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Detection of Mediterranean Fruit Fly Larvae *Ceratitis capitata* (Diptera: Tephritidae) in Different Types of Fruit by HS-SPME GC-MS Method

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Abstract

Timely detection of Mediterranean fruit fly (Medfly) is very important so that eradication action can be taken on time. The larvae stage of this insect is the most dangerous stage as it is within the pulp of the fruit, making it hard to detect by visual inspection. In most countries at ports of entry the inspector check a small sample of fruit by visual inspection or by cutting the produce and searching for fungus and pests. This paper will investigate a quick, reliable and sensitive method to determine the presence of fruit flies. Our research focuses on developing the technology for detecting hidden infestations by using the Head Space-Soild Phase Micro Extraction (HS-SPME) method coupled with Gas Chromatography-Mass Spectrocopy (GC-MS) technique. Five different types of fruit were infested with an early stage of Medfly *Ceratitis capitata* Wiedemann (Diptera: Tephidae). We investigated to detect the differences in volatile organic compounds (VOC's) between infested and non-infested fruits by using HS-SPME with (GC-MS). The results indicated that for few chemicals no significant differences between infested and non-infested fruit can be seen, especially in the fruits with first instar. However, in case of third instar larvae infested fruits significant differences in the chemicals can be seen as compare to non infested fruits and other instar infestations. These chemicals include ethyl (Z)-2 butenoate, 2-heptanone, anisole, β-cis-ocimene, 1,3,7-nonatriene,4,8-dimethy-,ethyl octyate, isoamyl caproate and 1β,4βh,10βh-guaia-5,11-diene, in apple. Ethyl (Z)-2-butenoate, (+)-2-bornanone, (-)-trans-isopiperitenol, methyl caprate, caryophyllene and farnesene in orange. Butanoic acid, 3-methyl-,2-methylbutul acetate, sabinene, β-myrcene, octanoic acid, methyl ester, dihydrocarvone, (-)-trans-isopiperitenol and ethyl laurate in mandarin. Butyl 2-methylbutanoate, terpinen-4-ol, P-menth-8-en-2-one,

E-,(3E,7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene and dodecanoic acid, ethyl ester in lemon. Decane, 3-methyl-, p-menth-1,4(8)-diene, 1-undecene and *α*-cubebene in avocado. Thus, the VOC's method could provide a possible tool for detecting tephritid larvae and this method could be adopted by industries importing and exporting fruit.

Keywords

Ceratitis capitata, Infested Fruits, SPME-GC-MS, Volatile Compounds, Apple, Orange, Mandarin, Lemon, Avocado

1. Introduction

Mediterranean fruit fly *Ceratitis capitata* Wiedemann is species of invasive pest that affects fruit production and export worldwide. *C. capitata* attacks approximately 250 different species around the world [1]. United States spends about $57 million per year on Medfly risk management [2]. Furthermore, over the period 2003-2008, Australian industry and government invested around $128 million in the management of Medfly *C. capitata* [3]. Currently, fruit fly control is almost exclusively carried out with chemicals that are harmful to human health and to the environment [4]. In organic fruit production, the issue is more serious, since the law regarding organic farming prohibits the use of synthetic substances that include pesticides [5]. For this reason, farmers are trying to limit the problems by avoiding infection by Medfly. Results obtained in field and laboratory tests demonstrate different susceptibilities to Medfly damage [6]. Several types of research have demonstrated how different varieties of fruit display a variety of chemical profiles and how the release of their VOCs increases or decreases during the maturation process. Also, much attention has been a focused on the development of trapping systems for detection and monitoring of hidden infection [7]. However, improved methods are needed for detection of the immature stages as well, such as first, second and third instars. Larvae feed and develop while hidden within the pulp, making infestation difficult to detect by the senses, especially for Medfly eggs and first instar larvae which are clear to pale white in colour and hence camouflage well with fruit pulp and only 2–3mm length inside the fruit [8]. In ports of entry, quarantine inspectors check import produce by taking small samples of fruit and checking for any signs of pests such as boring or feeding spots or by opening the fruit to search for Medfly larvae [9]. Research has shown that only 35% of fruit infested with fruit fly were detected by trained agricultural inspectors. If not checked by quarantine inspectors, infested fruits get distributed to consumers [8].

This paper will evaluate the use of Head Space-Solid Phase Micro Extraction (HS-SPME) method coupled with Gas Chromatography-Mass Spectroscopy (GC-MS) technique as a potential technology for improving detection of hidden insect infestation inside fruit. According to [10], medfly can change the volatile

compounds of the fruits. It also has been shown that chemical changes can occur within host fruit as a result of insect infestation [11]. In this research, we examined different types of fruits infested with *C. capitata* to determine if infested fruit give us different chemicals profiles from non-infected fruits. Samples volatiles were collected at various stages of infections (first, second and third instars) of larvae, and chemical analysis was performed by GC-MS equipment.

2. Material and Methods

2.1. Insects

Medfly colony were obtained from the Department of Agriculture and Food, Western Australia (DAFWA) and reared in the Murdoch University Laboratory, in Perth Australia. All the flies were reared under conditions: 23˚C ± 2˚C and 75% ± 5% RH, and 12:12-h (L:D) [12]. Adults are placed in screen cages (40cm length ×40 cm height ×40 cm depth) and each cage contained medfly food made from crystaline sugar from (Bidvest, Australia), yeast hydrolysate from (Australian Biosearch) in ratio of 4:1 and water 50 mL. About 10-12 days after adult's emergence from pupae and mating of adults flies, eggs were collected every day, which are deposited on to mesh side and fallen into the water tray kept adjacent to the cage.

