discrimination between the control and the three treatments (PC1/2:69.7%/24.3% and 53.1%/28.9% for *T. castaneum* and *R. dominica,* respectively). However, no obvious class separation was found among the HL/HB-SAS and DE treatments. The supervised multivariate analysis and the sparse Partial-Least Squares Discriminant Analysis (sPLS-DA) algorithm were applied to elucidate the different effects of the two SAS powder and DE dust treatments by reducing the number of variables (features) in the untargeted metabolomics data. The sPLS-DA classes were separated across Components 1 & 2: 28.2% & 10.3% and 32.5% & 11.6% for *T. castaneum* and *R. dominica,* respectively. The 95% confidence intervals of the HB-SAS partially overlapped the HL-SAS and DE treatments. Only features extracted by One-way ANOVA with a significant ($P<0.05$) difference and distinct fold changes ($FC>1.5$) were qualified from the Feihn metabolites database and the lipids database through MS-Dial. The metabolites identified belong to a broad range of metabolic pathways, such as the TCA/Krebs cycle, amino acids metabolism, glycolysis and gluconeogenesis, pentose phosphate, and nucleotide metabolic pathway (Figure 4.6).

Analysing the differences between the control and two SAS powders and the DE treatments showed that all three treatments had similar trends of energy metabolic changes in both insect species. After the three treatments, monosaccharides, glucose and fructose, and amino acids, including glycine, L-Tyrosine, L-Valine, L-Serine, L-Asparagine, L-Glutamine, L-Threonine, lysine, phenylalanine, beta-Alanine, and tryptophan, were reduced significantly. However, trehalose, fructose-6-phosphate (F6P), oleic acid, glycerol, triacylglycerol lipids (TAGs), and other structural lipids were over-expressed for all treatments in *T. castaneum* and *R. dominica*. Decreasing sugar alcohols, ribitol and xylitol, and increasing tryptophan and beta-alanine were only observed in *T. castaneum*. Two disaccharides, maltose and trehalose, presented opposite trends in *T. castaneum* and *R. dominica,* respectively.



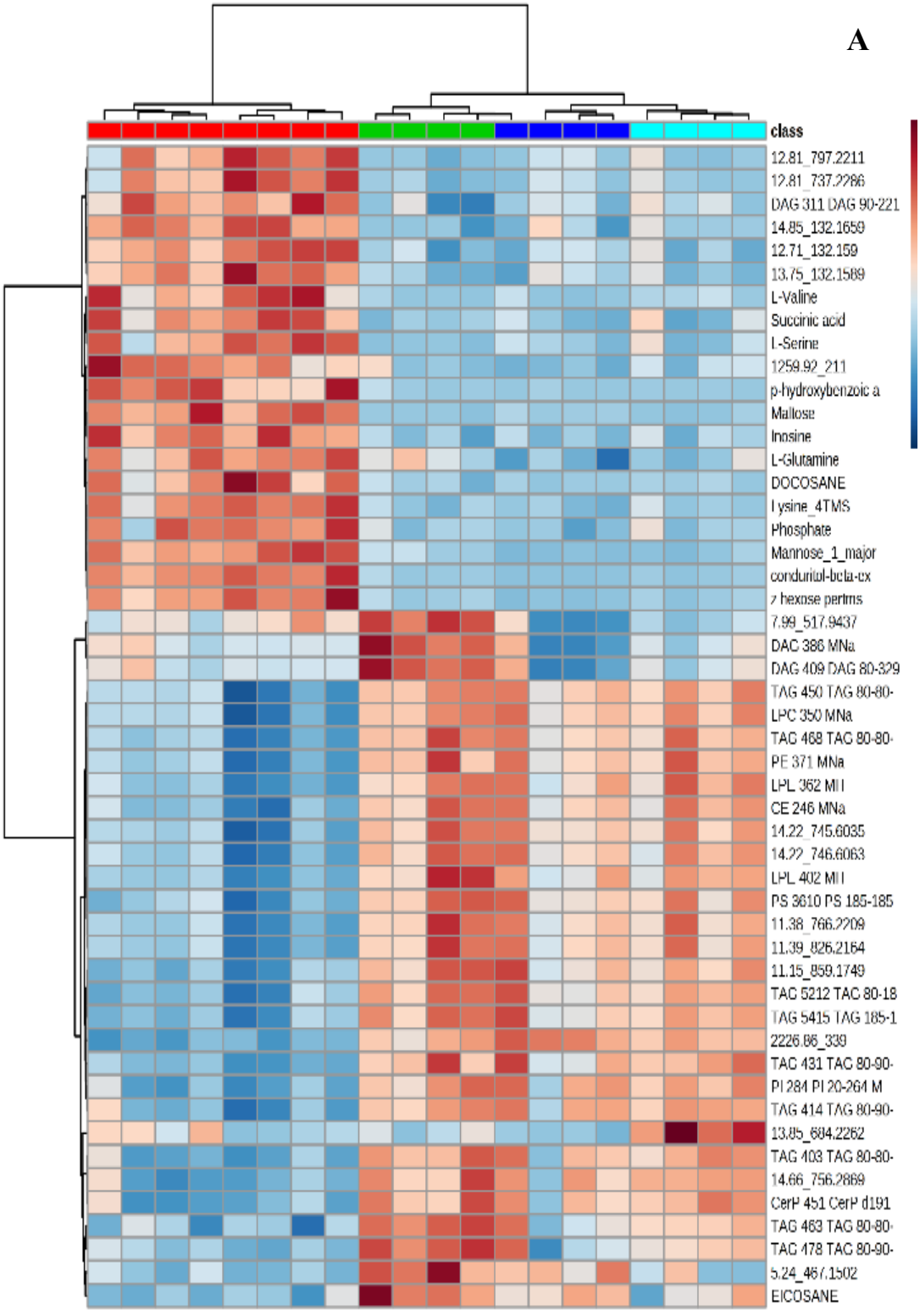
Principal Component Analysis (PCA)



Sparse PLS Discriminant Analysis (sPLS-DA)

Figure 4.4. Unsupervised and supervised multivariate analysis of *T. castaneum* and *R. dominica* exposed to hydrophobic synthetic amorphous silica (HB-SAS), hydrophilic synthetic amorphous silica (HL-SAS), and diatomaceous earth (DE) relative to the control (Ctrl). Unsupervised multivariate analysis: principal component analysis (PCA) PC1/PC2 score plots of global metabolites profiles of whole T. castaneum (A) and R. dominica (B). Supervised multivariate analysis: sparse partial least squares-discriminant analyses (sPLS-DA) Component 1/Component 2 score plots of global metabolites profiles of whole *T. castaneum* (C), and *R. dominica* (D). Four biological replicates were performed for each treatment. The replicate samples of the control group were collected twice from before and after treatments and combined for data quality control. The ellipses represent the 95% confidence intervals per sample group. Red represents the control group; blue represents HL-SAS; purple represents HB-SAS; green represents DE dust

.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12.81_737.2286
DAG 311 DAG 90-221
14.85_132.1659
12.71_132.159
13.75_132.1589
L-Valine
Succinic acid
L-Serine
1259.92_211
p-hydroxybenzoic a
Maltose
Inosine
L-Glutamine
DOCOSANE
Lysine_4TMS
Phosphate
Mannose_1_major
conduritol-beta-ex
7 hexose pertms
7.99_517.9437
DAC 386 MNa
DAG 409 DAG 80-329
TAG 450 TAG 80-80-
LPC 350 MNa
TAC 468 TAG 80-80-
PE 371 MNa
LPL 362 MH
CE 246 MNa
14.22_745.6035
14.22_746.6063
LPL 402 MH
PS 3610 PS 185-185
11.38_766.2209
11.39_826.2164
11.15_859.1749
TAC 5212 TAC 80-18
TAG 5415 TAG 185-1
2226.86_339
TAC 431 TAC 80-90-
PI 284 PI 20-264 M
TAG 414 TAG 80-90-
13.85_684.2262
TAG 403 TAG 80-80-
14.66_756.2869
CerP 451 CerP d191
TAG 463 TAG 80-80-
TAC 478 TAC 80-90-
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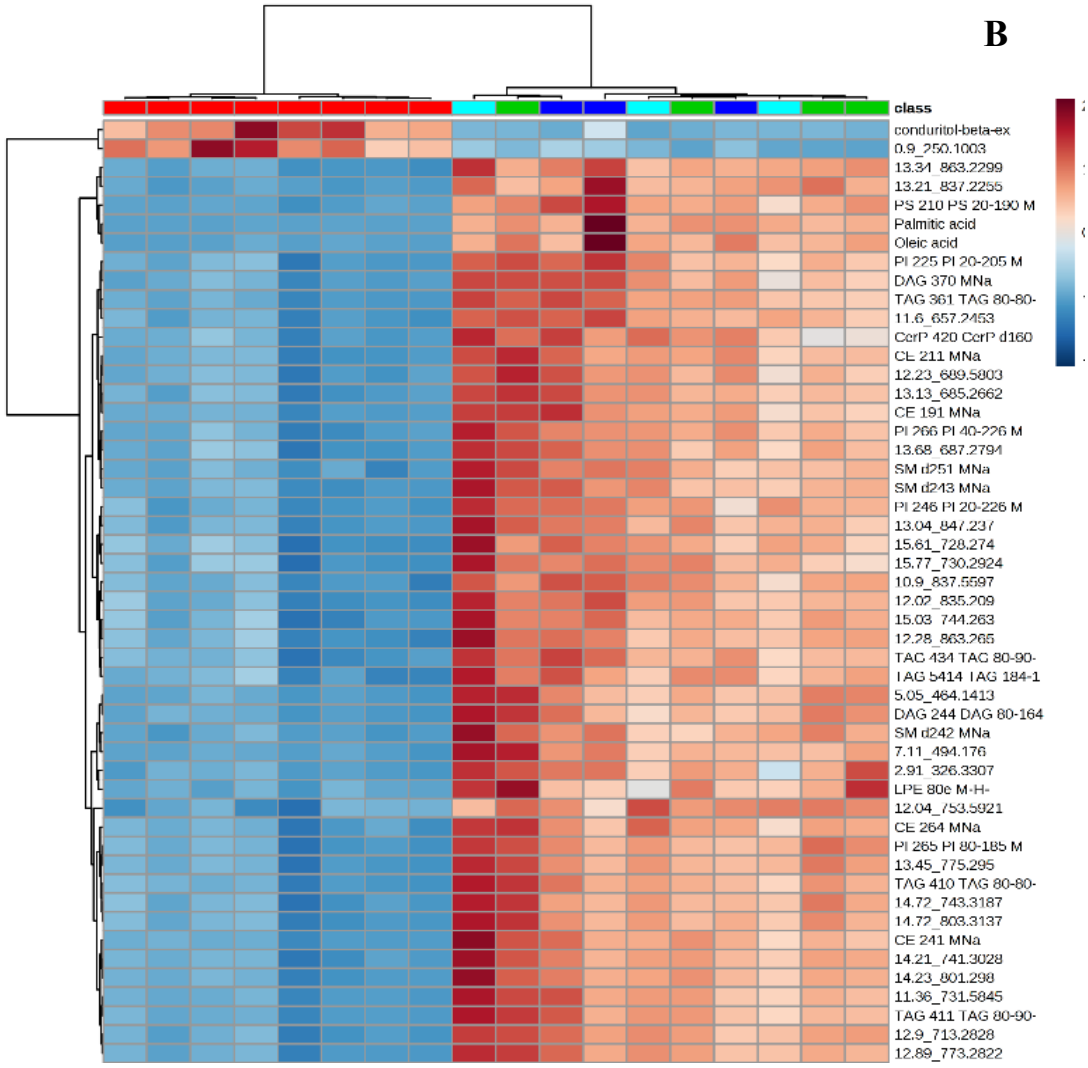
EICOSANE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13.34_863.2299
13.21_837.2255
PS 210 PS 20-190 M
Palmitic acid
Oleic acid
PI 225 PI 20-205 M
DAG 370 MNa
TAG 361 TAG 80-80-
11.6_657.2453
CerP 420 CerP d160
CE 211 MNa
12.23_689.5803
13.13_685.2662
CE 191 MNa
PI 266 PI 40-226 M
13.68_687.2794
SM d251 MNa
SM d243 MNa
PI 246 PI 20-226 M
13.04_847.237
15.61_728.274
15.77_730.2924
10.9_837.5597
12.02_835.209
15.03_744.263
12.28_863.265
TAC 434 TAC 80-90-
TAG 5414 TAG 184-1
5.05_464.1413
DAG 244 DAG 80-164
SM d242 MNa
7.11_494.176
2.91_326.3307
LPE 80e M-H-
12.04_753.5921
CE 264 MNa
PI 265 PI 80-185 M
13.45_775.295
TAG 410 TAG 80-80-
14.72_743.3187
14.72_803.3137
CE 241 MNa
14.21_741.3028
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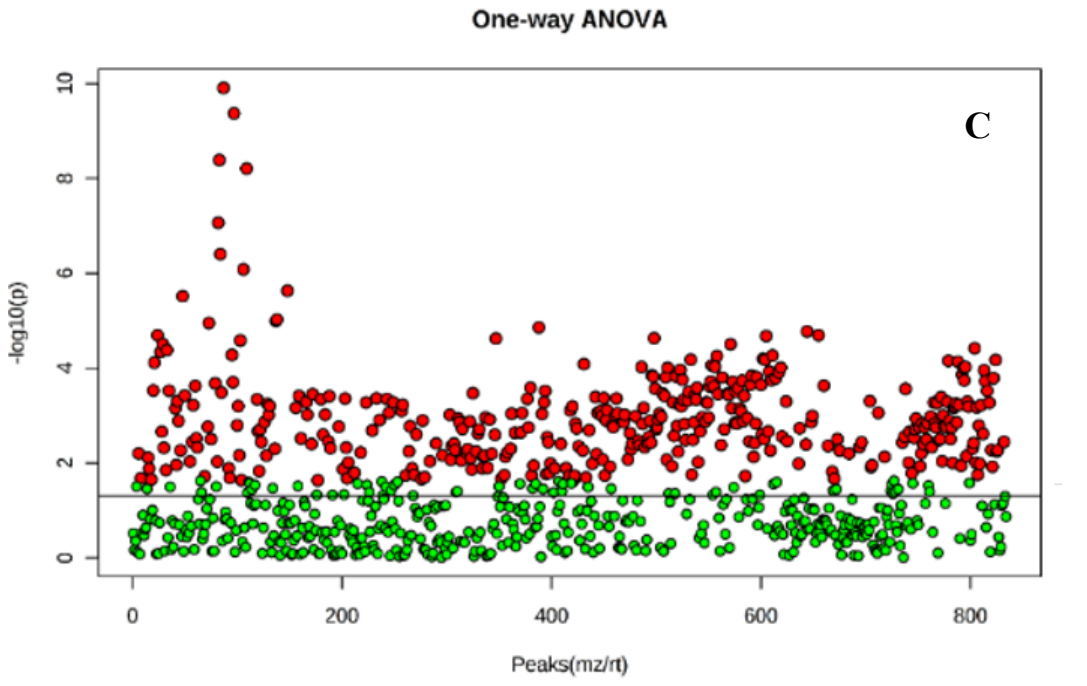
12.9_713.2828
12.89_773.2822


One-way ANOVA
C
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0
2
4
6
8
10
0
200
400
600
800
Peaks(mz/rt)

