Optimization and validation for identification of volatile organic compounds released from *Trogoderma variabile* Ballion using headspace solid-phase microextraction and GC-FID/MS

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Abstract

Volatile organic compounds (VOC) emitted by *Trogoderma variabile* at different life stages (larvae, female and male) can help us to understand the chemical signals that are released by the beetle which can serve as biomarkers for diagnostic purpose. There are several factors that effect on the optimization of VOC extraction including, temperature, jar size, number of insects, duration of extraction, and gas chromatography (GC) conditions. This study used headspace solid phase microextraction (HS-SPME) fibre coupled with flame ionization detection (FID) and gas chromatography with mass spectrometry (GC-MS) to determine the optimal method for accurate, rapid and cost-effective extraction and identification of VOC from different life stages of T. variabile. The HS-SPME technique and the analytical conditions with GC and GCMS were optimized and validated for the determination of VOCs released from *T. variabile*. Selection of the number of insects was based on the height and the number of peaks. Results showed that 15 and 20 larvae and adults respectively gave the best number of peaks. Sixteen hours were optimized as the best extraction time for larvae and adults to get maximum number of emitted VOCs. The number of compound released from *T.variabile* different stages was 7, 9 and 11 for male, female and larvae respectively. Furthermore, some compounds were identified in all stages such as oxime-, methoxy-phenyl, 2(3H)-furanone, 5-ethyldihydro- and nonanal. Also, most of the compounds were significantly different in peak area between different life stages.

Keywords: *Trogoderma variabile*, warehouse beetle, VOCs, SPME, GC-MS, GC-FID.

Introduction

Grains, especially wheat is the most important crop around the world. The economic importance of grains and its contribution to the diets of humans and livestock cannot be disputed. However, there are many problems with grains especially in the storage process; of which pest problem is a critical issue [1, 2]. One of these insects is *Trogoderma spp*.; which has more than 134 species including *T. granarium, T. glabrum, T. inclusum and T. variabile* [3]. In Australia, there are over 50 *Trogoderma* described species including *T. variabile* which is morphologically closest to T. *granarium. T. granarium,* is a quarantine pest in Australia. Suspected *Trogoderma* specimens found in grain products are usually the larvae which are difficult to diagnose morphologically [3]. Adult specimens are usually scarce and damaged and need expert dissection for identification [4-5]. Diagnostically the warehouse and khapra beetle can only be reliably identified by a limited number of skilled taxonomists. Many times, *T. variabile* and a comprehensive range of WA's native *Trogoderma* species and related Dermestid species could potentially be mistaken for *T. granarium*. Misidentification of *Trogoderma* and related Dermestids has the potential to seriously compromise Australian grain exports [6]. Early detection and monitoring of insects in the stored food grains become necessary for applying corrective actions. The capability of in-situ early detection, monitoring, cost, reliability, and labor requirements are the major factors considered during for selection of the method. Detection of hidden infestation, whose population may be many times higher than the free-living insects is an important concern to mitigate the losses in bulk storage warehouses, so as to enable the early actions for fumigation or to dispose of the grain [7]. Several detection techniques have been developed for the internal and external detection of insects in stored food grains such as detection probe, staining of the kernel, Berlese funnel method, acoustic techniques, uric-acid method, X-ray imaging, nuclear magnetic resonance imaging, thermal imaging and solid-phase micro-extraction method [8]. Some of these techniques are time-consuming, expensive, have potential health hazard, and less efficient. Manual sampling traps and probes are the most common methods used on farms, while manual inspection, sieving, and Berlese funnel method are used in grain storage and handling facilities [8]. The HS-SPME technique is a new, fast, simple, and highly sensitive and solvent-free sample preparation technique for the extraction of volatile compounds [9-12]. SPME is a good technique to identify and analyses compounds released by insects [13-15]. The solid phase microextraction (SPME) technique coupled with GC-MS has been used in other studies to collect volatile from grain insects, such as fruit fly, rhinoceros beetle and cerambycid beetle pheromones [16-18]; it was also used to detect the aggregation pheromone and other volatile metabolites of the lesser grain borer, *Rhyzopertha dominica* (F.) and the red flour beetle *T. castaneum* [19-20]. In the recent years, studies showed that using head-space solid phase microextraction (HS-SPME) coupled with gas chromatography–flame ionisation detection (GC-FID), gas chromatographic electroannographic detection analysis (GC-EAD) and gas chromatography–mass spectrometry (GC-MS) are a good technique for identifying volatiles in stored grains to