2.2. Fruits

Royal Gola Apple (*Malus domestica*) from New South Wales, Valencia orange (*Citrus spp.*) from Western Australia, Hass avocado (*Persea Americana*) from South-West of Western Australia, Hicksons Mandarin (*Citrus reliculata*) from New South Wales and Eureka Lemon (*Citrus limon*) from New South Wales were obtained from the local fruit and vegetable market. The fruits were stored for 2 days under 2˚C; twenty ripe fruits from each variety were used in our experiment.

2.3. Infestation and Sample Preparation

Fruits were cleaned with distilled water to remove any surface contaminants. Then, 30 eggs with 0.5 ml of water were transferred to each single fruit by using a sterile syringe. Fruits were divided into three groups (5 fruits in each group) for volatiles analysis at different stages of larvae, first, second and third instars. Each group used two fruits to monitor the progress of larval development by cutting the fruit to determine the larvae stage and to evaluate the level of infestation by using a microscope to see the larvae stages and remaining three fruits were used for volatile analysis. In addition, one group was sampled as non-infested fruits. All the fruits were placed and stored in laboratory at a temperature of 24˚C for 7 - 9 days; for the development of the larval stage. After collecting the volatile compounds as described in the next section, the various types of fruits were cut into small pieces and the number of larvae inside each fruit was counted.

2.4. Collection/Extraction of Volatile Compounds

The analysis of compounds was focused on whole fruits. Various types of fruit were placed individually into 2 litre jars. One whole fruit was analysed in each jar. Volatiles were collected by solid phase micro extraction (SPME) fibre with 50/30 µm Carboxen/DVB/PDMS (2 cm) (Sigma-Aldrich, Bellefonte, USA) coating. The samples were collected by inserting the fibre into the jar and exposing to the headspace. VOC's were collected on different times depending on the level of larvae inside the fruit. After sealing the jars for 16 hours at a temperature of 24˚C, the fibre was exposed to headspace for 2 hours which the optimized the HP-SPME extraction time. The desorption time of SPME fiber was 10 min in the injection port.

2.5. Analysis of Volatile Compounds

VOC's were analyzed with (Gas chromatography Agilent GCMS 7820A equipped with a mass spectrometer detector 5977E (Agilent Technologies, USA) and a DB-35ms column (30 m × 250 µm × 0.25 µm) (Santa Clara, CA 95051, USA). The carrier gas was 99.999% helium supplied by (BOC, gas, Sydney, Australia). The GC-MS operation conditions were as follows: The temperature of the injector port was 270˚C. The initial oven temperature was 50˚C and increased to 250˚C by (5˚C/min). The column Flow rate was 1:1 ml/min and splitless was 20 ml/min at 1.5 min. The total GCMS run time was 45 min. Three experimental replicates were taken for each type of fruit. Compound peaks were deconvoluted by AMDIS version 2.72 and identified by searching the NIST 2014 MS database (the US National Institute of Standards and Technology) with retention index confirmation. Three replications for each type of fruit were analysed, and the experiment were repeated two times to confirm the chemicals.

2.6. Method Sensitivity and Limit of Detection (LOD)

The limit of detection was evaluated with alkane standard C7-C30 (Supelco, Bellefonte, USA). One litre Erlenmeyer flasks (Bibby Sterilin, Staffordshire, Cat. No. FE 1 L/3 equipped with cone/screw-thread adapter (Crown Scientific, Code ST 5313) with 1.1 cm blue septa (Grace Davison Discovery Sciences, catalog: 6518) were used make stock and diluted standard. The stock standard of concentration was prepared by adding 4 µl of standard into sealed 1L Erlenmeyer flasks. Then, samples were diluted to ppb from ppm, ppt from ppb and ppq from ppt levels by transferring 1 mL of head space by syringe into another flask. After 1 hour of extraction time with 50/30 µm Carboxen/DVB/PDMS (2 cm) (Sigma-Aldrich, Bellefonte, USA) fibre at room temperature, the SPME fibre was injected into GC-MS with 270˚C injection port. Each level was repeated two times.

2.7. Statistical Analysis

The number of larvae inside the fruit was analysed by one way (ANOVA). For the comparison of volatile compounds between different instars, the peak area was analysed by software using the two way (ANOVA) test [13]. Differences in

the result were compared by using the least significant differences test (LSD $P \leq 0.05$) for determining the means between different instars with non-infested fruits. The peak area was divided by 10^6 for each single compound. The peaks left after subtracting from the blank run were only analysed.

3. Results and Discussion

3.1. Level of Infestation

The fruits were dissected immediately after collection of the volatiles compounds for finding out the level of infestation. For the first instar, it was hard to calculate the number of larvae; so the data was calculated by counting second instar larvae. The results indicated there were significant differences in the level of hatching in avocado compared to other types of fruit, like apple, lemon, orange and mandarin in laboratory conditions. Average ± SD number of larvae per fruit was as follow: 8.06 ± 1.58 apple, 13.13 ± 1.21 orange, 11.93 ± 1.77 mandarin, 9.06 ± 1.81 lemon and 18.93 ± 1.10 avocado (**Figure 1**). The non-infested fruit was also dissected to confirm there are no natural infestations by Medfly.

3.2. Limit of Detection (LOD)

The GC-MS response of the stock and diluted alkanes standard decreased form ppm (Parts per million) level to ppt (parts per trillion) level (**Table 1**). Some of the alkanes can be detected less than ppt level with SPME method. Octane, decane, undecane, pentadecane, hexadecane and heptadecane can be detected in ppt level. Octane can be detected in small amount even at ppq level.