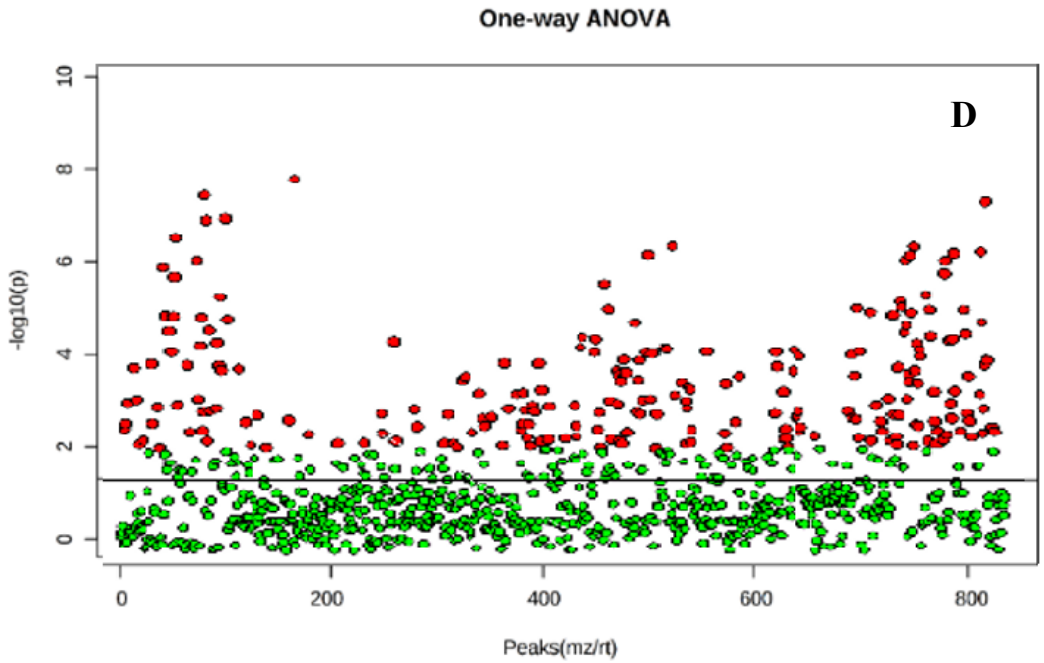
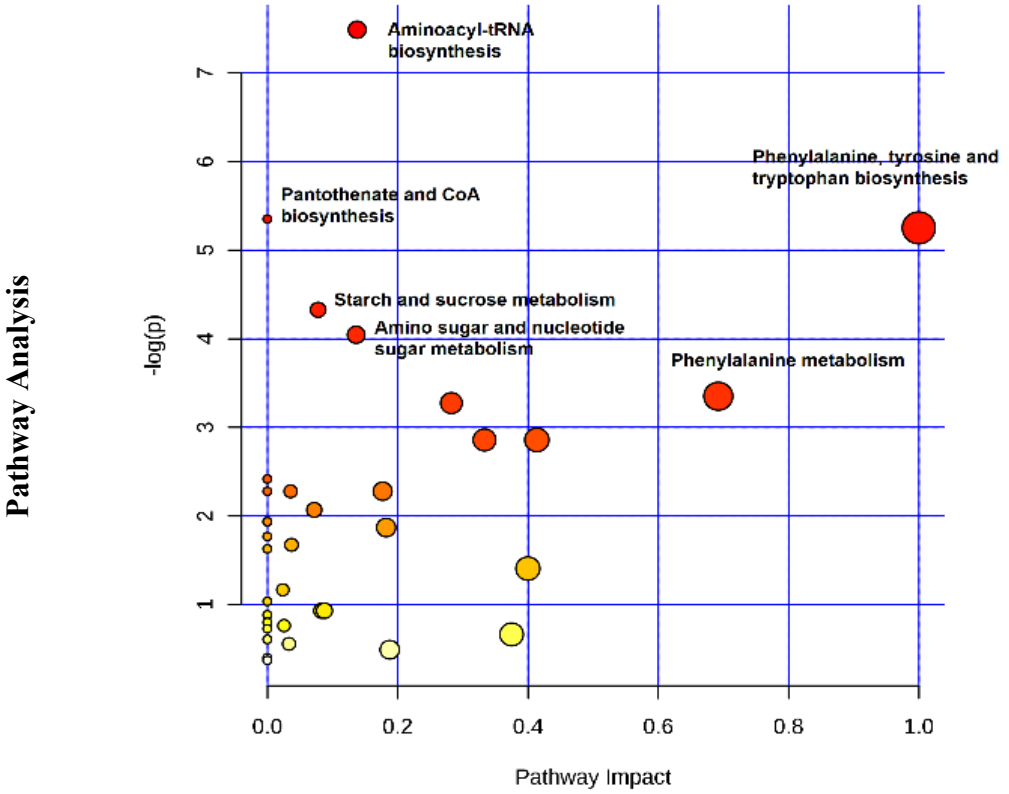
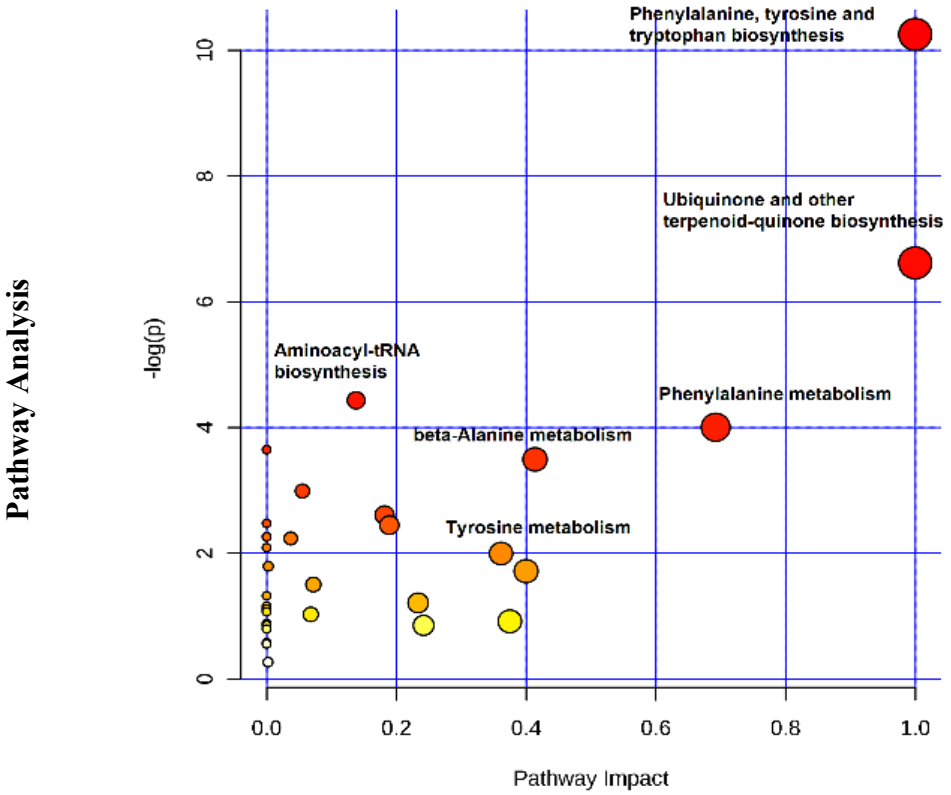


Figure 4.5. A heat map with two-dimensional hierarchical cluster analysis of *T. castaneum* (TC) (A), and *R. dominica* (RD) (B) exposed to hydrophobic (HB/blue) synthetic amorphous silica, hydrophilic (HL/light blue) synthetic amorphous silica, and diatomaceous earth (DE/green colour) relative to the control (Ctrl/red colour). The heat map legend shows group (class) labels in the top right corner and depicts high (red) and low (blue) relative levels of metabolite variation on a normalised scale from -2.0 to 2.0. Four biological replicates were performed for each treatment. Control samples were collected twice before and after treatments and combined for data quality control. One-way ANOVA analysis was used to find metabolite variants with significant ($p<0.05$) differences in *T. castaneum* (C) and *R. dominica* (D). The red dots represent the GCMS and LCMS features with significant ($p<0.05$) differences, and the green dots represent the unsignificant features. The X-axis is the peak ID (mz/rt, mass to charge ratio/retention time) of each feature. Y-axis is -log 10 based of the p-value.

A


Pathway Analysis
Aminoacyl-tRNA biosynthesis
Phenylalanine, tyrosine and tryptophan biosynthesis
Pantothenate and CoA biosynthesis
Starch and sucrose metabolism
Amino sugar and nucleotide sugar metabolism
Phenylalanine metabolism
-log(p)
Pathway Impact

B


Pathway Analysis
Phenylalanine, tyrosine and tryptophan biosynthesis
Ubiquinone and other terpenoid-quinone biosynthesis
Aminoacyl-tRNA biosynthesis
Phenylalanine metabolism
beta-Alanine metabolism
Tyrosine metabolism
-log(p)
Pathway Impact

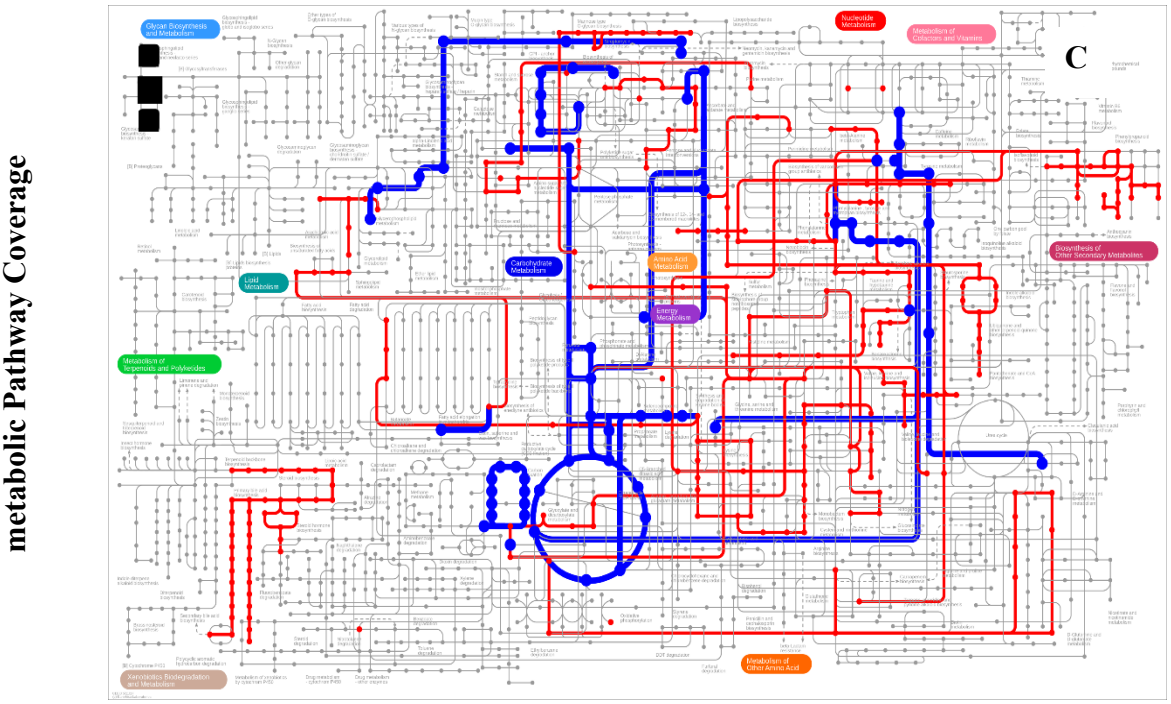


Figure 4.6. Summary of the metabolic pathways and enrichment analysis of *T. castaneum* and *R. dominica* treated with SAS powder. The pathway analysis is based on the data combined from HB-SAS and HL-SAS. (A) Pathway enrichment analysis of SAS treated and control groups of *T. castaneum*. (B) Pathway enrichment analysis of SAS treatment and control groups of *R. dominica*. Key metabolic pathways are named. (C) Overview of metabolic pathways for *T. castaneum* and *R. dominica* exposed to SAS powders compared to untreated ones. Metabolic pathways are shown in different colours: blue, down-regulated pathways; red, upregulated pathways. Statistical significance was evaluated using ANOVA with p value = 0.05.

4.3.3 Changes of Energy Metabolites Related to Phosphine Resistance

There were significant ($p<0.05$) differences in the GC-MS responses of the chromatographic peak areas between the phosphine-susceptible and resistant *T. castaneum*. Ten energy metabolites were revealed, eight decreased, and two increased (Figure 4.7). Two monosaccharides, glucose and fructose, were overexpressed by 9.0 and 2.7-fold (Figures 4.7A and B). At the same time, two disaccharides, trehalose and sucrose, decreased by 4.5 and 4.2-fold in the phosphine-resistant strain, respectively (Figures 4.7C and D). Based on SPME lipids analysis, six saturated and unsaturated free fatty acids (FFAs) with even carbon numbers ranging from 14 to 18 were upregulated by at least 7.2-fold. Palmitic acid (C16:0) and Oleic acid (C18:1), the two most abundant constituents of the FFAs pool, gave GC-MS responses 7.2 and 11.0

times more in the phosphine-resistant strain than in the susceptible strain (Figures 4.7 f and i). Similarly, significant ($p<0.05$) responses were observed in other FFAs with lower abundance (Figures 4.7 e, g, h and j).

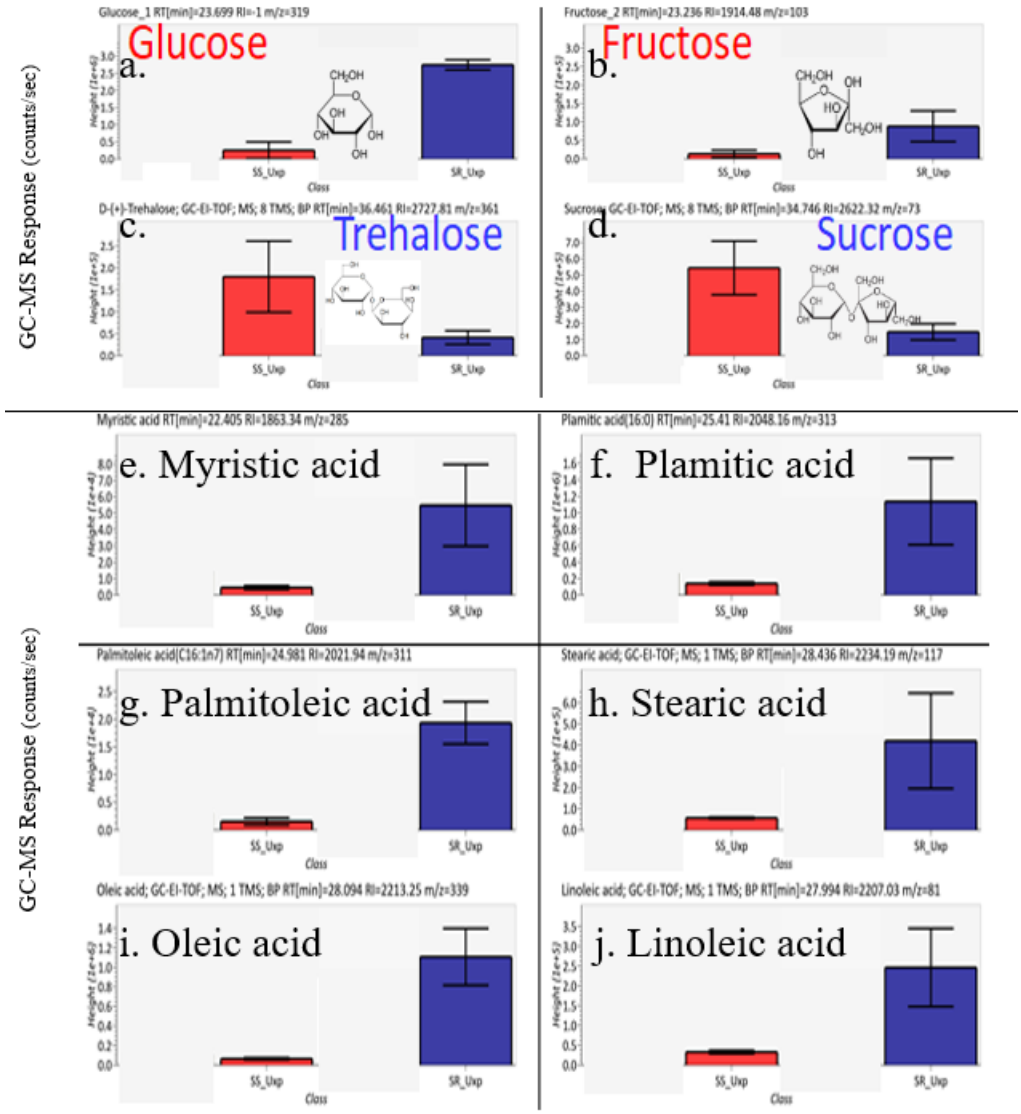


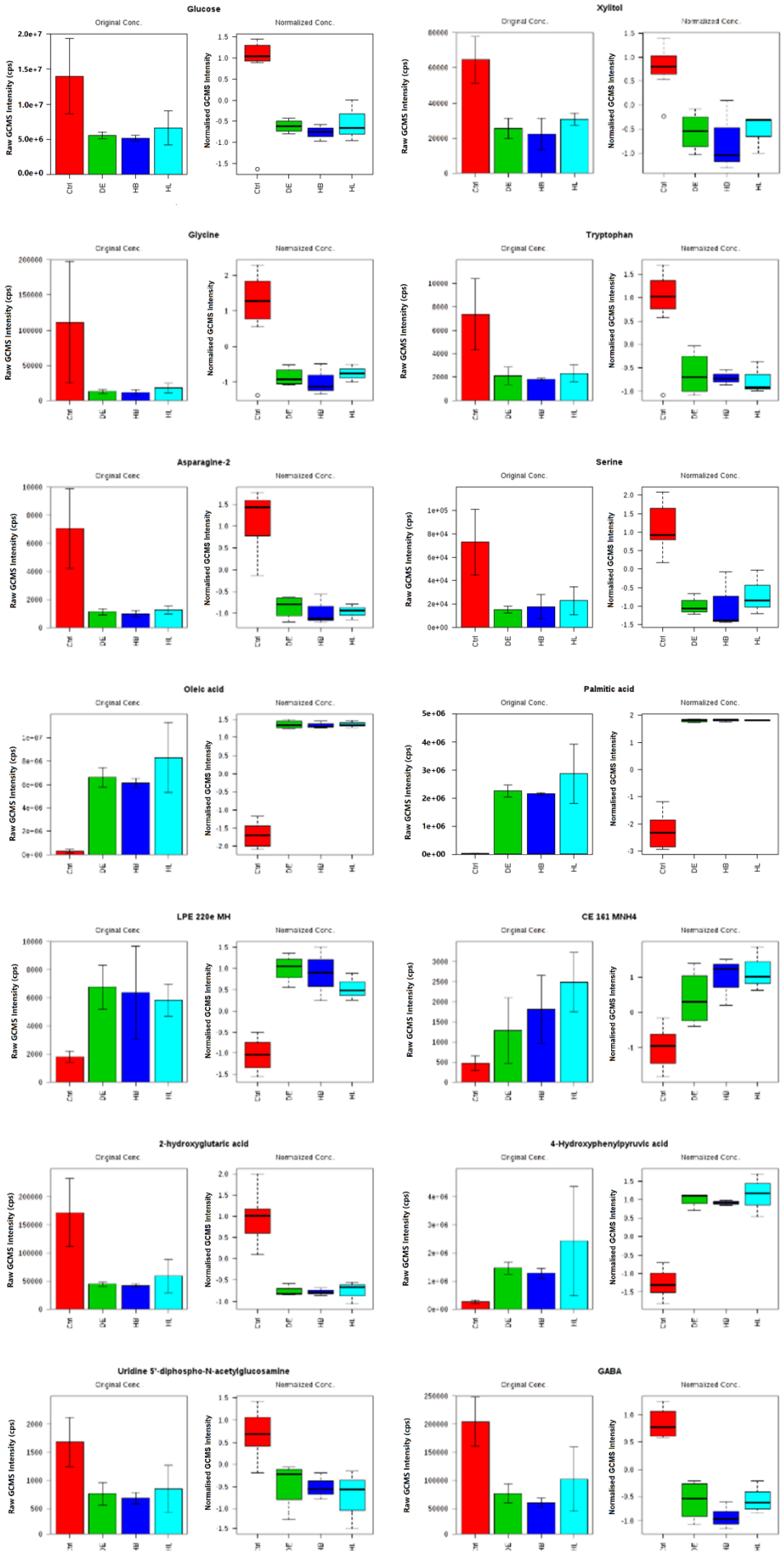
Figure 4.7. Boxplots of four carbohydrates (a-d) and six free fatty acids (e-j) were extracted and analysed in the adults of the phosphine-susceptible strain unexposed to phosphine (SS, MUWTC-CH-S) and strongly resistant strain unexposed to phosphine (SR, MUWTC-SR-500) of the red flour beetle, *T. castaneum*. Two monosaccharides (glucose and fructose) are in red; two disaccharides (trehalose and sucrose) are in blue; six FFAs are in black. GC-MS peaks of derivatised metabolites from whole-body extracts of phosphine-susceptible *T. castaneum* adults (red bars, SS) were compared with peaks observed for strong resistant *T. castaneum* adults (blue bars, SR). GCMS

responses are the counts per second (cps) received by the electron multiplier. Energy substances listed here were significantly ($p<0.05$) different from the phosphine-susceptible strain. All bars are means +/-2SD of 10 biological replicates; some SD bars are too small to be seen.