detect infestation with insects [21- 22]. Gas chromatography (GC) combined with flame ionization detection (FID) or mass spectrometry (MS) are known methods in detecting food flavors or insect metabolite using HS-SPME technique [23,24]. Using SPME fibre combined with GC-FID and GC-MS technique give us an accurate, rapid, efficient and non-destructive method to extract volatile organic compounds from insects [16,11]. Many factors might affect the optimization of extraction conditions which include an optimum extraction time and the correct fibre for capturing the whole range of VOCs, the temperature during extraction and the fibre absorption time from the headspace [25]. Identification of the volatile organic compounds emitted by grain insects in future can be used in early detection of insects in stored grains using headspace analysis [15, 24]. Based on previous studies, the aim of this study is to focus on developing optimal condition to collect volatile organic compounds from *Trogoderma variabile* including a number of insect and extraction time, followed by identification of unique peaks which can be used as early infestation detection tool for *Trogoderma variabile*.

Materials and Methods

Insect culture

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

Apparatus and equipment

Solid phase microextraction (SPME) fiber Divinyl benzene/carboxen/polydimethylsiloxane DVB/CAR/PDMS fibre, 50/30 µm (Sigma-Aldrich Australia, catalog number 57299-U), was used in this study to collect volatile organic compounds (VOCs). An Agilent Technologies gas chromatograph 7829A (serial number CN14272038) fitted with an HP-5MS column (30 m x 0.25 mm, film thickness 0.25 µm, RESTEK, catalogue number 13423) non-polar, with a flame ionization detector (FID) was used. For identification of VOC's GC Agilent GCMS 7820A equipped with a DB-35ms column (30 m × 250 µm × 0.25 µm) and MS detector 5977E (Agilent Technologies, USA) (Santa Clara, CA 95051, USA) was used. GC-MS operation conditions were as follows: Injector port temperature 325°C. The initial oven temperature kept at 50°C with an increase to 250°C (increment of 5 °C/min). The flow rate of the column was 1.1 ml/min, while the splitless mode was 20 ml/min at 1.5 min. The run time of GC-MS was 46 min. The glass vials 5 ml with screw tight cap with septa (SUPLICO, USA Lot: 82742) was used for collection of T. variabile VOCs by SPME.

Three experimental replicates were taken during the optimization process and also during identification of peaks.

Optimization of number of insects

Different number of insects (15, 20, 25, and 30) were tested for each life stage to get the optimal number of insects. larvae (mixed instars) and adults (mixed male and female) were placed into 5 ml glass vial (SUPLICO, USA Lot: 82742) and kept in 35°C in thermostatic and humidity chamber (HWS, Ningbo southeast Dongnan Instrumental Ltd) for four hours to enhance the release of VOCs.

Optimization of extraction time

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

Preparation of diluted standard for determination of limit of detection (LOD)

Limit of detection was tested using n-alkanes standard C7-C30 (supleco-USA) at ppm and ppb levels. An Erlenmeyer flask (1Liter, Bibby Sterilin, Staffordshire, Cat. No. FE 1 L/3) equipped with cone/screw-thread adapter (Crown Scientific, Code ST 5313) with 1.1 cm septa (Grace Davison Discovery Sciences, catalog: 6518) were used make stock and diluted standard. The stock standard of concentration 100mg/l was prepared by adding 4 µl of standard solution (C7-C30) using 10 µL syringe into sealed 1L Erlenmeyer flasks. Then, samples were diluted to ppb from ppm level by transferring 1 mL of head space by syringe into another 1L Erlenmeyer flask. The SPME fibre was then suspended into the headspace of Erlenmeyer flask. The extraction time used in this study was one hour at 25°C. After that, the SPME fibre was injected in GC-MS and the components were identified using mass spectrometer (GC-MS). Each sample was replicated three times.

Analysis and identification of real samples using optimized method

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

Data analysis

The GC data including retention time and peak area were collected and integrated into the chromatography software Agilent Chemstation (Mass Hunter Quantitative Analysis Software, B.07.00), and then exported to Microsoft Excel for further analysis.