Table 1. Limits of detection (LOD) of C7-C30 standard at three levels by using 50/30 μm Carboxen/DVB/PDMS fibre.

Standard	Formula	RI[a]	LOD (ppm)[b]	LOD (ppb)[c]	LOD (ppt)[d]	Linearity (r^2)[e]
Octane	C8H18	729.1	100.061	33.394	5.050	0.948
Nonane	C9H20	899.9	141.900	57.492	n.d	0.988
Decane	C10H22	1000.8	224.999	74.246	4.395	0.957
Undecane	C11H24	1100.6	164.794	77.475	2.983	0.997
Dodecane	C12H26	1200.8	24.028	6.819	n.d	0.941
Tridecane	C13H28	1300.6	56.533	27.538	n.d	0.998
Tetradecane	C14H30	1399	22.945	7.431	n.d	0.960
Pentadecane	C15H32	1500.5	17.307	10.262	1.068	0.994
Hexadecane	C16H34	1600.6	6.798	3.440	2.070	0.944
Heptadecane	C17H36	1700.5	6.499	2.521	0.861	0.946
Octadecane	C18H38	1800.1	7.309	3.487	n.d	0.999
Nodaecane	C19H40	1900	13.734	n.d	n.d	-
Eicosane	C20H42	2000.8	15.983	4.987	n.d	0.955
Heneicosane	C21H44	2100	18.651	n.d	n.d	-
Tricosane	C23H48	2299.5	20.503	n.d	n.d	-
Tetracosane	C24H50	2400.8	15.119	n.d	n.d	-
Pentacosane	C25H52	2499	10.042	n.d	n.d	-

a = retention index; b = parts per million; c = parts per billion; d = parts per trillion; e = Regression coefficient.

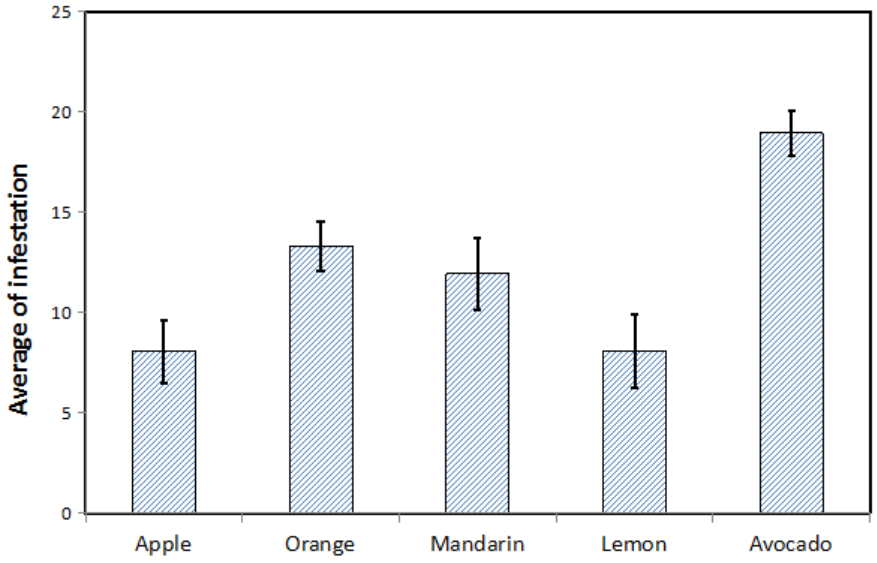

Figure 1. Infestation level (Mean ± SD) in different fruits by *C. capitata*.

3.3. The Volatiles Compounds from Fruit

There were many differences between compounds for each type of fruits and also between infested and non infested fruits. Some of the compounds were detected in one type of fruit, was found to be absent in another type. From the GC analysis, about 33 compounds from apple, 45 compounds from orange, 45 compounds from mandarin, 44 compounds from lemon, and 40 compounds from avocado were identified. All these compounds were identified by comparing with the retention index in the literature (NIST) and mass spectra in the NIST. We analysed 23 compounds each from apple (**Table 2**), orange (**Table 3**), mandarin (**Table 4**), and lemon (**Table 5**), while 17 compounds were analysed from avocado (**Table 6**). The comparison of main compounds between different types of fruit infested with three different instars is explained in **Figure 2**.

3.3.1. The Volatile Compounds from Apples

In case of non infested apples, the main peaks were hexyl acetate, n-butyl 2 methylbutyrate, n-hexyl propionate and isobutyl caproate. Many compounds were detected in fruits infested with third instar larvae but not in fruits infested with first or second instar larvae or in non infested fruit and these included ethyl (Z)-2 butenoate, 2-heptanone, anisole, β-cis-ocimene, 1,3,7-nonatriene,4,8-dimethy-, ethyl octyate, isoamyl caproate, ethyl decylate and 1β,4βh,10βh-guaia-5,11-diene (**Table 2**). The most significant compounds comparing with non infested fruits in third instar were propyl isobutyrate, 1-hexanol, hexyl acetate, n-butyl 2 methylbutyrate, n-hexyl propionate and isobutyl caproate. In the second instar, they were propyl isobutyrate, 1-hexanol, hexyl acetate, n-butyl 2 methylbutyrate and n-hexyl propionate, while the significant chemicals which were recorded in first instar were propyl isobutyrate, hexyl acetate and isobutyl caproate (**Table 2**). The concentration of 1-hexanol and styrene increased with increase in instars, however, propyl isobutyrate, hexyl acetate, n-butyl 2 methylbutyrate and

Table 2. GC peak area (one unit corresponds to a 10^6 area) of volatile compounds in apple infested with *C. capitata* detected by GC-MS.