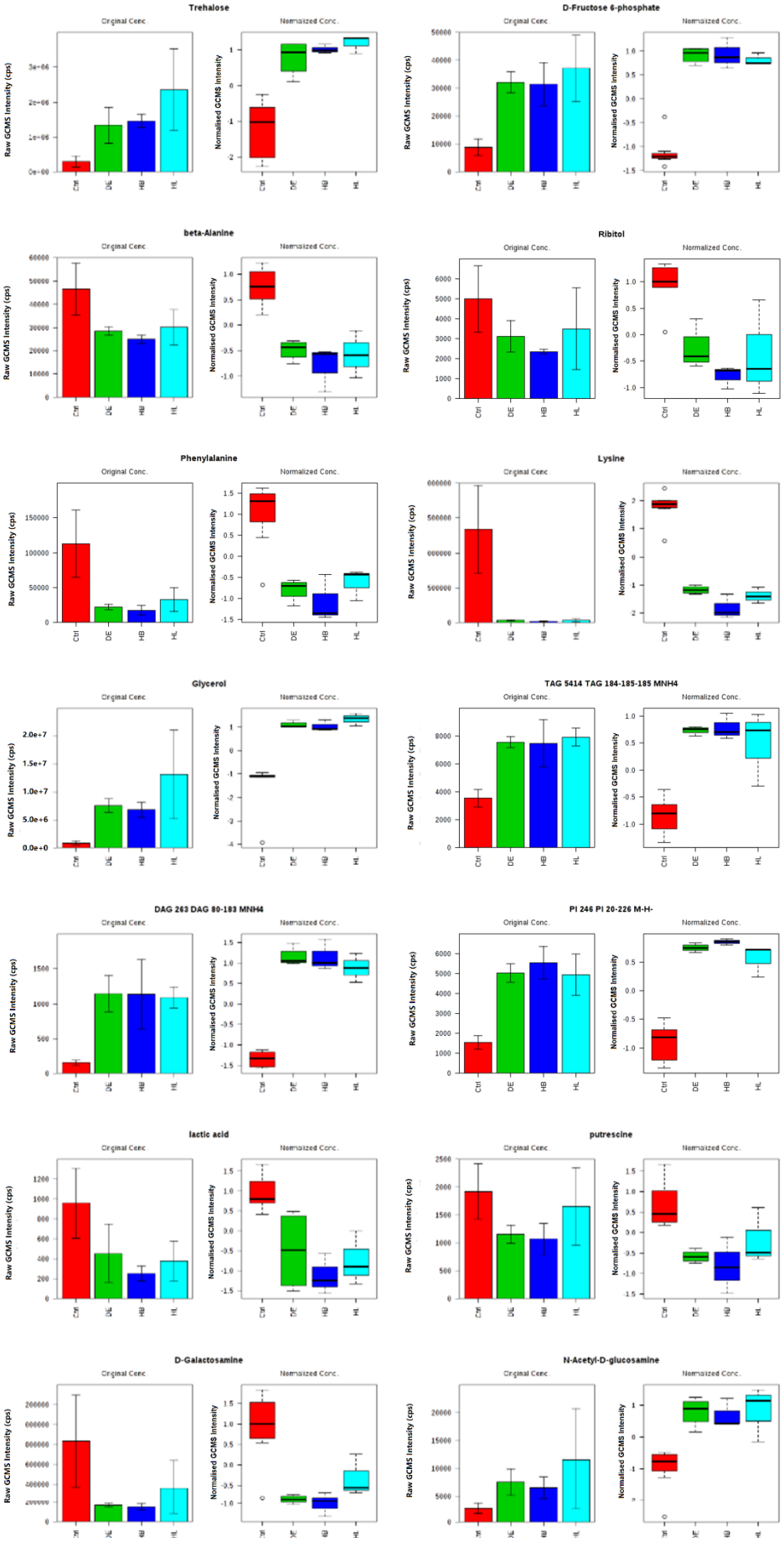
4.3.4 Changes of Energy Metabolites Related to Exposure of SAS Powders and DE Dust

ANOVA analysis applied to the GC-MS responses of the chromatography peaks isolated from control live adult *T. castaneum* exposed to the DE dust and two SAS powders revealed four constituents of the energy metabolites pool, which were altered significantly ($p<0.05$, ANOVA). Treated *T. castaneum* exhibited an overall pattern in the reduction of trimethylsilyl derivatives of mono-/di-saccharides and an elevation of free fatty acids when compared to the untreated control (Figure 4.8 a). *R. dominica* showed the same changes in its energy metabolites (Figure 4.8 b). Maltose is a disaccharide formed from two units of glucose joined with an α (1→4) bond. Both glucose and maltose decreased dramatically by at least 1.7 and 5.5-fold, respectively, while oleic acid increased in response to the treatments, particularly the HB-SAS (Figure 4.8).

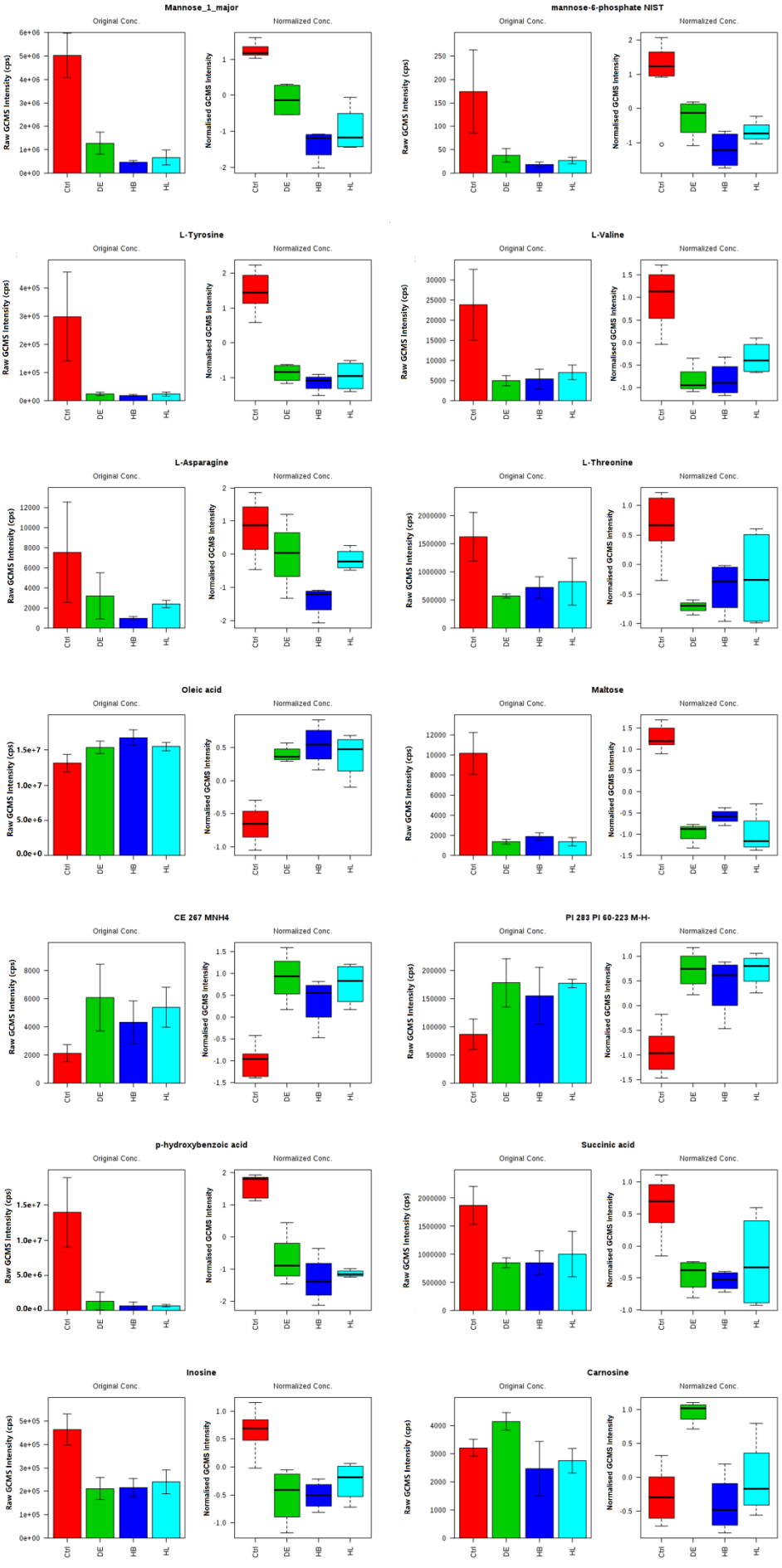
In addition, the high beta-alanine content may contribute to carnosine synthesis (beta-alanyl-L-histidine), a neurotransmitter in the brain and muscle when *T. castaneum* adults are exposed to relatively slow DE dehydration (Figure 4.8 a). Tryptophan, a precursor of neurotransmitter and building block of proteins, increased in *T. castaneum* and decreased in *R. dominica* (Figure 4.8 b).


Glucose
Xylitol
Glycine
Tryptophan
Asparagine-2
Serine
Oleic acid
Palmitic acid
LPE 220e MH
CE 161 MNH4
2-hydroxyglutaric acid
4-Hydroxyphenylpyruvic acid
Uridine 5'-diphospho-N-acetylglucosamine
GABA
Original Conc.
Normalized Conc.
Raw GCMS Intensity (cps)
Normalised GCMS Intensity
Ctrl
DE
HB
HL

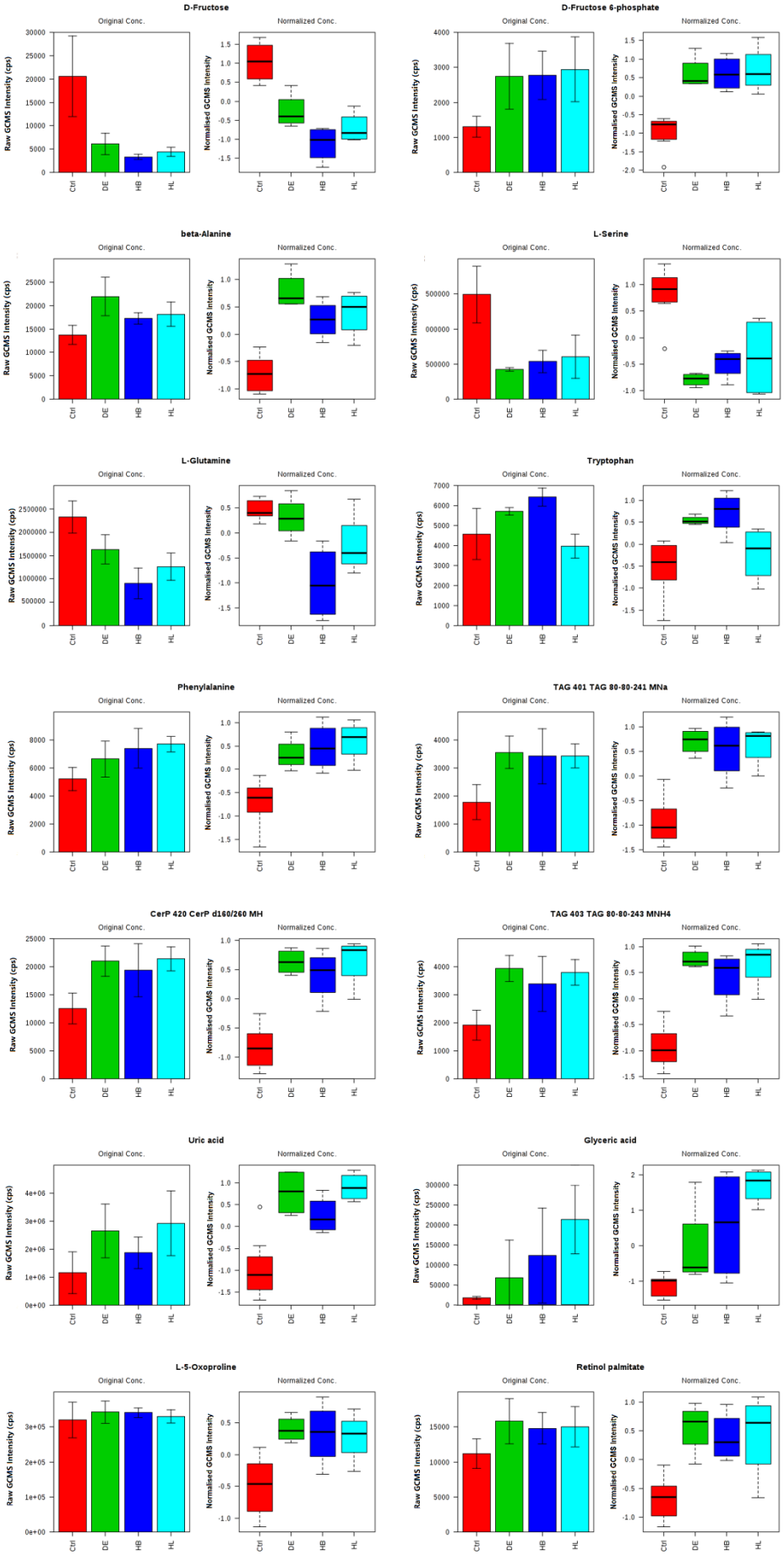
a-1.


Trehalose
D-Fructose 6-phosphate
beta-Alanine
Ribitol
Phenylalanine
Lysine
Glycerol
TAG 5414 TAG 184-185-185 MNH4
DAG 263 DAG 80-183 MNH4
PI 246 PI 20-226 M-H-
lactic acid
putrescine
D-Galactosamine
N-Acetyl-D-glucosamine
Original Conc.
Normalized Conc.
Raw GCMS Intensity (cps)
Normalised GCMS Intensity
Ctrl
DE
HB
HL

a-2.


Mannose_1_major
mannose-6-phosphate NIST
L-Tyrosine
L-Valine
L-Asparagine
L-Threonine
Oleic acid
Maltose
CE 267 MNH4
PI 283 PI 60-223 M-H-
p-hydroxybenzoic acid
Succinic acid
Inosine
Carnosine
Original Conc.
Normalized Conc.
Raw GCMS Intensity (cps)
Normalised GCMS Intensity
Ctrl
DE
HB
HL

b-1.


D-Fructose
D-Fructose 6-phosphate
beta-Alanine
L-Serine
L-Glutamine
Tryptophan
Phenylalanine
TAG 401 TAG 80-80-241 MNa
CerP 420 CerP d160/260 MH
TAG 403 TAG 80-80-243 MNH4
Uric acid
Glyceric acid
L-5-Oxoproline
Retinol palmitate
Original Conc.
Normalized Conc.
Raw GCMS Intensity (cps)
Normalised GCMS Intensity
Ctrl
DE
HB
HL

b-2.