The repeatability of replicates from the same sample was verified by checking the chromatogram pattern features such as detected peak retention times and peak areas.

Results and Discussion

Number of insects

Number of GC peaks and total peak area from different insect densities (15, 20, 25 and 30) for two life stages including larvae and adults (mix male and female) were compared. As per figure 1 the total peak area was not very significant between 15, 20, 25 and 30 larvae. But as per figure 2there is significant difference between 15, 20 ,25 and 30 larvae with respect to number of peaks. Twenty five larvae gave maximum number of peaks in comparison to 15, 20 and 30 larvae, therefore in this study, 25 larvae were selected as the optimal number to collect the emitted VOCs of T. variabile. For selecting the optimal number of adults also depend on total peak area and the number of compounds. When optimizing number of adults (mixed male and female) the total peak area between 15, 20 and 25 adults were much higher in comparison to 30 adults (Figure 1) but number of peaks from 20 adults was significantly higher than 15, 25 and 30 adults (Figure 2). hence 20 adults were finally optimized for further identification study. Different number of insects can affect the amount of volatile released by these insects. This result showed that the less numbers of T. variabile insects gives more volatile compared with the high numbers and that could be due to the overcrowding in the small vial (8 mL). The overcrowding might have caused a reduction in the metabolism of insects due to an increase in the CO2 quantity which has a critical effect on the biological and physiological processes of insects [26,28]. Additionally, the amount of sample strongly affects the amount of the extracted analyte [29].

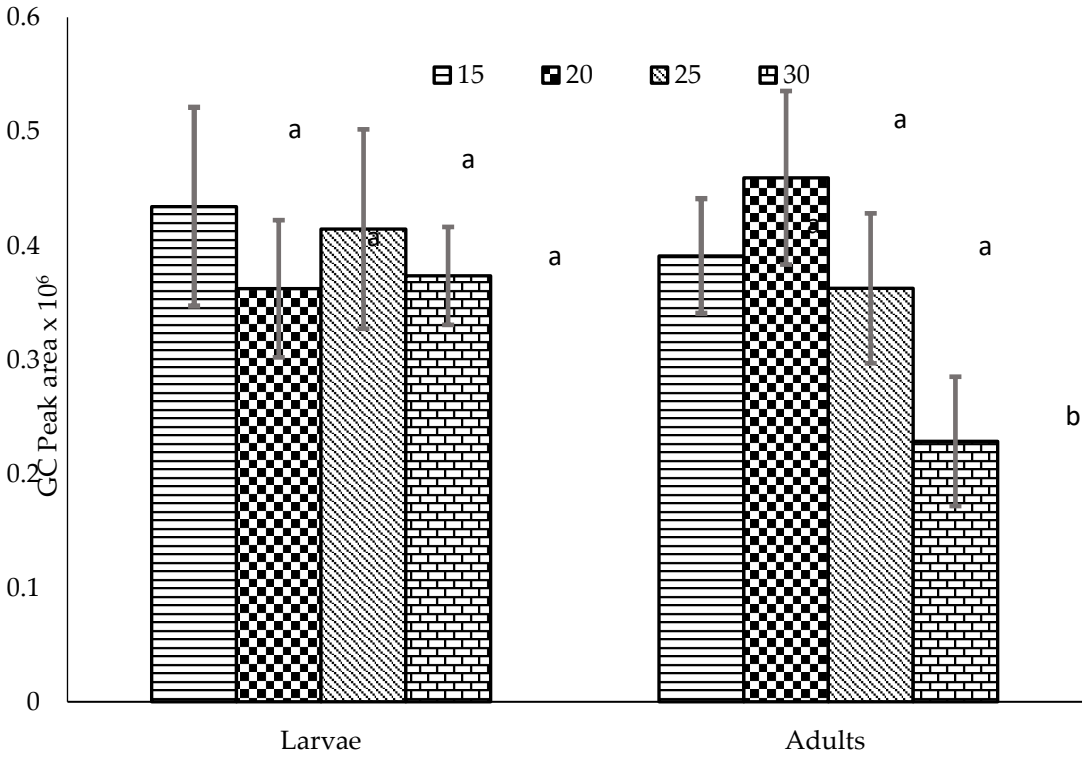

Figure (1): Total Peak area showing the effect of different densitie of insects on the VOCs emitted from two stages from *T. variabile* (larvae and adults). Error bars are standard error: (n=3). Different letters are significant (LSD 0.05)