Compounds	RT	RI	RIL	Prob.%	Infested			Non-infested
					1 instar	2 instar	3 instar	
Ethyl(Z)-2 butenoate	5.45	848.9	830	89	n.d.	n.d.	0.77	n.d.
Propyl isobutyrate	5.85	856.8	861	86	9.35*	3.89*	2.23*	24.07
1-Hexanol	6.26	872.3	860	89	35.51^{ns}	50.69*	78.72*	10.58
Styrene	6.95	801.7	883	87	0.24	0.12	2.04	n.d.
2-Heptanone	6.97	891.7	871	83	n.d.	n.d.	17.72	n.d.
Anisole	7.91	916.9	898	92	n.d.	n.d.	0.55	n.d.
Benzaldehyde	9.44	895.7	982	79	0.33	n.d.	1.18	n.d.
Hexyl acetate	11.42	973.7	984	88	419.33*	411.08*	396.33*	544.26
n-Butyl 2 methylbutyrate	12.41	1044.9	1019	84	210.68^{ns}	88.18*	75.64*	215.87
β-cis-Ocimene	12.60	1049.9	1041	89	n.d.	n.d.	0.46	n.d.
2-Methylbutyl 2-methylbutyrate	14.45	1105.3	1090	80	14.53	n.d.	19.05	n.d.
n-Hexyl propionate	14.56	1085.3	1083	94	151.50^{ns}	81.41*	80.19*	139.94
1,3,7-Nonatriene,4,8-dimethy-,	14.84	1108.3	1089	83	n.d.	n.d.	21.08	n.d.
Methyl caprylate	15.11	1126.5	1108	76	0.6	0.35	5.23	n.d.
Dodecane	17.40	1201.7	1200	81	1.19	5.22	n.d.	n.d.
Ethyl octyate	17.39	1199.8	1175	95	n.d.	n.d.	48.4	n.d.
Decanal	17.61	1199.6	1204	85	1.19	1.01	1.20	n.d.
Isoamyl caproate	18.90	1252.4	1253	90	n.d.	n.d.	1.76	n.d.
Ethyl decylate	22.86	1390.4	1381	88	n.d.	n.d.	1.07	n.d.
1*β*,4*β*h,10*β*h-Guaia-5,11-diene	24.93	1462.1	1469	93	n.d.	n.d.	0.85	n.d.

*Means there are significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). Prob% means percent of probability. ns means there are no significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). n.d. means compounds are not detected. (RT) retention time, (RI) retention index, (RIL) Literature retention index (NIST).

Table 3. GC peak area (one unit corresponds to a 10^6 area) of volatile compounds in orange infested with *C. capitata* detected by GC-MS.

Compounds	RT	RI	RIL	Prob.%	Infested			Non-infested
					1 instar	2 instar	3 instar	
Ethyl (Z)-2-butenoate	5.45	849.6	830	89	n.d.	n.d.	2.04	n.d.
β-phellandrene	8.26	850.2	964	81	0.95	n.d.	11.38	n.d.
n-Butyl butyrate	9.28	957.6	939	74	29.13^{ns}	57.79^{ns}	n.d.	32.76
L-*β*-Pinene	10.00	975.6	970	31	n.d.	7.29^{ns}	38.72^{ns}	n.d.
Myrcene	10.63	992.5	979	89	19.16^{ns}	40.7^{ns}	242.02*	12.58
3-Carene	11.21	1010.7	1005	89	124.85*	252.90*	115.88*	22.92
D-Limonene	11.88	1031.4	1018	86	389.93*	614.20*	799.27*	49.73
β-cis-Ocimene	12.60	1049.9	1041	81	1.22	5.34	24.95	n.d.
E-4,8-Dimethyl-1,3,7-Noatriene	14.96	1121.2	1116	89	297.84*	334.00*	271.89^{ns}	180.519
Methyl caprylate	15.11	1124.2	1134	91	4.35^{ns}	244.38*	123.45*	2.23
(+)-2-Bornanone	15.67	1146.7	1141	87	n.d.	n.d.	0.76	n.d.
Isobutyl caproate	15.94	1137.3	1118	78	348.61^{ns}	313.61*	144.03*	391.66

Continued

Ethyl octyate	17.40	1153.8	1152	96	155.85*	188.26*	208.95*	49.67
(-)-trans-Isopiperitenol	17.57	1206.0	1206	82	n.d.	n.d.	15.50	n.d.
Butyl(2E)-2-hexenoate	18.63	1243.1	1243	92	n.d.	50.13	22.16	n.d.
Isoamyl caproate	18.90	1252.6	1253	82	7.49	40.48	37.81	n.d.
Methyl caprate	20.96	1324.4	1309	80	n.d.	n.d.	0.90	n.d.
limonene-1,2-dial	21.38	1340.8	1342	84	4.59	13.56	57.27	n.d.
Octanoic acid n-butyl ester	21.58	1346.5	1348	87	n.d.	66.55	3.22	n.d.
Eugenol	21.88	1356.0	1337	90	n.d.	0.25	9.79	n.d.
Caryophyllene	23.54	1414.4	1424	87	n.d.	n.d.	52.50	n.d.
Valencen	25.38	1478.2	1474	86	885.93^{ns}	755.80*	636.74*	903.59
Farnesene	25.70	1488.9	1499	84	n.d.	n.d.	42.70	n.d.