Figure 4.8. Comparative analysis of key metabolites, including amino acids, carbohydrates, free fatty acids, and active biological signalling chemicals, in adult *T. castaneum* (a) and *R. dominica* (b) exposed to diatomaceous earth, Dryacide (Green, DE), hydrophobic SAS (Blue, HB) and hydrophilic SAS (Light blue, HL) relative to the control (Red). The x-axis is the type of treatment; raw intensity represents GC-MS peaks of derivatised metabolites in the y axis on the bar charts. Normalised concentration is the normalised GCMS responses with the Pareto scale by Metaboanalyte 4 in the y-axis on the boxplots. Raw intensity and normalised metabolite concentration are expressed with error bars (±1SD, Standard deviation) for each compound. Statistical significance was evaluated using ANOVA with 0.05 cut-off p-value (FDR) with Fisher's LSD post-hoc analysis.

4.3.5 Targeted Analysis of Fatty Acids Using DI-SPME-GCMS

Overlaid DI-SPME-GCMS total ion chromatographs (TICs) of saturated and unsaturated C16 and C18 fatty acids of *T. castaneum* and *R. dominica* exposed to HB-SAS, HL-SAS, and DE relative to the control (Ctrl) are shown in (Figure 4.9). Saturated and unsaturated C16 and C18 fatty acids include palmitoleic acid (C16:1), palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (18:1), and stearic acid (C18:0) (Figure 4.9). Five free fatty acids, palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (18:1) and linoleic acid (C18:2), increased dramatically in the homogenised bodies of both *T. castaneum* and *R. dominica* after the two SAS powder treatments (Figure 4.9).

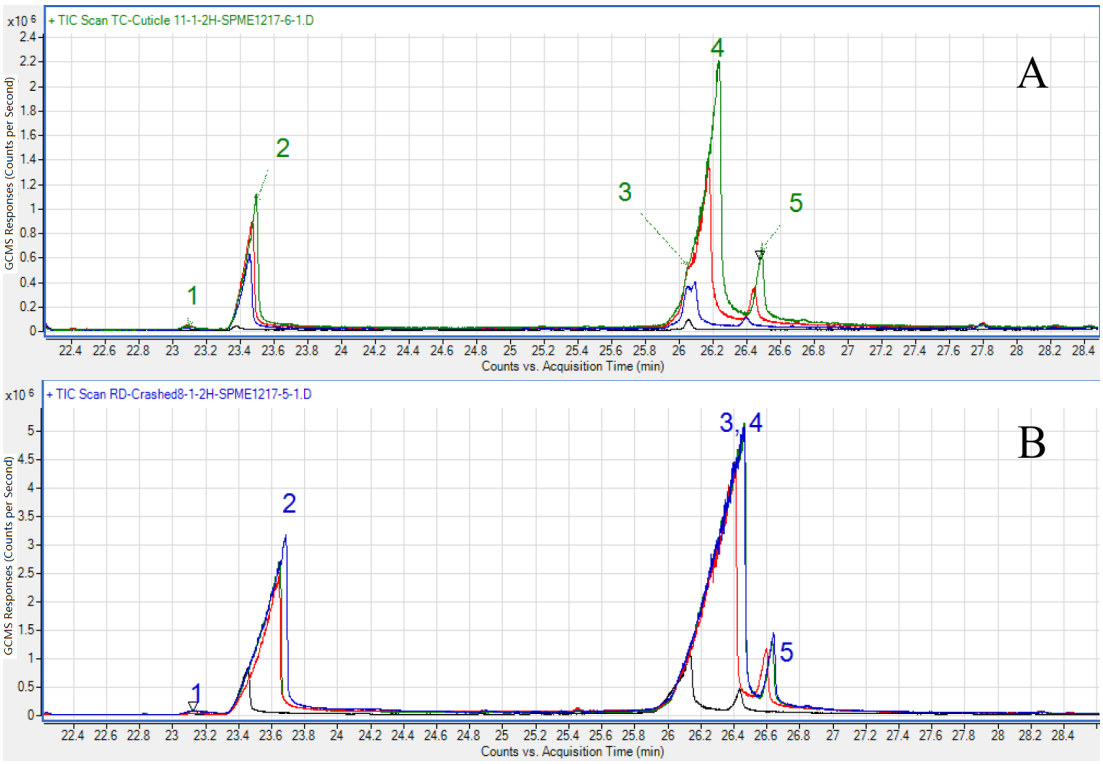


Figure 4.9. Overlaid GC-MS total ion chromatographs of *T. castaneum* (A) and *R. dominica* (B) exposed to hydrophobic synthetic amorphous silica (HB-SAS), hydrophilic synthetic amorphous silica (HL-SAS), and diatomaceous earth (DE) relative to the control (Ctrl). Black - control group (Ctrl), blue - hydrophilic synthetic amorphous silica (HL-SAS), red - hydrophobic synthetic amorphous silica (HB-SAS), green - diatomaceous earth (DE). (1) Palmitoleic acid (C16:1); (2) Palmitic acid (C16:0); (3) Linoleic acid (C18:2); (4) Oleic acid (18:1); and (5) Stearic acid (C18:0)

4.3.6 Mortality Responses of *T. castaneum* and *R. dominica* to SAS Powders

T. castaneum and *R. dominica* showed completely different mortality responses to the two SAS powders (Figure 4.10). *R. dominica* was more sensitive to both SAS powders than *T. castaneum*. The HB-SAS powder performed better than the HL-SAS in terms of higher toxicity to *T. castaneum*, but not to *R. dominica*.

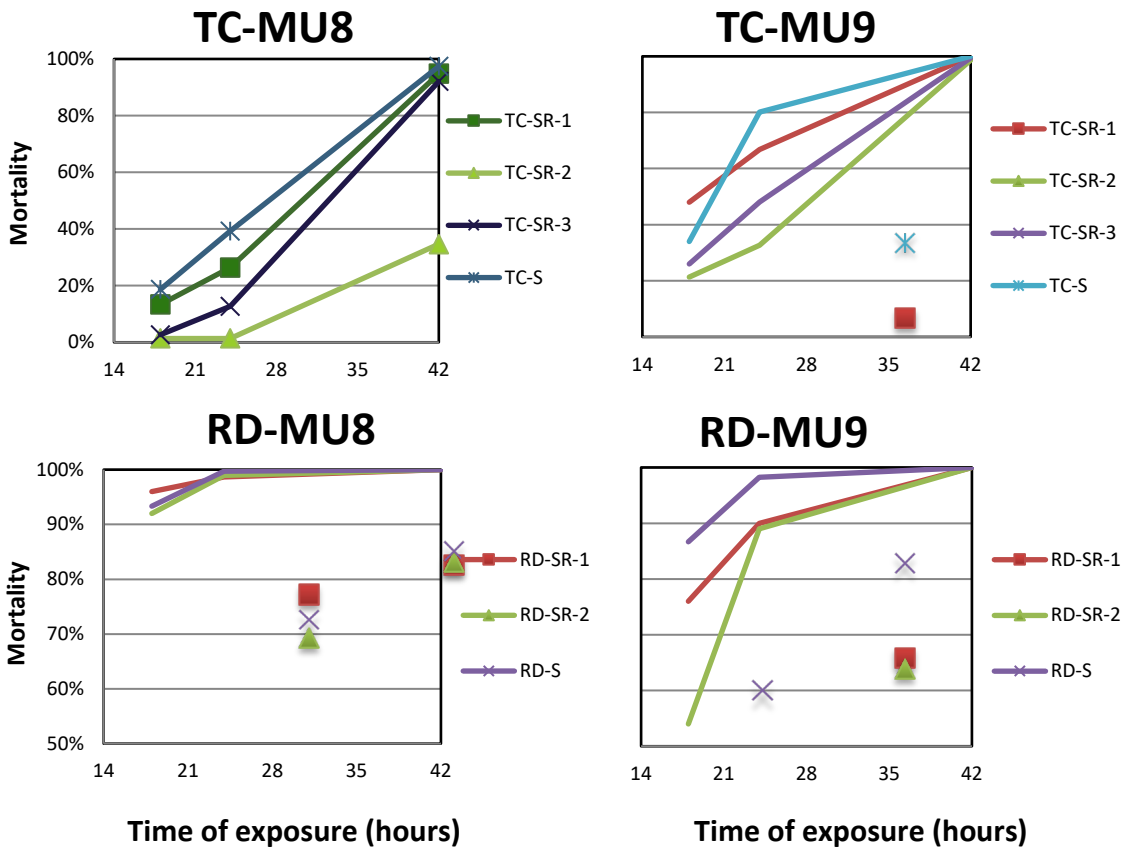


Figure 4.10. The efficacy of HL-SAS (MU8) and HB-SAS (MU9) powder treatments on *T. castaneum* (TC) and *R. dominica* (RD) in percentage mortality; SR=Strong Phosphine Resistance; S=Phosphine-susceptible. The data of TC-SR-2 is outlined in the TC-MU8 dataset.

4.3.7 Effect of Powder on The Hatching of *T. castaneum* Eggs

There were no significant differences in the hatching ratio between the two SAS treatments and the control. The average hatching ratio of control *T. castaneum* and *T. castaneum* treated with HL-SAS and HB-SAS ranged from 89.6%, 88.8% and 97.6%, with relative standard deviations (RSD) of 4.3%, 5.2% and 4.4%, respectively. The F-ratio value of ANOVA is 6.48175 with the p-value is .012338. This implies the result is significant at $p < 0.05$ (Table 4.1). The HL-SAS and HB-SAS powders functioned as drying agents (absorbent and dehydrating) during the application. As a result, the microenvironment would have changed with a decrease in relative humidity.

Table 4.1. Hatching ratio of *T. castaneum* eggs and ANOVA with Post Hoc Tukey HSD results. 2SD= two times standard deviation; RSD=relative standard deviation.

HL-SAS=Hydrophilic Synthetic Amorphous Silica; HB-SAS=Hydrophobic Synthetic Amorphous Silica. T1=Control; T2=HL-SAS; T3=HB-SAS.

Type	Hatching Ratio (%)	2SD	RSD
Control	89.6	7.7	4.3%
HL-SAS	88.8	9.2	5.2%
HB-SAS	97.6	8.7	4.4%

Result Details

Source	*SS*	*df*	*MS*	
Between-treatments	236.8	2	118.4	$F = 6.48175$
Within-treatments	219.2	12	18.2667	
Total	456	14		

The *f*-ratio value is 6.48175. The *p*-value is .012338. The result is *not* significant at $p < .01$.

Pairwise Comparisons		$HSD_{.05} = 7.2114$ $HSD_{.01} = 9.6446$	$Q_{.05} = 3.7729$ $Q_{.01} = 5.0459$
T_1:T_2	$M_1 = 89.60$ $M_2 = 88.80$	0.80	Q = 0.42 (p = .95305)
T_1:T_3	$M_1 = 89.60$ $M_3 = 97.60$	8.00	Q = 4.19 (p = .02980)
T_2:T_3	$M_2 = 88.80$ $M_3 = 97.60$	8.80	Q = 4.60 (p = .01753)

4.3.8 The Efficacy of SAS Powder on the Nascent Larvae of *Tribolium castaneum*

The results of the nascent larvae of *T. castaneum* treated with the SAS powders show that the newly emerged larvae can be 100% controlled in six and seven days with HB-SAS and HL-SAS, respectively. As nascent larvae are vulnerable, the natural mortality in the controls reached 31% on day seven. When the mortality was more than 20%, the relative standard deviation (RSD) was reduced to less than 45%. Lower mortality leads to higher RSD (Table 4.2).

Table 4.2. Mortality (%) over seven days and and ANOVA with Post Hoc Tukey HSD results of *T. castaneum* nascent larvae treated with HL-SAS and HB-SAS powders compared with the untreated control insects. 2SD= two times standard deviation; RSD=relative standard deviation. T1=Control; T2=HL-SAS; T3=HB-SAS.

Type	D1	D2	D3	D4	D5	D6	D7
Control	0	0	2	5	15	21	31
HL-SAS	0	6	17	27	53	99	100
HB-SAS	0	19	30	36	77	100	100
2SD							
Control	0	0	8	12	8	19	13
HL-SAS	0	20	20	21	24	2	0
HB-SAS	0	34	14	15	21	0	0
RSD							
Control	N/A	N/A	237%	116%	29%	45%	21%
HL-SAS	N/A	173%	57%	40%	23%	1%	0%
HB-SAS	N/A	89%	23%	21%	14%	0%	0%
ANOVA with Post Hoc Tukey HSD							
F	N/A	2.9199	12.9504	11.4644	41.1424	181.7672	283.5641
P value	N/A	0.1196	0.004442	0.006188	1.349e-4	9.267e-7	2.001e-7
T3:T2-P	N/A	0.3146	0.1315	0.36414	0.03307	0.9906	1
T1:T3-P	N/A	0.7708	0.0735	0.04229	0.002458	2.201e-6	3.933e-7
T2:T1-P	N/A	0.1072	0.003623	0.005849	1.17e-4	2.066e-6	3.933e-7

* Green numbers mean the P value <0.05

4.3.9 Efficacy of SAS Powder on the 1st instar *T. castaneum* Larvae

The first instar larvae of *T. castaneum* treated with SAS powders could be controlled to 100% in three and four days with HB-SAS and HL-SAS, respectively. Because the first instar larvae are more metabolically active than nascent larvae, the former are more vulnerable to the experimental conditions. Even the natural mortality of the control group increased to 58% on day three. When the mortality was more than 80%, the relative standard deviation (RSD) was reduced from 112% to 7% (Table 4.3).

Table 4.3. Mortality (%) and ANOVA with Post Hoc Tukey HSD results of first instar *T. castaneum* larvae treated with HL-SAS and HB-SAS powders compared to untreated insects as the control group. 2SD= two times standard deviation; RSD=relative standard deviation. T1=Control; T2=HL-SAS; T3=HB-SAS.

Type	D1	D2	D3	D4
Control	2	12	58	58
HL-SAS	39	92	99	100
HB-SAS	80	95	100	100
2SD				
Control	4	14	8	8
HL-SAS	16	6	2	0
HB-SAS	12	7	0	0
RSD				
Control	112%	56%	7%	7%
HL-SAS	20%	3%	1%	0%
HB-SAS	7%	3%	0%	0%
ANOVA with Post Hoc Tukey HSD				
F	56.2643	127.6493	75.0172	83.668
P value	8.007e-7	8.187e-9	6.567e-6	4.334e-6
T3:T2 P value	3.37e-4	0.7988	0.9289	1
T1:T3 P value	7.754e-4	3.573e-8	2.314e-5	1.317e-5
T2:T1 P value	5.3e-7	2.099e-8	1.711e-5	1.317e-5

* Green numbers mean the P value <0.05

4.4 DISCUSSION

4.4.1 Effect of Metabolic Changes of Carbohydrates, Amino Acids, and Their Intermediates

Exposure to SAS was correlated with the rapid death of insects. This may be due to SAS interfering with the metabolism of carbohydrates, amino acids, and their intermediates to kill the insects. Carbohydrates, including monosaccharides, were elevated in the phosphine-resistant *T. castaneum* strain but were significantly reduced when the insects were treated with the two SAS powders. Saccharides perform numerous essential roles in biological organisms. In insect biology, monosaccharides and disaccharides are important energy biomolecules in energy utility and storage. The reduction in monosaccharides and elevation in disaccharides as observed in the phosphine-resistant *T. castaneum* tally with the observation that glycolysis was downregulated in phosphine-resistant rice weevil, *S. oryzae* (Kim *et al.* 2019).

Glycolysis is the downstream metabolic pathway that converts glucose into high-energy adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Therefore, the survival strategy of phosphine-resistant *T. castaneum* is to upregulate the conversion rate of their storage sugars, such as trehalose and sucrose, to monosaccharides, which could benefit rapid energy utilisation under the anaerobic metabolism caused by phosphine fumigation.

Conversely, reducing the abundance of simple sugars in the SAS powder-treated insects, including mono and disaccharides, could be driving all the glycolysis-related alterations, thus depleting the energy needed for survival. No matter how well phosphine-resistant insects manage energy substances, the SAS powders forced *T. castaneum* to choose anaerobic metabolism rather than the aerobic pathway. In conclusion, sugar energy depletion is a potential insecticidal mechanism of the two SAS powders.

All detected carbohydrates, including monosaccharides, disaccharides, and sugar alcohols, were maintained significantly lower than the controls in both insect species, except for trehalose which accumulated during the SAS treatments. Possibly, this is because trehalose has lower polarity than glucose and can be stored within the fat body. Trehalose represents the insects' major energy source, which can be broken down into glucose by the catabolic enzyme for labour-intensive muscle, e.g., flight muscle, and other activities, like surviving freezing and desiccation (Erkut *et al.* 2011).

The significant decline in the content of most amino acids in *T. castaneum* and *R. dominica* could be attributed to the activation of defence mechanisms by reducing biosynthetic activity. Conversely, the metabolic changes of individual amino acids are varied and fragmented in *T. castaneum*. The precursors of neurotransmitters (e.g., beta-alanine and tryptophan) increased in *T. castaneum* and decreased in *R. dominica*. When treated with the SAS powders, these exciting findings could plausibly explain the different physiological responses between the excited *T. castaneum* and relatively inactive *R. dominica*. Decreased p-hydroxybenzoic acid and succinic acid in *T. castaneum* indicate a depletion of the benzoquinone derivatives reservoir and an inactive TCA cycle metabolism. Additionally, the increased uric acid content was due to the degradation of purine nucleotides due to the polarity change of the haemocoel's circulatory fluid (haemolymph).