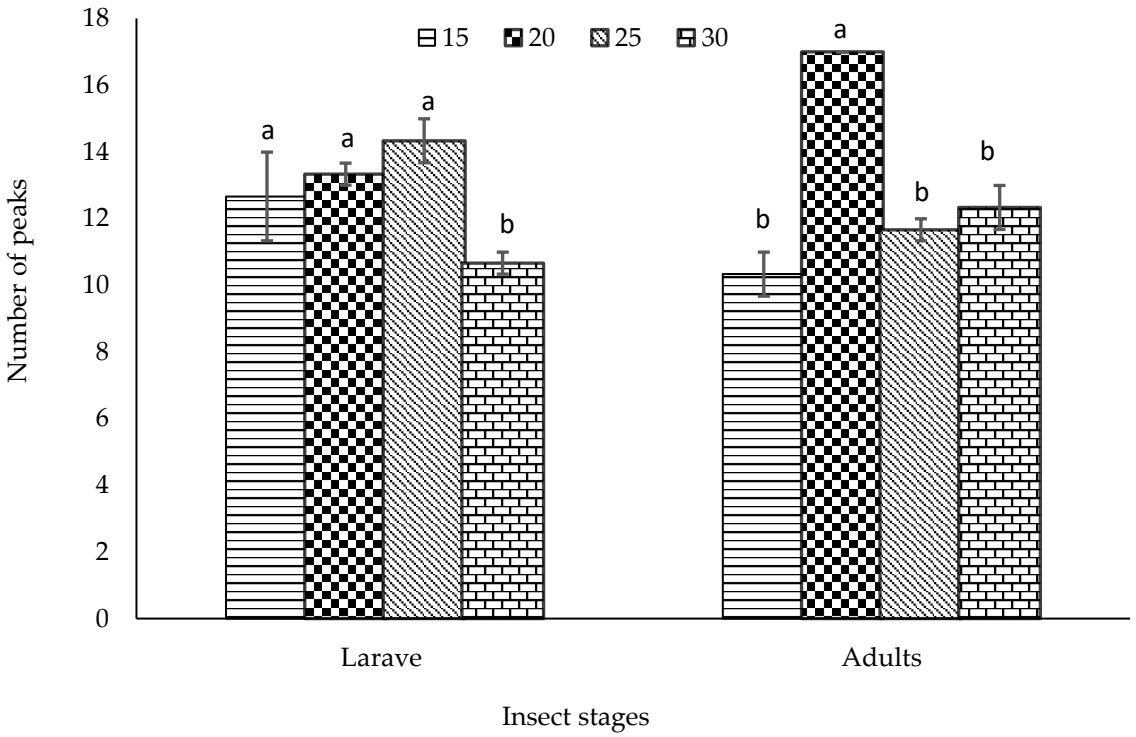

Figure (2): showing the number of compounds emitted from different insects' densities of *T. variabile* at different stages (larvae and adults). Error bars are standard error: (n=3). Different letters are significant (LSD 0.05).

Extraction time

Once the insect densities were selected, the effect of extraction time on the VOCs emission from T. variabile (larvae and adults) was studied. In this study, four different extraction times (4h, 8h, 16h, and 24h) were tested to collect volatile organic compounds from two different T. variabile stages larvae (mixed instars) and adults (mix male and female). Based on figures 3 and 4 the best extraction time for larvae was 16 hours because it gave higher peak are and more number of peaks. Sixteen hours extraction time showed significant difference with respect to peak area in comparison to 4, 8 and 24hrs (Figure 3). In case of adult also, the optimum time selected was 16 hours based on significant difference with respect to peak area and number of peaks (Figure 3 and 4). Extraction time is a significant parameter in hand space solid phase microextraction. It is an important step to determine extraction time using SPME fibre method [15,30]. The amount of extracted volatile depends on the sampling method such as extraction time [20,31]. In this regard, other studies focused on the importance of extraction time, finding it as a crucial factor in recovering VOCs from a range of sample types [14,32,33]. The temperature and extraction time significantly affect HS-SPME methodology because they effect equilibrium during extraction of volatile organic compounds [13].