*Means there are significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). Prob% means percent of probability. ns means there are no major differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). n.d. means compounds are not detected. (RT) retention time, (RI) retention index, (RIL) Literature retention index (NIST).

Table 4. GC peak area (one unit corresponds to a 10^6 area) of volatile compounds in mandarin infested with *C. capitata* detected by GC-MS.

Compounds	RT	RI	RIL	Prob.%	Infested			Non-infested
					1 instar	2 instar	3 instar	
Butanoic acid, 3-methyl-	5.42	849.8	830	88	n.d.	n.d.	2.86	n.d.
2-methylbutul acetate	6.64	882.0	868	81	n.d.	n.d.	1.2	n.d.
1R-a-Pinene	8.47	932.8	922	85	1.16	5.87	n.d.	n.d.
Mesitylene	9.74	968.0	956	82	0.71^{ns}	n.d.	n.d.	4.26
Sabinene	9.96	973.9	975	89	n.d.	n.d.	129.64	n.d.
Terebenthene	10.00	970.0	975	87	n.d.	49.52	168.13	n.d.
β-Myrcene	10.63	992.5	979	89	n.d.	n.d.	86.54	n.d.
3-Carene	11.21	1010.7	1005	89	3.24^{ns}	25.88*	25.99*	0.24
D-Limonene	11.88	1031.4	1018	89	625.23*	751.71*	907.88*	518.48
Moslene	12.90	1059.5	1047	85	5.08*	17.68^{ns}	57.21*	22.13
p-menth-1,4(8)-diene	13.64	1088.3	1080	83	3.27	9.92	32.33	n.d.
Octanoic acid, methyl ester	15.14	1126.5	1109	84	n.d.	n.d.	3.75	n.d.
(-)-Terpinen-4-ol	16.74	1178.9	1161	84	n.d.	6.62	51.19	n.d.
α-Terpineol	17.11	1192.5	1172	88	n.d.	3.22	20.46	n.d.
Dihydrocarvone	17.37	1197.4	1189	80	n.d.	n.d.	43.36	n.d.
Dodecane	17.40	1200.1	1200	89	4.03^{ns}	n.d.	n.d.	4.62
(-)-trans-Isopiperitenol	17.44	1206.0	1206	85	n.d.	n.d.	8.03	n.d.
p-Mentha-1(7),8(10)-dien-9-ol	20.03	1340.5	1340	82	n.d.	n.d.	2.64	n.d.
Tridecane	20.28	1299.9	1300	81	3.59^{ns}	2.79^{ns}	n.d.	4.01
1,2-Cyclohexanediol, 1-methyl-4-(1-	21.37	1338.8	1342	96	n.d.	3.25	19.49	n.d.
(-)-β-Elmene	22.80	1388.1	1387	83	n.d.	2.55	3.97	n.d.
(+)-epi-Bicyclosesquiphellandrene	23.79	1421.7	1428	81	0.41	0.87	1.98	n.d.
Ethyl laurate	27.73	1598.7	1580	90	n.d.	n.d.	1.23	n.d.

*Means there are significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). Prob% means percent of probability. ns means there are no significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). n.d. means compounds are not detected. (RT) retention time, (RI) retention index, (RIL) Literature retention index (NIST).

Table 5. GC peak area (one unit corresponds to a 10^6 area) of volatile compounds in lemon infested with *C. capitata* detected by GC-MS.

Compounds	RT	RI	RIL	Prob.%	Infested			Non-infested
					1 instar	2 instar	3 instar	
Sabinene	9.96	973.2	975	89	21.21^{ns}	238.72	399.21	n.d.
D-Limonene	11.88	1031.4	1018	89	352.62*	625.74*	767.11*	95.4
Butyl 2-methylbutanoate	12.40	1044.8	1026	85	n.d.	n.d.	0.99	n.d.
Benzene,1-methyl-3-(1-methylethenyl)-	13.91	1089.2	1099	84	6.26^{ns}	n.d.	n.d.	10.88
(E)4,8-Dimethyl-1,3,7-Noatriene	14.88	1119.0	1116	86	277.41	136.05	183.96	n.d.
Methyl caprylate	15.11	1126.5	1109	86	3.87^{ns}	55.20*	317.42*	2.81
Limonene oxide, trans-	15.51	1139.4	1130	88	7.88^{ns}	n.d.	n.d.	10.09
(+)-2-Bornanone	15.67	1146.7	1141	86	n.d.	8.11	18.83	n.d.
Terpinen-4-ol	16.79	1178.9	1161	84	n.d.	n.d.	86.01	n.d.
α-Terpineol	17.11	1192.5	1172	83	5.97	36.04	179.05	n.d.
P-Menth-8-en-2-one,E-	17.57	1088.3	1080	82	n.d.	n.d.	5.79	n.d.
Hexyl 2-methylbutyrate	18.51	1238.6	1232	90	29.81*	14.20^{ns}	46.60*	11.84
Tridecane	20.28	1299.9	1300	81	2.13^{ns}	n.d.	n.d.	3.15
Decanoic acid, methyl ester	20.96	1324.1	1309	81	n.d.	13.41	31.97	n.d.
Limonene-1,2-diol	21.37	1340.8	1342	93	4.16^{ns}	42.17*	7.96^{ns}	4.4
(-)-β-Elmene	22.80	1388.1	1387	88	122.59*	106.96^{ns}	35.50^{ns}	77.31
7-epi-a-selinene	26.05	1501.0	1503	85	87.3	190.56	n.d.	n.d.
E-Nerolidol	27.02	1534.9	1548	85	65.53*	31.74^{ns}	47.65^{ns}	0.87
(3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	27.38	1547.3	1557	55	n.d.	n.d	1.98	n.d.
Dodecanoic acid, ethyl ester	27.72	1559.2	1566	87	n.d.	n.d.	0.8	n.d.
Intermedol	29.20	1610.9	1630	81	n.d.	7.52^{ns}	122.58*	4.23

*Means there are significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). Prob% means percent of probability. ns means there are no significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). n.d. means compounds are not detected. (RT) retention time, (RI) retention index, (RIL) Literature retention index (NIST).