4.4.2 Effect of HL-SAS and HB-SAS Powders on Structural Lipids and Storage Lipids

There were fatty acids and storage lipids, including monoacylglycerides, diacylglycerides and triacylglycerides (M/D/TAGs), gathered in response to the SAS powders in *T. castaneum* and *R. dominica*. The evidence of reducing glucose and fructose levels plus increasing fructose-6-phosphate (F6P) in the HL-SAS and HB-SAS treated insects indicated that the glycolysis pathway was mobilised towards pyruvate. The major metabolites involved in the TCA (tricarboxylic acid) cycle, which produce energy ATP, are polar. The elevated levels of storage lipids associated with overloaded fatty acids and glycerol implied a plausible MoA hypothesis that SAS powder suppressed the polar metabolites to produce lower polar substances (Figure 4.11).

The polarities of fatty acids and glycerol are higher than storage lipids, and water has the highest polarity index of all other major metabolites. As water is lost through the cuticle layer during SAS treatments, the polarity of the haemolymph decreases accordingly until the bio-fluid is no longer fluid. This metabolic change trend was observed in *T. castaneum* and *R. dominica* treated with diatomaceous earth (DE). This suggests that the two SAS powders and the inert dust, desiccated and imposed energetic stress on the primary (*R. dominica*) and secondary (*T. castaneum*) stored grain insects, by ceasing the fatty acid β-oxidation pathway.

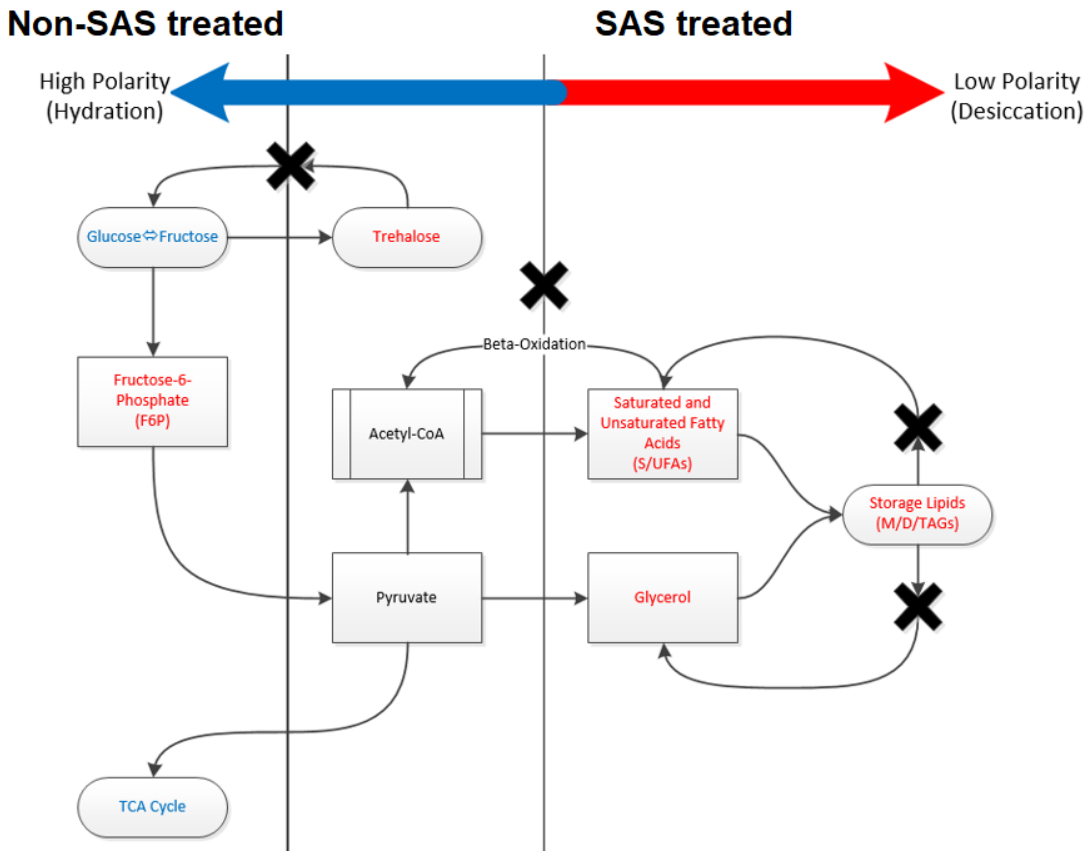


Figure 4.11. A hypothetical diagram outlining the relationships of the principal metabolic pathway related to the primary (*T. castaneum*) and secondary (*R. dominica*) grain storage insects' lipid metabolisms due to desiccation following exposure to the SAS powders. The metabolites and pathways are shown in different colours: blue - down-regulation, red - up-regulation, black - not detected or no significant change. Statistical significance was evaluated using ANOVA with a 0.05 cut-off p-value (FDR). The horizontal-coloured two-way arrow indicates the gradient polarity of the insects' haemolymph. The blue arrow illustrates the higher polarity of the haemolymph in control samples from right to left. While the red arrow illustrates the lower polarity of the haemolymph due to desiccation following treatment with SAS from left to right. The X symbol means the SAS treatment blocks the pathway. M/D/TAGs=Mono/Di/Triglycerides

4.4.3 The Efficacy of SAS Powder on the Internal Life Stages of Stored Grain Insects

Ren and Agarwal (2015) provided a comprehensive efficacy dataset of SAS powders on stored grain insect adults. However, this study is the first time to report the internal life stages of *T. castaneum* and *R. dominica* had different mortality responses. *R. dominica* was more sensitive to both SAS powders than *T. castaneum*.

The HB-SAS powder had better performance than HL-SAS in terms of higher mortality efficacy to *T. castaneum*. The HL-SAS and HB-SAS powders had no significant effect on the hatching of eggs in *T. castaneum*. The efficacy of SAS powder on the nascent *T. castaneum* larvae showed that HB-SAS was a better insecticide than HL-SAS on the newly emerged *T. castaneum* larvae, because 100% mortality was achieved in 6 and 7 days with HB-SAS and HL-SAS, respectively. Although the nascent larvae could survive in the powders for 2 to 3 days, the newly hatched larvae kept dying, and 100% mortality was reached within seven days. Both HL-SAS and HB-SAS powders efficiently control *T. castaneum* 1st instar larvae within 3-4 days.

4.4.4 Alignment of Metabolomics to Ascertain the Mode of Action of SAS Powder Treatments

Among the five popular proposed MoAs of DE powders, three are related to water loss leading to dehydration. The two powders caused asphyxiation by blocking the spiracles (see Chapter 3), which correlated to the anoxic metabolic process observed. As in Chapter 3, there was no evidence from Micro-CT imaging to show that SAS powder particles were found inside the digestive system of *T. castaneum* and *R. dominica*. It is possible that the mode of silica oxides acting inside the insects is negligible and most likely plays no role in insect mortality.

Based on the mode of desiccation, it is most likely that the loss of polar compounds, such as water and other undetected polar semi-volatile chemicals, was directly caused by a reduction in the polarity of the haemolymph. Increasing the low polar biofluid can significantly reduce solubility and the distribution/partition coefficient of the high polar metabolic energy substances and their conversion to low or non-polar chemicals, such as triglycerides. Given that the energy substances involved in TCA cycle and fatty acid β-oxidation are polar, the insects' lack of polar energy substances in response to SAS treatment can induce mortality. Eventually, the insects died due to a loss of energy sources and an accumulation of self-poisoning chemicals.

Although the HB-SAS powder was modified from the HL-SAS, it has similar physical properties, including purity, particle size and pore diameter. The metabolic change in the insects in response to HB-SAS can indicate the effect of the chemical properties of the inert powder. Although both the DE and HL-SAS powder are hydrophilic, HL-SAS was more effective than the DE powder. The comparative

analysis emphasised that the physical properties of the SAS powder played a more important role, particularly concerning its smaller particle size and greater oil absorbing capacity. Therefore, the relatively low levels of polar energy metabolites and the higher levels of low/non-polar energy metabolites under the HB-SAS treatment indicate that hydrophobicity supplements the existing modes of action, namely through desiccation and anoxia. In addition, the better pesticide performance of HL-SAS than the DE treatment could be interpreted as due to their contrasting physical properties, which play an essential role in interrupting the central metabolic pathways.

4.5 CONCLUSION

This study is the first metabolomics analysis of stored product insects placed under stress following synthetic amorphous silica dioxides treatment. Synthetic amorphous silicon dioxides with appropriate physicochemical properties are lethal to both primary and secondary stored product insects.

The mass spectrometry-based metabolite profiling of *T. castaneum* and *R. dominica* treated with the SAS powders showed that although phosphine-resistant adults can downregulate or slow their consumption of energy substances to survive phosphine fumigation, the HL-SAS and HB-SAS powders can deplete monosaccharides and block the fatty acid β-oxidation pathway leading to a substantial overabundance of free fatty acids (FFAs). The overabundance of FFAs, including saturated and unsaturated FFAs, eventually becomes toxic and lethal to the insects. The upregulated synthesis of fatty acids and storage lipids is consistent with the currently recognised modes of action: dehydration and asphyxiation. In contrast, the decline in the levels of most carbohydrates, amino acids and TCA cycle metabolites indicate the energy metabolism was interrupted and ceased because of the reduced polarity of the haemolymph during SAS powder treatment. Therefore, food-grade SAS powders kill phosphine-resistant adult *T. castaneum* by depleting sugar energy and stopping the β-oxidation of FFAs to energy substances (Figure 4.12). In addition, varied trends of the neurotransmitter precursors found in *T. castaneum* and *R. dominica* indicated that the two grain insect species' responses to SAS powder are contrasting in respect to the neurological excitation.

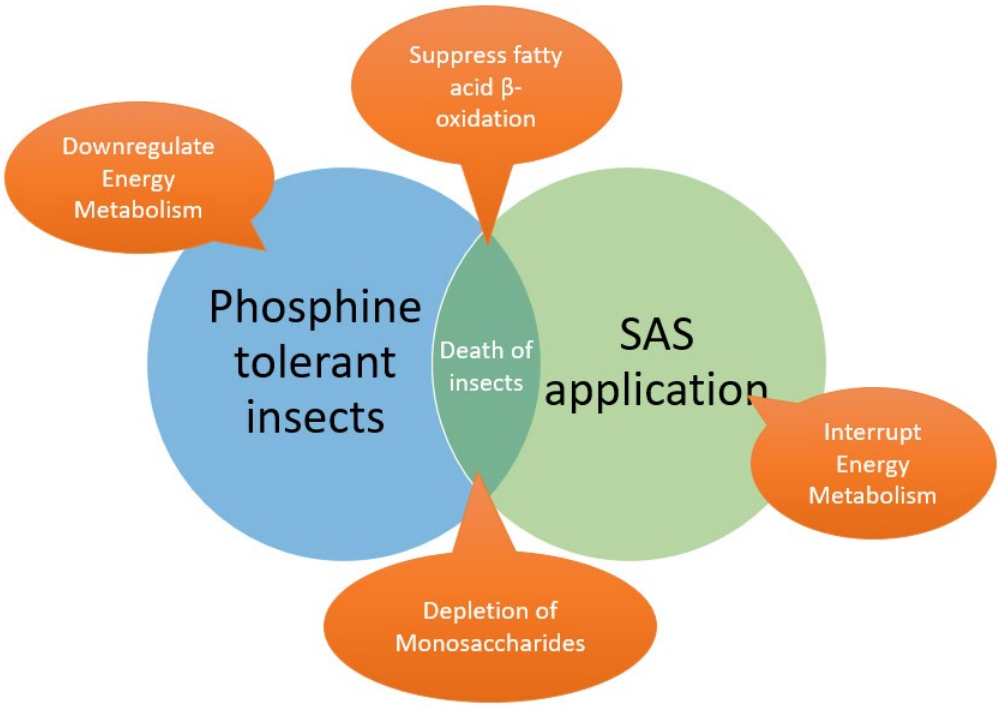


Figure 4.12. Venn diagram illustrating the overlap of the changed metabolic pathways between the mechanisms of phosphine resistance and insecticidal actions of SAS powders on the model organism.

Due to limited resources, the metabolic research with HL-SAS and HB-SAS was not validated on the immature life stages of grain insects, including eggs, larvae and pupae. However, the performance of the SAS powders on these life cycle stages in *T. castaneum* was conducted as part of this study. Both SAS powders were as effective on nascent (<1 day) and first instar larva (>2 days) as on the adults but were not effective on the hatching eggs. Depleting primary metabolic energy substances and blocking pathways/supply chains were found as the key MoAs of the HL-SAS and HB-SAS powders which were directly lethal to the two insect species in this study. Finally, as the HB-SAS powder was more efficient than the HL-SAS powder on the nascent, first instar larva and adults, there may likely be other pesticide mechanisms behind HB-SAS, which enhanced the killing power of the HB-SAS powder.

This research has significantly enhanced our understanding of the pesticidal mechanisms of inert powders. However, further studies should integrate more 'omics' (e.g., genomics and proteomics) approaches to systematically discover and validate the potential insecticidal MoAs throughout the insect life cycle. This knowledge will

help identify vulnerable characteristics of target pests and optimise the efficacy of non-chemical pre- and post-harvest pest management.

5 The Respiratory and Excretory Responses of Phosphine-resistant *Tribolium castaneum* and *Rhyzopertha dominica* to Treatment with Synthetic Amorphous Silica Powders

ABSTRACT

Synthetic amorphous silica (SAS) powder is used as an insecticide to damage the cuticle layer as a means of managing grain storage insect pests, particularly phosphine-resistant strains. The volatile organic chemicals (VOCs) naturally excreted from the red flour beetle (*T. castaneum*) and lesser grain borer (*R. dominica*) together with respiration and excretion were evaluated in response to treatment with hydrophilic and hydrophobic SAS powders. The respiration rate of the insects monitored by mass spectrometry (MS) varied with SAS treatments. Hydrophobic SAS stimulated the *T. castaneum* to increase respiration and produce benzoquinones and derivatives, causing death within two hours of treatment. Volatile organic chemicals were identified and quantitated from *T. castaneum* by headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GCMS). In response to HB-SAS, *T. castaneum* released significant amounts of the benzoquinone derivatives, ethyl p-Benzoquinone, methyl p-Benzoquinone, and ethyl 1, 3-Benzenediol by 133.1, 43.1 and 41.9 times more than the control group without SAS, respectively. However, these ratios were found at 1.9, 2.5, and 1.7 folds higher in the phosphine-resistant *T. castaneum* than the susceptible strain. Hydrophobic SAS was more efficacious than hydrophilic SAS on insect pests, and *T. castaneum* was more sensitive to hydrophobic SAS than *R. dominica.* Therefore, the optimised HS-SPME-GCMS method may be used to determine the resistance of *T. castaneum* to phosphine.

5.1 INTRODUCTION

The red flour beetle, *T. castaneum* and the lesser grain borer, *R. dominica* are two major economically important insect pests worldwide, with different life cycles and habits. *R. dominica* lives and feeds inside grain kernels from egg to adult, whereas *T. castaneum* only feeds on flour outside kernels. Several researchers have studied the respiratory mechanisms of *T. castaneum* and *R. dominica* at normal and modified atmospheric conditions (Lord 1950; Emekci *et al.* 2005; Lu *et al.* 2002). Fumigation with phosphine for managing *T. castaneum* and *R. dominica* has been integral to postharvest commodity protection across the globe for several decades (Nayak *et al.* 2020). Phosphine's significance for disinfestation of stored products has been enhanced in recent years, primarily due to the phase-out of the ozone-depleting methyl bromide and the lack of suitable alternatives. Phosphine is also cheap and versatile in its application and effectiveness against a range of insect pests (Nayak *et al.* 2020). A disadvantage of this over-reliance by industry on phosphine as a single treatment has resulted in resistance in key stored product pest species (Lorini *et al.* 2007; Nayak *et al.* 2013, 2020; Afful *et al.* 2018; Kocak *et al.* 2015; Agrafioti *et al.* 2019). In Australia, for example, strong resistance to phosphine has been well documented for major pest species, including *R. dominica* (Collins *et al.* 2017), and *T. castaneum* (Nayak *et al.* 2017). Therefore, there is an urgent need to develop alternative methods to manage resistance to phosphine in storage insect pests.

Inert dust has been evaluated to control stored grain insect pests for many years. The diatomaceous earth-based formulations are natural products that can reduce the use of chemical pesticides to some extent. However, the low impact of inert dust on insect mortality and being non-food grade has limited its wider application (Korunić 1997; Korunić *et al.* 1988). Also, the high recommended dosage (500 to 3500 mg/kg=0.5-3.5kg/ton grain) has resulted in several adverse effects on grain, including a reduction in its flowability into storage facilities and its bulk density, visible residue, and extra dust generation during processing (Clark-Cooper 1977; IDPA 1990). As a result, diatomaceous earth dust is not used for bulk grain treatment.