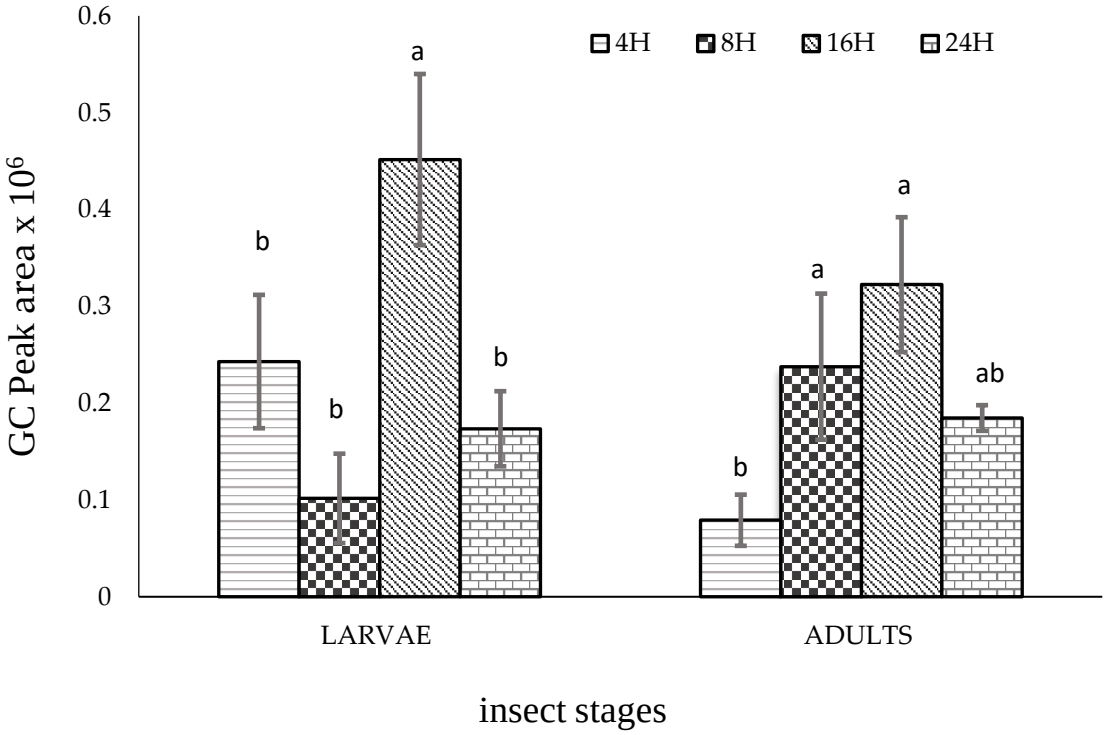

Figure (3): Total Peak area showing the effect of different extraction times on the VOCs emitted from two stages from *T. variabile* (larvae and adults). Error bars are standard error: (n=3). Different letters are significant (LSD 0.05).