Table 6. GC peak area (one unit corresponds to a 10^6 area) of volatile compounds in avocado infested with *C. capitata* detected by GC-MS.

Compounds	RT	RI	RIL	Prob.%	Infested			Non-infested
					1 instar	2 instar	3 instar	
1-Heptanal	7.36	898.8	882	88	1.04^{ns}	0.81^{ns}	n.d.	0.44
Sulcatone	10.48	973.9	964	80	0.58^{ns}	33.85*	1.35^{ns}	0.53
Hexyl acetate	11.42	973.7	984	84	0.51^{ns}	n.d.	n.d.	0.23
D-Limonene	11.88	1031.4	1018	90	23.56*	114.96*	131.75*	94.70
Moslene	12.90	1058.3	1047	86	0.75*	51.88*	2.87^{ns}	7.19

Continued

Decane, 3-methyl-	13.31	1070.5	1072	82	n.d.	n.d.	0.22	n.d.
p-menth-1,4(8)-diene	13.88	1088.3	1080	81	n.d.	n.d.	0.38^{ns}	1.49
1-undecene	14.00	1091.7	1088	79	n.d.	n.d.	0.13	n.d.
E-4,8-Dimethyl-1,3,7-Noatriene	14.88	1119.0	1116	86	16.19 ^{ns}	14.31*	2.02*	11.69
α-Terpineol	17.11	1192.5	1172	81	n.d.	6.41	0.19	n.d.
1,2,6-Dimethylundecane	17.81	1214.6	1216	81	0.45	1.21	0.55	n.d.
Limonene glycol	21.38	1338.2	1342	83	n.d.	2.69	n.d.	n.d.
α-Cubebene	21.66	1348.4	1350	82	n.d.	n.d.	0.22	n.d.
Meraneine	22.03	1361.3	1342	85	n.d.	0.41	n.d.	n.d.
(4R,4aS,6S)-4,4a-Dimethyl-6-(prop-1-en-2-yl)-1,2,3,4,4a,5,6,7-octahydronaphthalene	24.79	1457.4	1475	82	0.32^{ns}	10.64*	2.54^{ns}	0.61
1β,4βh,10βh-Guaia-5,11-diene	24.93	1462.0	1469	83	1.55^{ns}	n.d.	n.d.	1.64
Valencen	25.38	1479.9	1492	89	61.82^{ns}	3.31*	14.60*	61.13

*Means there are significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). Prob% means percent of probability. ns means there are no significant differences between infested fruit and non-infested fruit (LSD mean P ≤ 0.05). n.d. means compounds are not detected. (RT) retention time, (RI) retention index, (RIL) Literature retention index (NIST).