Synthetic amorphous silica (SAS) consists of three types, namely pyrogenic, precipitated, and surface-treated SAS. These powders can be distinguished from natural amorphous silica such as diatomaceous earth by their high chemical purity and the particles' finely particulate nature and characteristics. SAS as a non-chemical

method for pest management is revolutionary and advantageous compared to traditional approaches (Ren and Agarwal 2014). SAS is a form of SiO_2, is a dry white powder, and as a food additive has been used for decades. The typical diameter of SAS particles is greater than 100 nm. Toxicological studies have generated extensive physiochemical, ecotoxicology, toxicology, safety, epidemiology, and genotoxicology data, proving that SAS is friendly to the environment and human health (Fruijtier-Polloth 2012 and 2016; Guichard *et al.* 2015; Ren and Agarwal 2014). As a non-hazardous substrate, SAS particles have been considered for use in the stored grain industry as a contact insecticide. However, their insecticidal mechanism(s) is poorly understood, and the optimal application protocol is not yet developed. Therefore, it is essential to investigate and understand the insecticidal mechanisms of SAS particles and their application as an alternative and practical stored grain pest control method.

Respiration indicates an insect's physiological response to the environment it is exposed to (Emekci *et al.* 2002). Oxygen levels have an essential role in the respiration rate of insects (Emekci *et al.* 2001), and studies have confirmed that the respiration of *T. castaneum* adults is higher in low oxygen environments than in normal atmospheric air (Emekci *et al.* 2001; Emekci *et al.* 2002). Seitz and Ren reported the characterization of volatile organic chemicals (VOCs) under different oxygen levels by *T. castaneum* in grains (Seitz and Ram 2004; Niu *et al.* 2016). Villaverde *et al.* (2007) identified and quantitated three major VOC components from *T. castaneum*, namely methyl-1,4-benzoquinone, ethyl-1,4-benzoquinone and 1-pentadecene (C15:1), by solid-phase microextraction-capillary gas chromatography (SPME-CGC); resulting in 742±93, 1672±244 and 946±237 ng/beetle, respectively.

This study aims to determine the physiological response to SAS powders using the respiration rate and volatile organic compounds (VOCs) of adult *T. castaneum* as indicators. The changes in respiration and excretion rates of VOCs by *T. castaneum* and *R. dominica* were qualified and semi-quantitated when subjected to hydrophobic and hydrophilic SAS treatments by two methods based on mass spectrometry. In addition, since SAS particles with different physical and chemical properties can affect the physiology and ecology of insects in grain storage systems, the risk of toxic VOCs were assessed.

5.2 MATERIALS AND METHODS

5.2.1 Insect Cultures

Four insect strains were cultured in the laboratory, namely strong phosphine-resistant and susceptible strains of *T. castaneum* and *R. dominica*. The phosphine-susceptible strain (MUWTC-CH-S) and the phosphine-resistant strain (MUWTC-SR-500) of *T. castaneum* were obtained from the Department of Primary Industries and Regional Development (DPIRD), Western Australia, Australia, and cultured over ten generations in the Post-harvest Biosecurity and Food Safety Laboratory, Murdoch University. *T. castaneum* insects were fed with 1kg wheat flour supplemented with 7.7% yeast in 2 L clear glass jars with mesh lids. The phosphine-susceptible strain (MUWRD-S-6000) and a resistant strain (MUWRD-SR-500) of *R. dominica* were also obtained from DPIRD and fed with 1 kg wheat kernels in 2 L glass jars at 29±1°C and 65±5% relative humidity (R.H.). The evaluation of phosphine resistance was conducted and determined by the FAO 1975 method (FAO 1975). The resistance status was determined with the FAO 1975 method (FAO 1975).

5.2.2 Synthetic Amorphous Silica (SAS) Powder

Hydrophilic (MU8) and hydrophobic (MU9) synthetic amorphous silica (SAS) powders were used (Ren and Agarwal 2014). Both powders are extremely fine, white powdered silicon dioxide with an average particle size of 118 and 117 nm, respectively. MU8 is hydrophilic (purity >99.9% w/w) with a Brunauer-Emmett-Teller (BET) surface area of 260-280 m^2/g, an oil-carrying capacity of 280-300 mL/100g, and an average diameter of 5µm. MU9 is hydrophobic (purity >99.9% w/w), and its surface is modified with polysiloxane with a surface area >230 m^2/g, an oil-carrying capacity >350 mL/100g and an average diameter 4.5-5.5 µm.

5.2.3 Chemicals and Solid Phase Microextraction (SPME) Fiber

Food grade carbon dioxide (CO_2) was purchased in a cylinder (BOC, Australia) and used for preparing calibration standards, purity >99.9% Moisture <100ppm. A 2 cm 50/30µm DVB/CAR/PDMS fiber was purchased from Supelco (Bellefonte, PA, USA) and conditioned according to the manufacturer's recommendations before use. The SPME fiber was performed in a manual holder (Supelco, Bellefonte, PA, USA).

5.2.4 Treatment of Insects with SAS Powder

One hundred milligrams of each insect strain (Approx. sixty *T. castaneum* adults and ninety *R. dominica* adults) were placed in a 9 cm diameter glass Petri dish without bias for gender and age. Every insect strain had three replicates. Ten grams of the SAS powder were added into the funnel of the powder applicator (Ryobi 20L Stainless Steel Wet Dry Workshop Vacuum), and then blown into a modified fume hood within 20 sec. Five seconds after blowing all the powder, uncovered Petri dishes were placed into the fume hood for 2 minutes. Then the insects were transferred and sealed into a 100 mL Erlenmeyer flask with a GC septum adaptor.

5.2.5 Analysis of VOCs by Headspace-Solid Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME-GCMS)

The HS-SPME-GCMS method was based on Du *et al.* (2016), where VOC/semi-VOC (volatile organic chemicals) were absorbed and concentrated by 2 cm DVB/CAB/PDMS SPME fibers at room temperature (23±1°C). The insects collected from section 5.2.4 were transferred into a 100 mL Erlenmeyer flask with a GC septa adaptor with the SPME fibers inserted for VOC/semi-VOC extraction. Then the analytes were desorbed from the fibers into the GCMS for identification and quantitative analysis.

The GCMS was conditioned as follows: An Agilent 5977B GCMSD system (Agilent Technologies, VIC, Australia) was used for quantitation and qualification analysis. The GC was equipped with a split/splitless injector and an SPME inlet, Supelco (Bellefonte, PA, USA), which operated under splitless mode during the analysis. The injection inlet was set at 300°C, and the GC purge valve was switched on at 1 min after injection. An Agilent HP-5MS 30 m × 0.25 mm × 0.25 μm was used for separation. Ultra-high purity helium was used as carrier gas at a constant flow rate of 1.2 mL/min. The initial oven temperature was held at 60°C for 5 min, then increased to 150°C at 6°C/min and 300°C at 8°C/min held for 5 min. The mass spectrometry detector (MSD) transfer line, ion source, and quad-pole temperatures were 200, 230 and 150°C, respectively. Agilent Mass Hunter Software was used for data acquisition and analysis, in which extracts were randomised and analysed in two independent iterations.

5.2.6 Monitoring Insect Respiration by Mass Spectrometer (MS)

A DSQII Mass Spectrometer (Thermo-Fisher, USA) was used to monitor the carbon dioxide in the headspace of an Erlenmeyer flask with a GC septa adaptor. A 23G syringe needle pierced the rubber septum to facilitate gas sampling. The instrument was calibrated by measuring five fortified CO_2 standards between 2,000 and 40,000ppm. High purity CO_2 (99.995%) was transferred from a gas cylinder into a 1 L Tedlar® gas sampling bag, screw-cap combo valve with the septum (CATALOG#: GSTP001-0707S, E.I. DuPont, USA). Then a 10 mL plastic syringe was used to dilute the CO_2 from the gas sampling bag in a 1 L Erlenmeyer flask with three 1 cm diameter glass beads. The Thermo-fisher DSQII MSD transfer line parameters, ion source and quad-pole temperatures were 200, 180 and 150°C, respectively. Thermo XCalibur software was used for data acquisition and analysis. The validation and verification procedures and calculations followed the National Association of Testing Authorities, Australia (NATA) technical note of ISO/IEC 17025 (NATA 2013).

5.2.7 Statistical Analysis and Identification

Each extract was analysed in duplicate, resulting in five technical replicates per biological replicate (e.g., each strain). Peak detection, deconvolution, filtering, scaling and integration were detected, extracted, and aligned using Mass Hunter Quantitative Analysis for the GCMS (Ver. 7.045.7). The software generated and exported to a data matrix consisting of retention time, m/z, and peak intensity based on peak area for all features. The mass spectra of unknown compounds were de-convoluted and identified by AMDIS_32 with the NIST MS database 2014. The retention index was also used for assisting compound identification. Unsupervised and supervised multivariate statistical analysis techniques (PCA and PLS-DA, respectively) were employed to evaluate and visualise the data, following with ANOVA for the features with significant difference, through MetaboAnalyst 3 (Xia and Wishar 2016). The calculated p values (raw.pval) were converted to -log10(p) by the MetaboAnalyst 3.

5.3 RESULTS

5.3.1 Validation of the Direct Mass Spectrometer (DMS) Method for CO2

Linearity was regressed with the correlation coefficient ratio R^2=0.9992 with lower than 8.7% relative standard deviation (RSD) in the range from 2,000 to

40,000ppm (Figure 5.1). The method detection limit (MDL) and method quantitation limit (MQL) were 29.5ppm and 98.3ppm, respectively, based on the standard deviation of the blank. The figures were calculated from three times and ten times the standard deviation of ten technical replicates of fresh air.

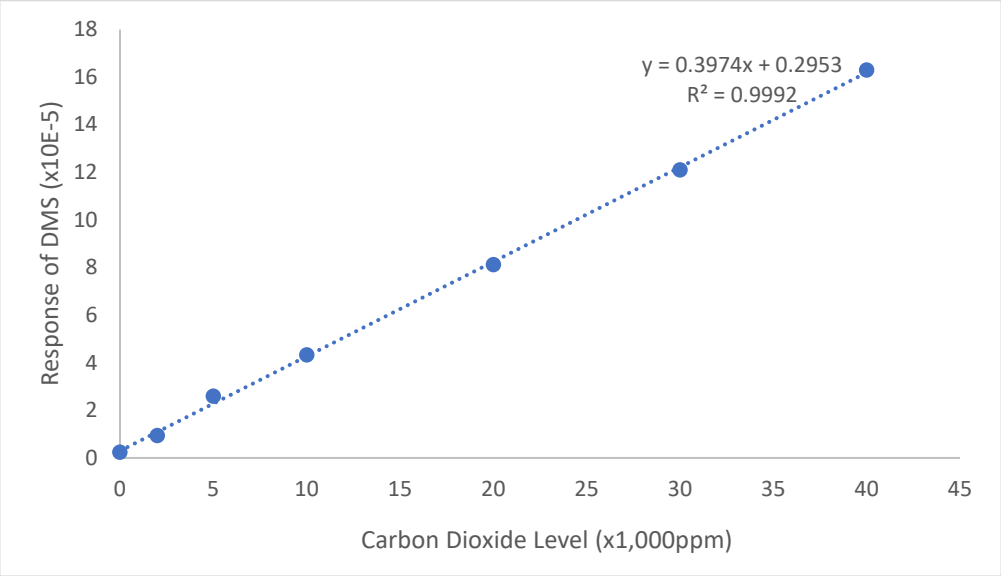


Figure 5.1. Linear calibration curve of carbon dioxide using a direct mass spectrometer (DMS).

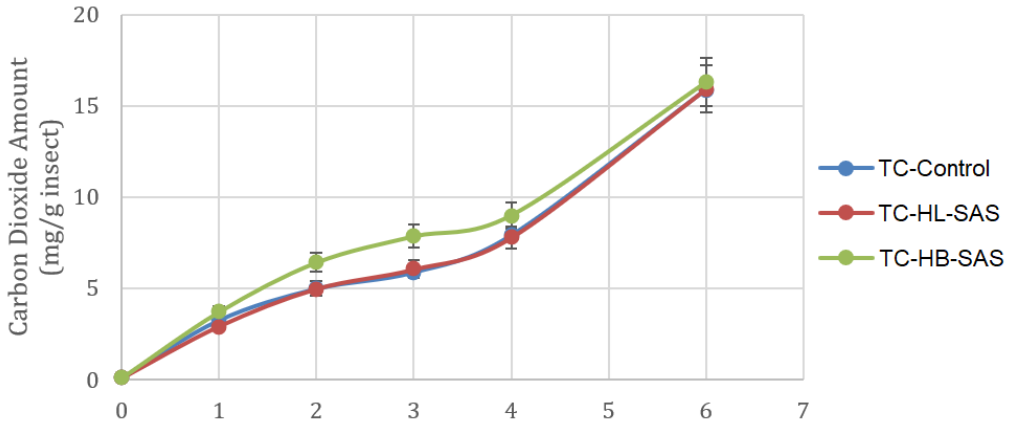
Reproducibility was determined to be 5.54% from headspace samples spiked at 2,000ppm (v/v). Accuracy was assessed from 93.78 % to 106.08 % by fortifying headspace samples of the *T. castaneum* and *R. dominica* at 2,000ppm (n = 3 intra-day, and n =2 inter-day). Ruggedness of the analytical method was not assessed in the biological sample because insects can simultaneously respond to spiked carbon dioxide, which is toxic to insects at high concentrations.

5.3.2 Effect on Initial Respiration of CO2

Direct mass spectrometry (DMS) for CO_2 analysis revealed two modes of action for SAS particles between *T. castaneum* and *R. dominica* (Figure 5.2). In the respective hydrophobic SAS treatments, the respiration rate of *T. castaneum* treated with hydrophobic SAS (HB-SAS) increased by 31% after three hours of exposure, and the respiration rate of *R. dominica* decreased by 27.5% after six hours of exposure (Figure 5.2). When the flask's top was opened, the *T. castaneum* and *R. dominica* took over 24 hours to reach the same mortality levels. Half of HB-SAS treated *T. castaneum*

adults were dead in two hours after closing the lid of 100 mL Erlenmeyer flask. The higher toxicity was observed and correlated to the high concentration of *T. castaneum* pheromones presented. The hydrophilic SAS particles were not as effective as the hydrophobic particles to irritate insect bodies, because the carbon dioxide exhale line of TC-HL-SAS is overlapping with one of TC-Control across the six hours observation window (Figure 5.2a). According to *R. dominica,* the carbon dioxide exhale line of RD-HL-SAS is closer to the one of RD-Control, than RD-HB-SAS (Figure 5.2b).

a.



b.

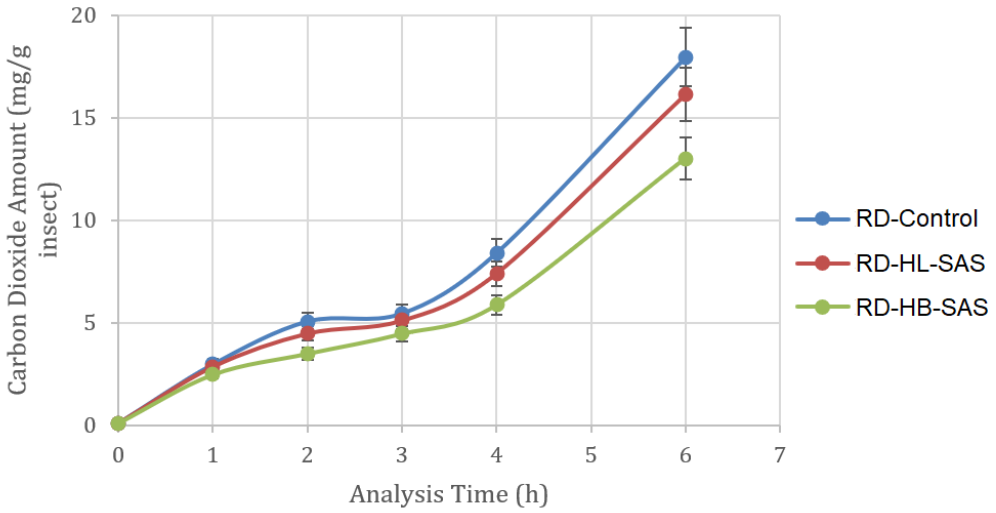


Figure 5.2. Amount of respiration rate change of carbon dioxide with error bars released from phosphine resistant strains of *T. castaneum* (a) and *R. dominica* (b) in the three treatment groups, control, hydrophilic SAS (HL-SAS) and hydrophobic SAS (HB-SAS). The data points are the mean amount of carbon dioxide, and the error bars represent standard deviation.

5.3.3 Effect of Physicochemical Properties of SAS Powder on Excretion of VOCs

T. castaneum can release volatile pheromones as attractants and repellents under different conditions. The physicochemical properties of the SAS powders were correlated to the changes of repellent pheromones affected by SAS particles.

The absorption time of the HS-SPME method was optimised to two hours. Eight VOCs were captured from *T. castaneum* after two hours, including four benzoquinone derivatives and four hydrocarbon chemicals. No VOCs were extracted from *R. dominica*. It was evident that the two-hour extraction time was not enough to absorb sufficient VOCs from *R. dominica* to be detected. The hydrophilic SAS powder stimulated *T. castaneum* to excrete seven VOCs with fold changes ranging from 2.5 to 756 more than the control group, excluding 1-(2-Hydroxy-4-methoxyphenyl) propan-1-one. The hydrophobic SAS induced a higher concentration of all VOCs than the hydrophilic SAS with fold changes ranging from 8.3 to 7046.3. Three out of the eight VOCs, including 1,3-Benzenediol, 4-ethyl, 1-(2-Hydroxy-4-methoxyphenyl) propan-1-one, and 1-Hexadecyne, were detected at trace levels in the control group of *T. castaneum* (Figure 5.3).