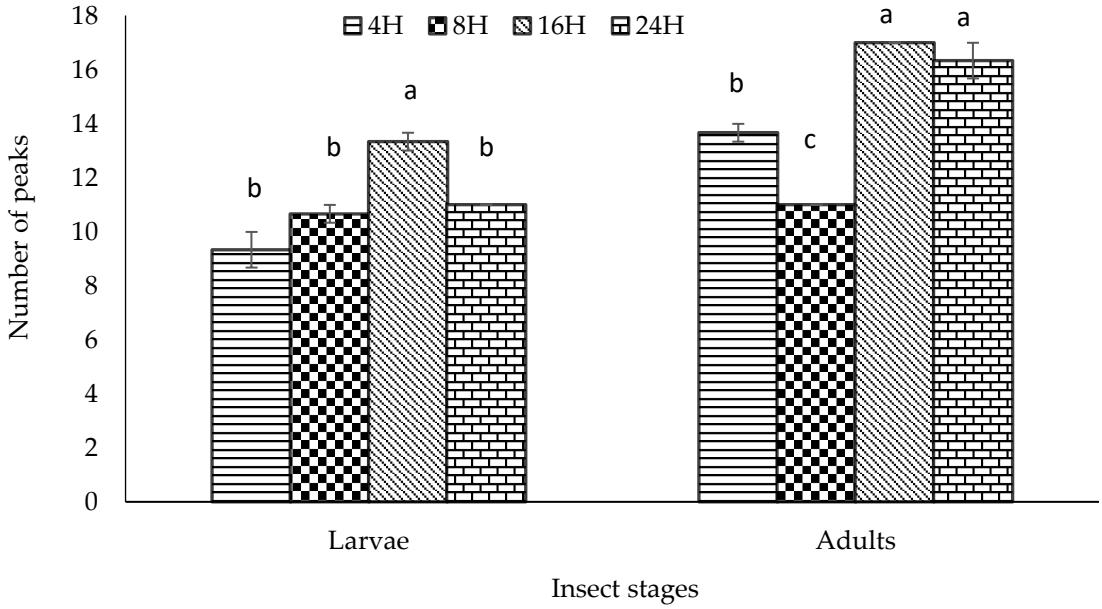

Figure (4): showing the number of compounds emitted from different extraction time of *T. variabile* at different stages (larvae and adults). Error bars are standard error: (n=3). Different letters are significant (LSD 0.05).

Analysis and identification of real samples using optimized method

The GC-MS was used to identify volatile compounds in larvae and adults. Results showed there are difference in the amount of each chemical. Volatile organic compounds were identified based on their retention index and mass spectra in comparison with external n-alkane standards. (Figure 5) shows the results of the heatmap analysis. Each value in heatmap represent three replicates. The lowest relative abundance of the compounds was specified as light color while the dark color represent the height of the peak. There were 11 compounds identified in larvae stage which was pentanoic acid; oxime-, methoxy-phenyl; 2,5-dihydroxybenzaldehyde, 2TMS derivative; naphthalene, 2-methyl-;2(3H)-furanone,5-ethyldihydro-; n-decanoic acid ;nonanal; dodecane,1-iodo-;decanal; n-hexadecanoic acid and m-camphorene. Results show that pentanoic acid was detected in larvae and adult male. However, dodecane, 1-iodo- and decanal were detected in larvae and female only. Compounds identified from T.variable male were slightly different from those identified from female such as ethyl bromide, pentanoic acid; butanoic acid, 2-methyl- and heptane, 1,1'-oxybis-. Two compounds were identified in all three different stages which were oxime-, methoxy-phenyl, and nonanal. Six compounds were significantly different between larvae, male and female adults such as ethyl bromide; naphthalene, 2-methyl-; nonanal; dodecane, 1-iodo-; decanal; n-hexadecanoic acid and m-camphorene. The compounds detected only from T. variabile larvae were identified as 2,5-dihydroxybenzaldehyde, 2TMS derivative; naphthalene, 2-methyl-; n-decanoic acid and n-hexadecanoic acid. Four compounds exclusively detected from female were dimethyl sulfone; nonane, 2,2,4,4,6,8,8-heptamethyl-; nonanic acid and undecanal. In case of male, three compounds can also be detected and identified which are ethyl bromide; butanoic acid, 2-methyl- and heptane, 1,1'-oxybis- (Table s1). Hence these compounds can act as biomarkers and as diagnostic compounds which can tell us the infestation stages. Identification of the volatile organic compounds released by insects can be used to detect insects' in stored grains [15]. There was an attempt to identify the VOCS compounds released by Cryptolestes ferrugineus (rusty grain beetle) and Tribolium castaneum (red flour beetle) by headspace analysis. Some of the reported compounds in this study were identified on different insects. Heaxanic acid; oxime-methoxy-phenyl-_; decanal; nonanal; dodecane and heptane were identified from Rhyzopertha dominica (F.) [24]. (Table 1). However, 9 compounds were detected at ppb level nonane, decane; undecane; dodecane; tridecane; tetradecane; pentadecane; octadecane and nonadecane (Table 1).