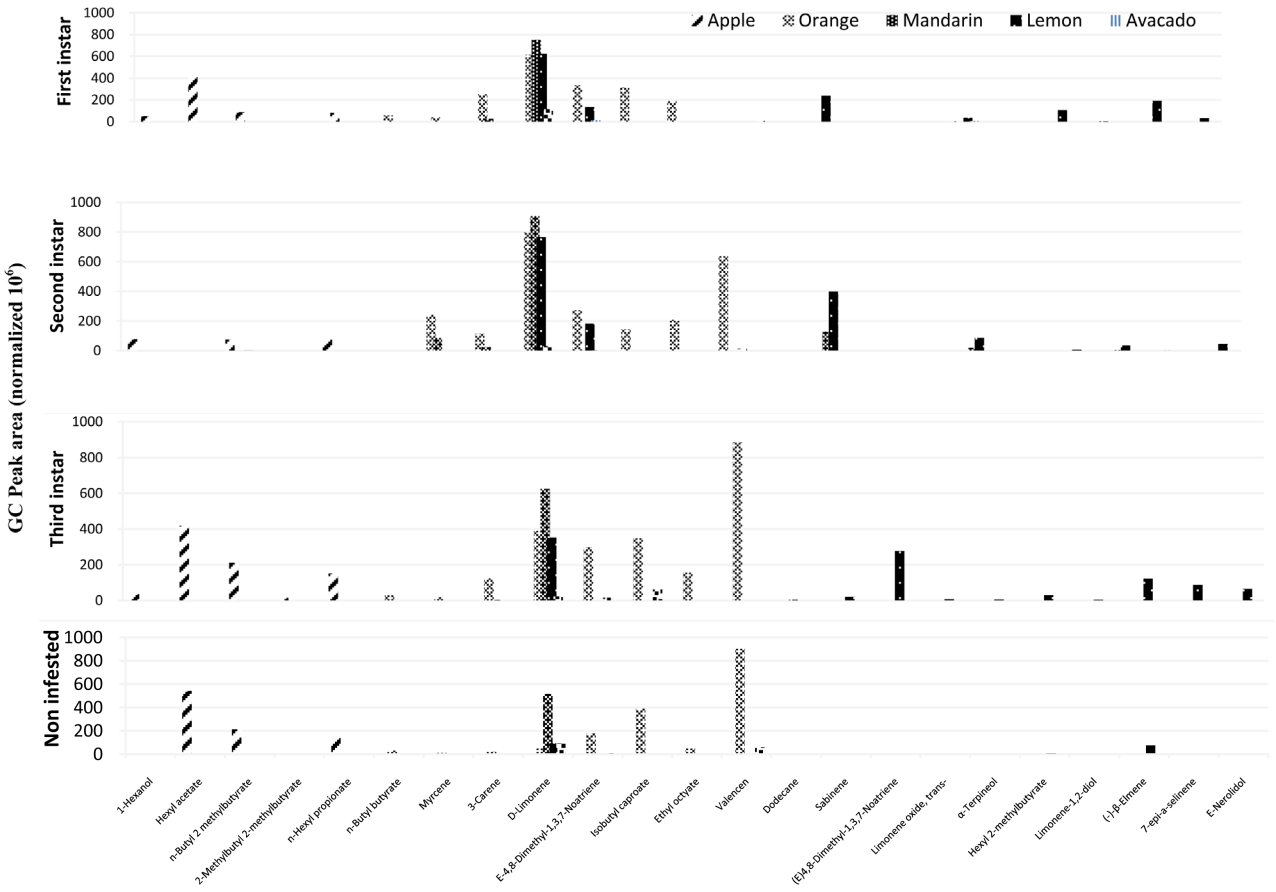

Figure 2. Comparison of main peaks between different types of fruits at three different instars.

n-hexyl propionate were decreased with increase in instars. From these results, it shows that the level of larvae can change the profile of compounds as also reported by [14]. Also, these compounds may impact on medfly larvae behaviour [15]. Infested apple with codling moth (*Cydia pomonella* L.) larvae gave a high level of esters and farnescene, from first instar infested and other instars, but the amount decreased in healthy fruits [16]. [11] Similar pattern in increase of esters can be found in our findings. In addition, some compounds were presented in high concentration in non-infested apple as compare to infested fruit such as propyl isobutyrate, n-butyl 2 methylbutyrate and others and also vice versa pattern is seen where high concentration of some compounds can be seen in infested fruits as compare to non infested fruits (**Table 2**). [11] [17] found that infested mango *Mangifera indica* (MI) with *Anastrepha ludens* (Loew) larvae extracts contain many compounds in higher level compared with the other two kinds of mangoes healthy mango (HM) and mechanically damaged mango (MDM). [18] Found nonanol, dodecane, tetradecane, 2-pinene, limonene, farnesene and hexyl carproate in five cultivars of peach infested with Medfly.

3.3.2. The Volatile Compounds from Orange

In case of non infested oranges, the main peaks were *β*-myrcene, 3-carene, D-limonene, E-4,8-dimethyl-1,3,7-noatriene, methyl caprylate, isobutyl caproate, ethyl octyate and valencen. Many compounds were detected in fruits infested with third instar larvae but not in fruits infested with first or second instar larvae or in non infested fruit and these included ethyl (Z)-2-butenoate, (+)-2-bornanone, (-)-trans-isopiperitenol, methyl caprate, caryophyllene and farnesene (**Table 3**). The most significant compounds compared with non infested fruits in third instar were myrcene, 3-carene, D-limonene, methyl caprylate, isobutyl caproate, ethyl octyate and valencen. In the second instar, they were 3-carene, D-limonene, E-4,8-dimethyl-1,3,7-noatriene, methyl caprylate, isobutyl caproate, ethyl octyate and valencen, While for first instar 3-carene, D-limonene, E-4,8-dimethyl-1,3,7-noatriene and ethyl octyate were of significant differences compare to non-infested orange (**Table 3**). Some chemical started to increase in concentration with increase in level of infestation as compare to non infested fruits such as myrcene, D-limonene, β-cis-ocimene, ethyl octyate and limonene-1,2-dial, while some compounds decreased with level of infestation as compare to non infested, like isobutyl caproate and valencen. In fact, some of these compounds come from peel oil, such as ethyl butanoate, *β*-myrcene, and *α*-pinene [19], and others compounds were found in fruit juice including, hexanal, *β*-myrcene, cis-*β*-ocimene, terpinolene and valencene and other compounds [20]. There were some volatile compounds identified by infested orange with *Thaumatotibia leucotreta* by using SPME-GC technique, these include *β*-myrcene, D-limonene, E-4,8-dimethyl-1,3,7-noatriene, caryophyllene and valencen [21]. D-limonene, n-butyl butyrate, 2-pinene, nonanal, decanal and valencen were detected by infested Grapefruits with immature stages of Caribben fruit fly *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) [22].

3.3.3. The Volatile Compounds from Mandarin

Table 3 show the differences between infested and non-infested mandarin fruits. D-limonene was the main peak in both infested and non infested fruit. The fruit infested with third instar of larvae recorded more number of chemicals than other stage of infestation. These new chemicals includes, butanoic acid, 3-methyl-,2-methylbutul acetate, sabinene, *β*-myrcene, octanoic acid, methyl ester, dihydrocarvone, (-)-trans-isopiperitenol, p-mentha-1(7), 8(10)-dien-9-ol and ethyl laurate which are present only in fruit with third instar larvae (**Table 4**). Some of these chemicals increase in concentration with increase in infestation like 3-carene, D-limonene, moslene, p-menth-1,4(8)-diene and (+)-epi-bicyclosesquiphellandrene increased from non infested fruit to fruits with third instar larvae. However, tridecane was decreased within first and second instar. From our results, larvae of Medfly have changed the profile of infested fruit especially third instar and this change in profile can be used as an identification tool. Similar results were found by [23] where change in the composition of host fruit odors (volatile profiles) was observed when fruit was infested with medfly and its parasitoid *Diachasmimorpha longicaudata* (Ashmead). These compounds provide a tool for detection of infestation with fruit fly (Medfly) in the early stage of larvae [21] [14].