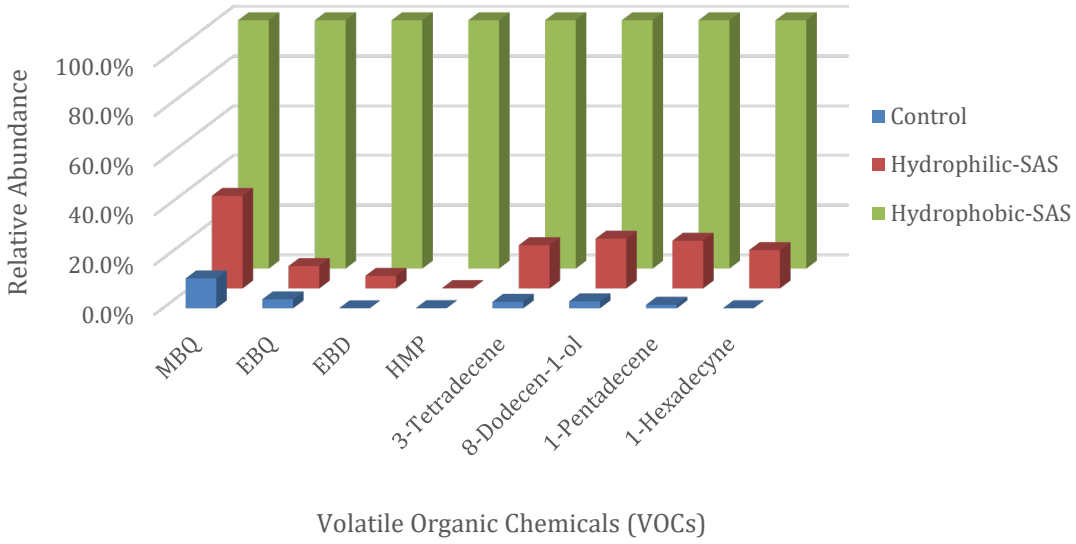


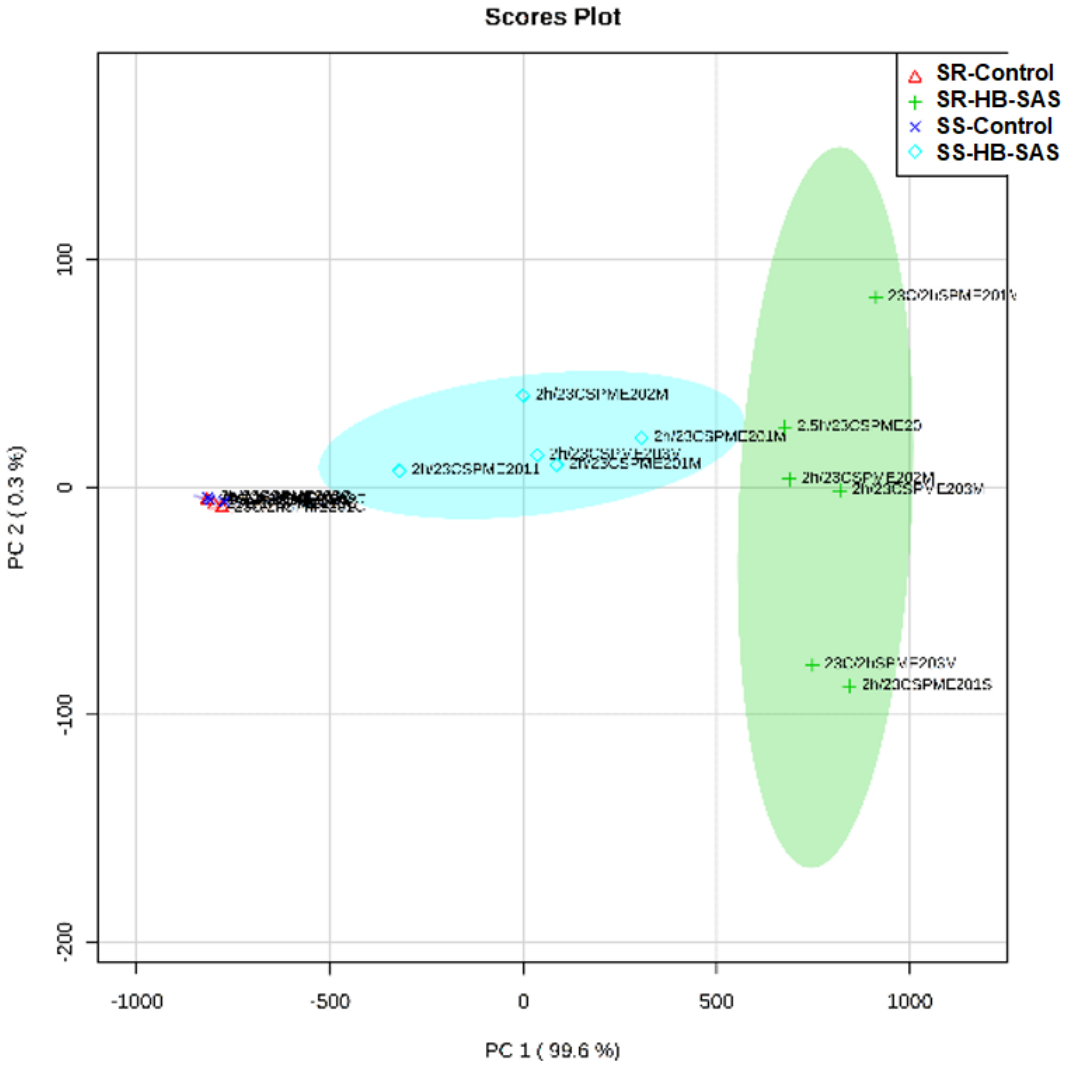
Figure 5.3. 3D column chart showing the relative abundance of volatile organic chemicals (VOCs) excreted from *T. castaneum* treated with hydrophilic and hydrophobic SAS particles compared to control insects using HS-SPME-GCMS with a two-hour extraction time.

5.3.4 Distinguishing the Phosphine-Resistant Strain of T. castaneum using HS-SPME-GCMS

There was no significant difference between methyl p-Benzoquinone and ethyl p-Benzoquinone between the phosphine-susceptible and resistant *T. castaneum* control groups. The VOCs were resolved between the phosphine-susceptible and resistant strains when treated with the two SAS powders. The PCA described 99.9% of the data variance between PC1 and PC2 (Figure 5.4; 99.6% and 0.3% for PC1 and PC2, respectively). The distinct clustering groups of biological replicates (n = 6) with 95% confidence interval indicated significant differences associated with phosphine resistance. Of most interest were the differences in benzoquinone derivatives between the phosphine-strongly resistant (SR) *T. castaneum* treated with the hydrophobic SAS (HB-SAS) and the treated phosphine-susceptible (SS) *T. castaneum* (Figure 5.5a). The concentrations of p-Benzoquinone, 2-ethyl and 1,3-Benzenediol, 4-ethyl from the phosphine-resistant *T. castaneum* were 1.90 and 2.45 times (n=5) higher than the

phosphine-susceptible *T. castaneum* with highly significant ($p<0.00003$ and $p<0.0004$) differences, respectively (Figure 5.5b).

a.



b.

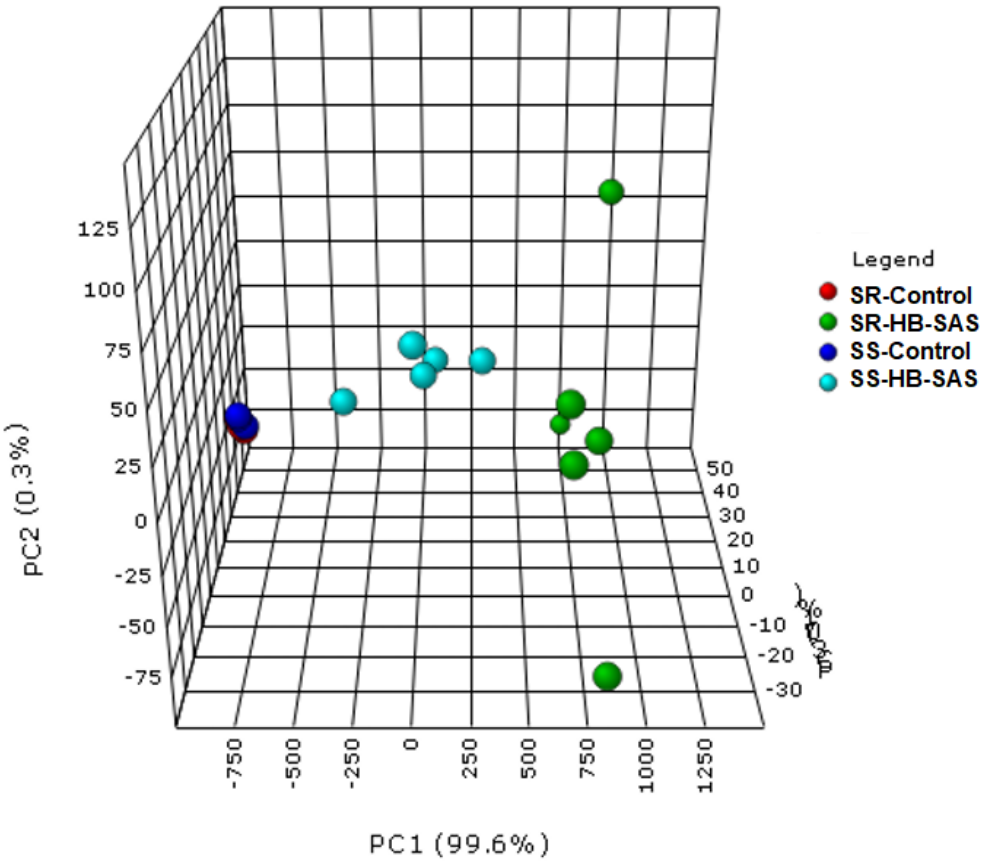
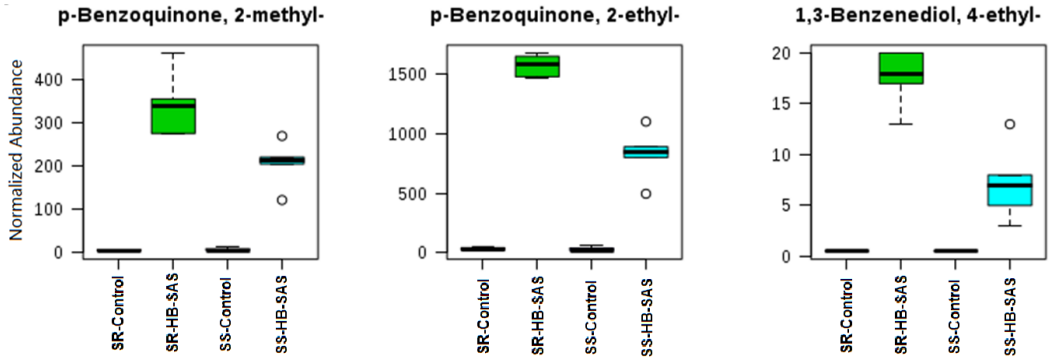


Figure 5.4. 2D (a) & 3D (b) principal component analysis plots of VOCs characterisation of phosphine-susceptible (SS) and strong resistant (SR) *T. castaneum* treated with hydrophobic SAS (HB-SAS) (n = 5). The total variation in secondary metabolites (99.9%) is represented in the first and second components. The SR-HB-SAS has 6 dots including one instrument repeat for data quality control. Red dots (SR-Control) are invisible in the 3D PCA (b) because they overlap with the blue dots (SS-Control).

a.



b.

Name	FC	log2(FC)	raw.pval	-log10(p)
p-Benzoquinone, 2-ethyl-	1.91	0.92	2.99E-05	4.52
1,3-Benzenediol, 4-ethyl-	2.45	1.30	3.99E-04	3.40
p-Benzoquinone, 2-methyl-	1.66	0.73	5.96E-03	2.22

Figure 5.5. Boxplot (a) of the GCMS response (×1,000) of three volatile organic chemical biomarkers found from the hydrophobic SAS (HB-SAS) treated *T. castaneum*. The whiskers indicate variability outside the upper and lower quartiles. The circles indicate the readings beyond the range of the whiskers; Table (b) of the fold change (FC) difference in the phosphine-resistant strain compared to the phosphine-susceptible strain of *T. castaneum* treated with HB-SAS shown for each biomarker with highly significant ($P < 0.001$) differences. raw.pval = raw P-value. -log10(p) = negative log base 10 of P-value.

5.4 DISCUSSION

This study has shown for the first time that high levels of the pheromones, namely benzoquinone family compounds, are rapidly produced in response to SAS, and become toxic within hours to both phosphine-susceptible and strong resistant *T. castaneum*. Also, the SAS treatment increased the respiration rate of the phosphine-resistant *T. castaneum* and decreased the respiration rate of the phosphine-resistant *R. dominica.*

There is very little literature correlating the physiochemical properties of SAS to the respiration and excretion responses of phosphine-resistant *T. castaneum* and *R. dominica*. The greater surface area should absorb and hold more chemicals. Although the hydrophobic SAS (MU9) had 88% of the surface area of the hydrophilic SAS (MU8) particles, MU9 was more lethal to both insects. This finding agreed with Ren and Agarwal (2015) that hydrophobic SAS powders are more lethal to various insect adults than hydrophilic SAS. Consequently, chemical properties in the hydrophobic SAS played a more critical role than surface area.

SAS powders provide a dry microclimate surrounding the treated insects. The larger surface area of SAS powders provided more kinetic energy to accelerate the evaporation of benzoquinones and other VOCs molecules from cuticle layer. Also, SAS powder may irritate the insects causing them to release more benzoquinones. Because of the rapid loss of organic chemicals and derivatives from its body, *T. castaneum* struggled to remove the SAS particles to prevent the draining of pheromone liquids from its glands and sacs. The glands and sacs absorbed the hydrophobic SAS particles and lost the biological function of maintaining moisture. In addition, the high concentrations of methyl p-Benzoquinone and ethyl p-Benzoquinone, which are the repellents for the self-defense of *T. castaneum* (Roth 1941, Roth and Stay 1958, Yezerski et al. 2004), induced strenuous physical activity to increase metabolic rate. Consequently, *T. castaneum* increased its respiration rate and produced more carbon dioxide than the control group. Methyl p-Benzoquinone and ethyl p-Benzoquinone have been used to indicate *T. castaneum* presence in flour (Villaverde *et al.* 2007). As the level of pheromones continued to increase, the tolerance of *T. castaneum* to toxins reached the lethal limit, resulting in death.

Within two hours, 100% mortality of *T. castaneum* adults treated with HB-SAS was observed in a sealed Erlenmeyer flask during the carbon dioxide experiment. In contrast, no dead insect adult was found in control groups, which indicated there was enough oxygen in the enclosed space for survive. Furthermore, *T. castaneum* adults treated with HL-SAS weren't dead in two hours as well, which corroborated the desiccation wasn't the cause of the rapid lethal effect. The correlation between respiration and the excretion of VOCs by *T. castaneum* was clear. Yezerski (2004) also reported that VOCs (benzoquinones) excreted from *T. castaneum* were toxic to

themselves. In contrast, it was ambiguous for *R. dominica*, due to the lack of VOCs produced by *R. dominica* in this study.

The levels of benzoquinone extracted from the headspace in this study compared well with the results from Villaverde *et al.* (2007), who reported 349±107 ng/beetle of methyl p-Benzoquinone and 780±290 ng/beetle of ethyl p-Benzoquinone. Unruh *et al.* (1998) reported that the total benzoquinone estimated from the solvent extract is about 15–21 μg methyl p-Benzoquinone and 22–32 μg ethyl p-Benzoquinone per adult *T. castaneum* using liquid chromatography (LC) with both photodiode array and electrochemical detection. Unruh's (1998) total benzoquinone ratio to Villaverde's (2007) gas phase data matches the multiplying ratio acquired from the present study. Such a matching observation revealed that SAS particles caused the insect to accelerate the excretion of pheromones. The hydrophobic SAS powders aggravated the cuticle layer of *T. castaneum* to release the benzoquinones as repellents to maintain the insect's social distance. However, the pheromones produced by individual insects increased dramatically, which stimulated neighbouring insects to release more pheromones for self-defence. This chain reaction continued, resulting in death.

In conclusion, the toxicity of the high levels of pheromones secreted by the phosphine-susceptible and resistant *T. castaneum*, can kill insects in hours. This observation reveals a new mode of action (MoA) for the use of SAS to manage phosphine-resistant *T. castaneum*. Unfortunately, this MoA did not affect the lesser grain borer, *R. dominica*. Because the pathways of energy metabolism are different between *T. castaneum* and *R. dominica*, including phosphine-susceptible and resistant strains, regardless of the energy efficiency in resistant strains of both species, the SAS powders can kill phosphine-susceptible and resistant strains of both species by manipulating their secondary metabolites, such as benzoquinones. This study also indicated that phosphine-resistant *T. castaneum* could be distinguished from phosphine-susceptible strains within two hours by HS-SPME-GCMS. This finding shines a light on a rapid and cost-effective resistance test method based on HS-SPME to increase the sensitivity and reduce the detection limits (LOD) for determining if *T. castaneum* is resistant or not to phosphine. However, further research is needed for the proposed method to include testing on various strains of *T. castaneum* with different fumigant-resistance levels, and full method validation according to the international standards, e.g., ISO/IEC 17025.