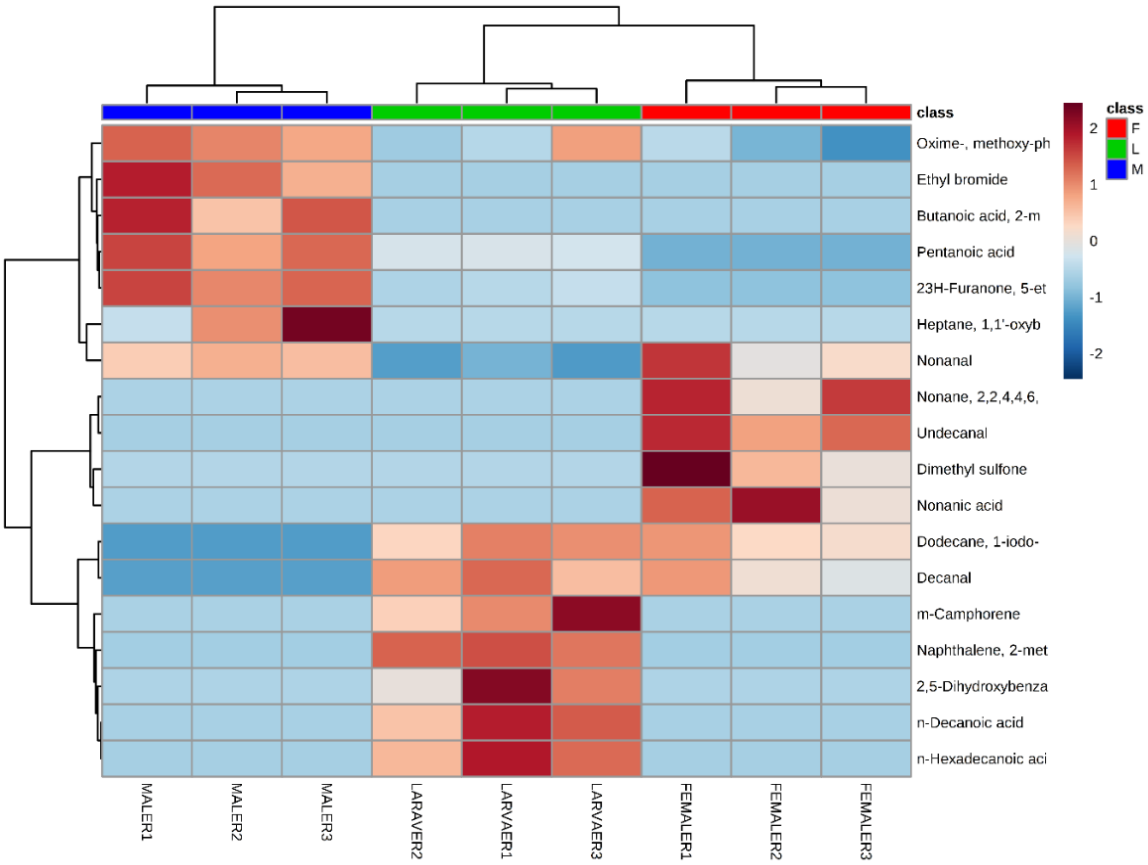

Figure (5): Heatmap shows the difference between three different life stages of *T.variabile* (Larvae, Male and Female). Three samples in each group. n=three replicants.

Limit of detection (LOD)

Some of the alkanes can be detected upto ppb level whereas others could only be detected to ppm level like octane; hexadecane; heptadecane and eicosane.

Table (1): Limits of detection (LOD) of C7-C30 standard at PPM and PPB level

Compounds	*RT (min)*	*RI NIST*	*LOD(ppm)*	*LOD (ppb)*
octane	**3.917**	**830**	**0.311**	
nonane	**7.081**	**938.6**		**1.812**
decane	**10.67**	**1007.6**		**0.142**
undecane	**14.09**	**1071.1**		**6.407**
dodecane	**17.144**	**1140.6**		**6.355**
tridecane	**20.019**	**1215.2**		**4.751**
tetradecane	**22.667**	**1296.4**		**1.442**
pentadecane	**25.166**	**1386.4**		**0.713**
hexadecane	**27.498**	**1472**	**1.73**	
heptadecane	**29.759**	**1560.5**	**2.33**	
octadecane	**31.911**	**1664.5**		**0.498**
nonadecane	**33.951**	**1774.1**		**0.157**
eicosane	**35.863**	**1880.6**	**0.254**	

RT is Retention time (min); RI NIST is Retention indices from NIST; LOD is Limit of Detection; ppm is Part per million, ppb is Part per billion. RI NIST is retention index from NIST.

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

Author Contributions: T.A., M.A. and M.T methodology and designed experiment. T.A, A.L., performed all experiment procedures and data analysis. T.A., M.A. and A.L. and P.K., Writing-Original Draft Preparation. All authors edited and accepted the final manuscript.

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