3.3.4. The Volatile Compounds from Lemon

The highest peaks which were recorded in non infested lemon are sabinene, D-limonene, (E)4,8-dimethyl-1,3,7-noatriene, methyl caprylate, *α*-terpineol, (-)-*β*-elmene, 7-epi-a-selinene and intermedol. The results indicated that fruit infested with third instar of larvae recorded a high number of target compounds. These compounds were butyl 2-methylbutanoate, terpinen-4-ol, P-menth-8-en-2-one,E-, (3E,7E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene and dodecanoic acid, ethyl ester (**Table 5**). There were significant differences between infested and non infested lemon fruit in different stages of larvae; these include D-limonene, methyl caprylate, hexyl 2-methylbutyrate and intermedol in third instar. In second instar, D-limonene, methyl caprylate and limonene-1,2-diol. In first instar, they were D-limonene, hexyl 2-methylbutyrate and E-nerolidol. Some of chemicals started to increase in concentration with increase the level of infestation such as sabinene, D-limonene, methyl caprylate and *α*-terpineol, while (-)-β-elmene decreased within instars. [23] recorded D-limonene, n-butyl butyrate, (-)-*β*-elmene and valencen from infested Grapefruits with immature stages of Caribben fruit fly Anastrepha suspensa (Loew) (Diptera: Tephritidae). In addition, [24] found Sabinene, D-limonene, 3-Carene compounds in lemon infested with *D. citri* vector.

3.3.5. The Volatile Compounds from Avocado

Sulcatone, D-limonene, E-4,8-dimethyl-1,3,7-noatriene and valencen were the main peaks in avocado fruits. There were some new peaks associated with avocado infestation especially with third instar infestation; these compounds included decane, 3-methyl-, p-menth-1,4(8)-diene, α-terpineol, 1,2,6-dimethylun-

decane and α-cubebene. There were significant differences between infested and non infested avocado fruit in different stages of larvae; these include D-limonene, E-4,8-dimethyl-1,3,7-noatriene and valencen for third instar infested fruit. In second instar of larvae, sulcatone, D-limonene, moslene, E-4,8-dimethyl-1,3,7-noatriene and valencen. In first instar, they were D-limonene and moslene. Some of chemicals increased in concentration with increase in the level of infestation like D-limonene. Similar results were observed in case of orange, mandarian and lemon. However, 1-heptanal, E-4,8-dimethyl-1,3,7- noatriene and *α*-terpineol decreased in concentration with increase in the level of infestation (**Table 6**). As mentioned before the number of larvae recorded on avocado fruit was much higher compared to other fruits under laboratory conditions (**Figure 1**), and this may be due to the volatiles contents which are favourable for the growth of larvae as compare to other types of fruits. Some of the compound detected by [25] includes, hexyl acetate, 2-pinene, valencen and hexyl carproate.

3.3.6. The Volatile Compounds from Different Fruits Infested with Third Instar Larvae

In summary, if we compare all five fruits infested with third instar larvae, the major identifying components for each fruits infested with third instar are 1-hexanol, hexyl acetate, n-butyl 2 methylbutyrate and n-hexyl propionate for apple; 3-carene, D-limonene, isobutyl caproate, E-4,8-dimethyl-1,3,7-noatriene and valencen for orange; 3-Carene and D-limonene for mandarin; Sabinene, D-limonene, (E) 4,8-dimethyl-1,3,7-noatriene, hexyl 2-methylbutyrate, (-)-*β*-elmene, 7-epi-a-selinene and e-nerolidol for lemon and finally D-dimonene and valencen were from avocado (**Figure 2**). D-limonene was present in orange, mandarin, lemon and avocado fruits, but not in apple. Isobutyl caproate were found in orange and avocado fruits. [26] Found that, many volatiles compounds were produced by fruit, which has the same molecular structure with other fruits including, D-limonene and hexyl 2-methylbutyrate

4. Conclusion

In conclusion, this paper showed that different types of fruit produce, different volatile organic compound profile as detected by GC-MS and with various larvae instars. Some of these compounds are specifically associated with Medfly infested fruit. In fruits infested with Medfly, the presence of volatile compounds like styrene, decanal in apple, l-*β*-pinene, *β*-cis-ocimene, isoamyl caproate, limonene-1,2-dial in orange, terebenthene, p-menth-1,4(8)-diene, (-)-*β*-elmene, (+)-epi-bicyclosesquiphellandrene in mandarin, sabinene, (E)4,8-dimethyl-1,3,7-noatriene, *α*-terpineol in lemon and 1,2,6-dimethylundecane, α-terpineol in avocado can demonstrate distinction between non-infested and infested fruits. We have shown how Medfly can increase or decrease some of the fruit volatiles. Our results indicate that these volatiles levels, emitted from fruit with an early stage of larvae infestation can be detected by the HS-SPME GC-MS method. Recently, volatiles compound detection technology has been successfully used in different postharvest cases for early infested detection of insects and fungus.

Fruit infested with Medfly or other insect eggs, release unique volatile compound. These compounds can be exploited to provide tools for improved pest detection. Finally, this research provides the basis for determining the larvae infested by the HS-SPME GC-MS method. Also, it can be used to assess the applicability of this technology for detection of other species of fruit fly, different type of fruits and different number of larvae. We recommend the use this technology in quarantine areas or prior of the importation of fruit for early detection of any infestation in the fruits by fruit flies.

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