6 General Discussion

This study has shown that food-grade SAS powders have tremendous potential to control phosphine-resistant insects in grain storage facilities. The new modes of action (MoA) found in the present study challenge the existing MoAs of inert dust (mainly, DE dusts). The key findings are listed below:

- The residual fumigant levels in heterogeneous food matrices can be detected and quantified by the HS-SPME-GCMSD method.
- Weight loss consists not just of water loss but also from the loss of semi-volatile chemicals from the insect's biological fluids.
- SAS powders are found the fragile trachea and sensory hairs on the insect's cuticle layer.
- The wax layer loss is not directly attributed to the weight loss rate.
- A reduction in monosaccharides and elevation of disaccharides was found in the SAS treated resistant strains.
- The FFAs accumulate due to a potential reduction in the enzyme activity of β-oxidation downstream and an increase in the anabolism of storage lipids in the upstream of FFAs metabolic pathway.
- The high concentrations of pheromones released by *T. castaneum* became toxic, leading to their death.
- The occurrence of empty desiccated spaces was observed within the bodies of *T. castaneum* and *R. dominica* after HL-SAS or HB-SAS treatments.
- SAS particles affect the respiration rate of grain insects. SAS powders can force *T. castaneum* to prefer the anaerobic rather than the aerobic pathway.

Each of the above is now discussed in more detail.

6.1 DEVELOPMENT OF ADVANCED ANALYTICAL METHODS

As fumigants are highly volatile chemicals, the headspace samples were collected by 2 cm 50/30µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) coated SPME fibers. This method provided high sensitivity, good reproducibility, and simultaneous confirmation of the identity of the individual fumigants and quantification of the fumigant residues, including phosphine, present in different food matrices. The limits of quantitation (0.08-6.64 ng/g) were, in all cases, significantly lower than the MRLs established for wheat, canola, oats, almonds and sultanas. The proposed method could be adapted to other fumigant residues with more accurate results and better performance than the syringe injection method, except for sulfuryl fluoride. This is the first report of a sorption study of residual fumigant levels in heterogeneous food matrices using an HS-SPME-GCMSD method.

6.2 MODE OF PHYSICAL ACTIONS OF SYNTHETIC AMORPHOUS SILICA (SAS) POWDERS

Based on the results of the physical effect of the SAS powders on the insects' bodies and behaviours, the weight loss due to water and organics caused by the SAS powders was significant. The SEM microscopy and micro-CT results combine to justify the observation that SAS powders targeted the fragile trachea and sensory hairs on the insect's cuticle layer (Chapter 3). It was satisfactory to see the insects' behavioural responses to the SAS treatments concerning respiration and pheromone excretion. The HB-SAS powder increased the respiration rate of the phosphine-resistant *T. castaneum*. In contrast, the phosphine-resistant *R. dominica* reduced their respiration activity in response to the SAS treatments. At the same time, *T. castaneum* excreted more biofluids containing attractants/repellents, particularly the benzoquinones (Chapter 5). Due to its hydrophobic properties, the HB-SAS is self-deagglomerating and lightweight, allowing it to penetrate the tracheal tubes quickly.

Given that the benzoquinones and other VOCs are emitted from the secretory glands, the sensory hairs, when blocked by the SAS powders, might have triggered the excretion of these compounds. The secretory glands are distributed across the insect’s entire body and help explain the empty desiccated spaces observed within the insect's body, including the legs, head, and antenna, after applying the HL-SAS and HB-SAS powders. Therefore, the desiccated and malfunctioning internal organs resulted from

the above processes. All of which leads to the death of the two grain storage pests following treatment with the SAS powders.

Given that high humidity and moisture contents significantly reduce the efficacy of DE dusts, the tiny amounts of HL-SAS and HB-SAS applied under the same conditions are adequate to control both *T. castaneum* and *R. dominica*. Even high relative humidity can increase the mortality of secondary grain insects using either HL-SAS or HB-SAS. This indirect evidence supports the hypothesis that the loss of organics is more crucial than water loss. These biochemicals may contribute to insect mortality more than water loss leading to desiccation (Figure 6.1).

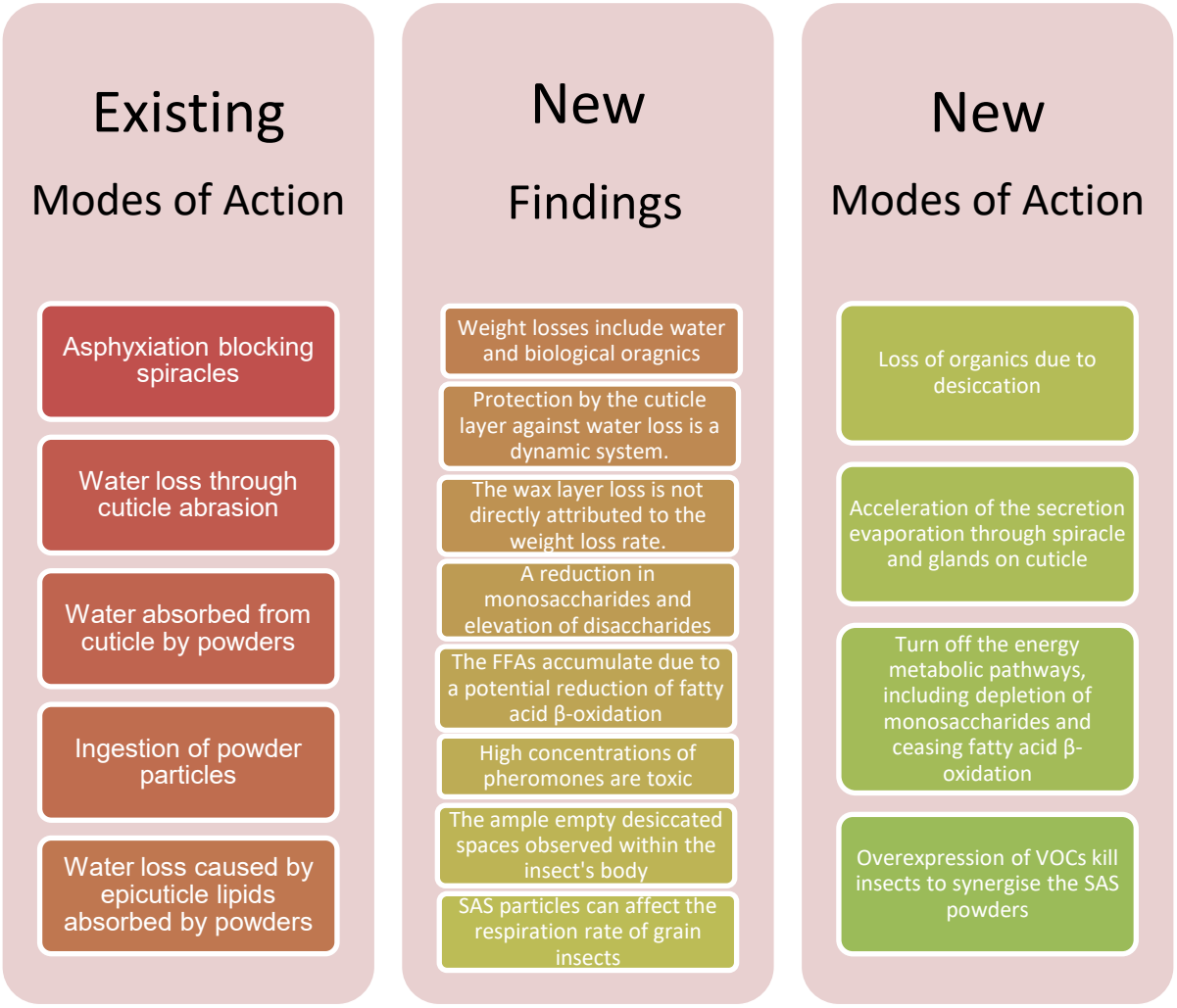


Figure 6.1. Conceptual diagram showing the acknowledged modes of action of SAS powders prior to this study, together with the new findings and new modes of action conceived during the current study.

6.3 MODE OF CHEMICAL ACTIONS OF SYNTHETIC AMORPHOUS SILICA (SAS) POWDERS

This study emphasises the key role of energy metabolism as a mechanism of insecticidal actions of food-grade synthetic amorphous silica powder on the phosphine-resistant *T. castaneum*.

The GC-MS based metabolomics data were used to successfully explore several energy metabolites and their derivatives, including sugars, amino acids and free fatty acids, responses to phosphine resistance, and desiccation caused by food-grade SAS powders in adult *T. castaneum*. The metabolism of *T. castaneum* and its metabolic products play an essential role in maintaining the water content in its body by triggering and sustaining the water retention capability of the cuticle layer. As major metabolic substances, saccharides perform numerous essential roles in biological organisms. In insect biology, monosaccharides and disaccharides are important energy biomolecules in energy utility and storage. The reduction in monosaccharides and elevation of disaccharides was seen in the phosphine-resistant *T. castaneum*, as was observed in the strong phosphine-resistant Rice weevil, *S. oryzae,* where glycolysis was downregulated (Kim *et al.* 2019). Glycolysis converts glucose into high-energy adenosine triphosphate (ATP) and the reduced nicotinamide adenine dinucleotide (NADH). Compared with the phosphine-susceptible *T. castaneum*, the survival strategy of the phosphine-resistant *T. castaneum* is to upregulate the conversion rate of the storage sugars, such as trehalose and sucrose, to monosaccharides. This could benefit rapid energy utilisation under anaerobic conditions caused by phosphine fumigation. Compared with susceptible populations, the lower activity rate and reproduction are the physiological basis of phosphine resistance (Pimentel *et al.* 2007). The molecular biological mechanisms of phosphine resistance of stored grain insects have been well studied (Schlipalius *et al.* 2012; Oppert *et al.* 2015; Kim *et al.* 2019). Phosphine-resistant insects can display many differential expressions of cytochrome P450, cuticle, carbohydrate, transporter, and mitochondrial genes (Oppert *et al.* 2015). The metabolic pathway of β-oxidation from free fatty acids to acetyl-CoA, which eventually forms energy substances (several equivalents of ATP), is downregulated in the strong phosphine-resistant rice weevil, *S. oryzae* (Kim *et al.* 2019). Therefore, the phosphine-resistant *T. castaneum* strain slows down their consumption rate of fatty acids, thereby storing more free fatty acids than the phosphine-susceptible strain.

Regardless of how efficient phosphine-resistant insects manage their energy substances, SAS powders can force *T. castaneum* to choose the anaerobic rather than the aerobic pathway. The depletion in sugar energy is an insecticidal mode of action of SAS powders. In addition, the overload of free fatty acids can induce biological toxicity due to the organism's impermeable membrane due to oxidative stress (Hoekstra *et al.* 2001). However, the *T. castaneum* results make it difficult to determine whether the lethal mechanism is due to the inhibition of the aerobic metabolism, fatty acids toxicity, or both.

In summary, phosphine-resistant adults survive phosphine fumigation by downregulating or slowing down the consumption of energy substances. In contrast, treating phosphine-resistant insects with food-grade SAS powders accelerates the carbohydrate metabolism to deplete monosaccharides and block the β-oxidation pathway to cause a substantial redundancy of free fatty acids (FFA's), including saturated and unsaturated FFAs, which possibly induce the lethal toxicity of overloaded fatty acids.

Based on the existing MoAs of inert dusts, the stored product insects have common physio-chemical responses to the stresses imposed by HL-SAS, HB-SAS and DE dust, such as blocking spiracles, absorption of epicuticle lipids and desiccation. Unlike DE dust, the HL-SAS and HB-SAS powders attach and stick to the insects' cuticle rather than abrase the hard surface. So, water loss through cuticle abrasion cannot be caused by SAS powders. Due to current analytical limitations, it was impractical to determine if the powder particles were ingested inside the insects' bodies. Given that DE dusts and HL-SAS are hydrophilic, their powders can absorb water from the cuticle. However, the dosage of HL-SAS was only 1/10 of DE dusts, so the amount of water absorbed from insects will not be critical to their survival. In reality, HL-SAS desiccates insects much faster than DE dusts. In addition, HB-SAS powder is hydrophobic and repels water. Therefore, the water absorption mechanism does not fit the SAS powders.

According to the new MoAs explored for the SAS powders, both HL-SS and HB-SAS induced the loss of organic molecules and shut down the insects' energy metabolic pathways equally (Figure 6.1). Although both SAS powders can also irritate *T. castaneum* to accelerate the secretion of its pheromones, HB-SAS is more efficient in the evaporation of VOCs and interruption of respiration and gas exchange (Chapter

5). So, this mechanism is more beneficial to HB-SAS than HL-SAS treatment. However, this thesis did not validate the mechanism on the eggs, larvae, and pupae with HL-SAS and HB-SAS.

6.4 FUTURE RESEARCH

This study focused on two model insects with similar body sizes (2-3mm in length). Rigaux *et al.* (2001) found DE-resistant strains from fourteen strains of *T. castaneum* (Herbst) with lethal dosage for 50% of population (LD50) of 413 ppm (mg/kg). Therefore, it will be beneficial to examine the SAS based research findings across a broader spectrum of insect strains and species with different body sizes, e.g., booklice ($\leqslant$ 1mm) and moths (10-20mm). It will be interesting to see the effect of the ratio between SAS particle sizes to insect size on the pesticidal efficacy experiments. Small insects may also be susceptible to SAS due to the high ratio of SAS particles to insect size. The large and small insects' physiochemical responses to the HL-SAS and HB-SAS may also vary at the molecule level. Further validation studies on other stored grain pests, e.g., flat grain beetle (*Cryptolestes* spp.) with extraordinarily high resistance levels, and khapra beetle larvae and adults, the world's worst stored grain pest, are also recommended.

Due to the small size of grain insects, most respiration experiments have been designed and conducted on populations (containing 20 - 100 insects of each population in a test) rather than individual insects (Lord 1950, Price 1980, Emekci 2001, Emekci 2002, Pimentel *et al.* 2007, Lu *et al.* 2009,). Based on the preliminary experimental data for a single grain insect, the gas exchange cycle is blended with continuous and discontinuous respiration. This means insects can adjust their respiration rate and even pause their breathing when exposed to an unpleasant and toxic environment. However, it is hard to use the carbon dioxide signals of a population of insects to distinguish the individual single insect's respiration pattern. Suppose the regular carbon dioxide exchange is interrupted somehow in the early stage of the SAS treatment. In that case, it will be additional direct evidence to prove the particles are targeting the insect's spiracles leading to respiratory failure.

The DE dust took 1-2 weeks to control grain insects in the grain storage systems (Perišić *et al.* 2018). In contrast, SAS only took several days under the same storage conditions. In the laboratory experiments, the two insect pests were killed by SAS

within 3 hrs to 2 days. The metabolomics data reported in this thesis only collected the insect samples before and after treatment. The period of these treatments ranged from 2-14 days. Therefore, a time series analysis with a shorter time interval, e.g., sampling every 1 or 2 hours, will be helpful and necessary to study the effects of SAS application in more detail. Multiple MoAs, e.g., desiccation and the malfunctioning of internal organs, lead to the death of the insects following treatment with the SAS powders. A dedicated analysis over time will provide solid evidence about the cause-and-effect, identifying and ranking the likely causes of insect mortality due to the SAS treatments. The loss of biological fluids proved to be a crucial physiochemical response in this thesis. However, the cause-and-effect relationship between the loss of biological fluids and desiccation needs to be investigated further.

The insects' self-defence behaviours, e.g., diapause and narcosis, accelerate the evolutionary process of chemical resistance development. SAS application is a physical treatment, not a toxic chemical one. Therefore, SAS powders will not induce insects to develop any chemical resistance to the product. Furthermore, they may break insects' diapause and narcosis by irritating their sensory hairs. There are pores on each hair, and these are connected to sensory neurons under the insect's cuticle layer. These can open and close and remain open during either diapause or narcosis. When SAS powders attack these sensory hairs, the insects may be forced to respond, e.g., clean their antenna and legs. Consequently, the grain industry can reduce the usage of lethal grain protectants and fumigants by introducing the new product to IPM (integrated pest management) programs. Further research is required to determine if SAS powder can work with all or specific fumigants synergistically.

6.5 CONCLUSION

This study summarised the pesticide action of SAS powder against primary and secondary grain insects in that they absorb the epicuticular lipid compounds causing excessive organic compounds and water loss through the epicuticle. Unlike DE dusts, SAS powder adhered to the insect's body rather than removing the epicuticle lipid layer due to the ultra-low application dosage of SAS treatment. In addition, SAS irritated *T. castaneum* to deplete its pheromones and interrupted the respiration and gas exchange of *T. castaneum* and *R. dominica*. These findings supported the metabolomics part in this thesis, which shows SAS powders accelerate the carbohydrate metabolism to deplete monosaccharides and block the fatty acid β-

oxidation pathway to cause a substantial redundancy of free fatty acids (FFA's). This indicated the lethal toxicity of overloaded fatty acids, possibly as primary mechanism of action of SAS powders.

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