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Optimization and Validation for Determination of VOCs from Lime Fruit *Citrus aurantifolia* (Christm.) with and without California Red Scale *Aonidiella aurantii* (Maskell) Infested by Using HS-SPME-GC-FID/MS

K. Mohammed, M. Agarwal, J. Mewman, Y. Ren

Abstract**—**An optimum technic has been developed for extracting volatile organic compounds which contribute to the aroma of lime fruit (*Citrus aurantifolia*). The volatile organic compounds of healthy and infested lime fruit with California red scale *Aonidiella aurantii* were characterized using headspace solid phase microextraction (HS-SPME) combined with gas chromatography (GC) coupled flame ionization detection (FID) and gas chromatography with mass spectrometry (GC-MS) as a very simple, efficient and nondestructive extraction method. A three-phase 50/30 μm PDV/DVB/CAR fibre was used for the extraction process. The optimal sealing and fibre exposure time for volatiles reaching equilibrium from whole lime fruit in the headspace of the chamber was 16 and 4 hours respectively. 5 min was selected as desorption time of the three-phase fibre. Herbivorous activity induces indirect plant defenses, as the emission of herbivorous-induced plant volatiles (HIPVs), which could be used by natural enemies for host location. GC-MS analysis showed qualitative differences among volatiles emitted by infested and healthy lime fruit. The GC-MS analysis allowed the initial identification of 18 compounds, with similarities higher than 85%, in accordance with the NIST mass spectral library. One of these were increased by *A. aurantii* infestation, D-limonene, and three were decreased, Undecane, α-Farnesene and 7-epi-α-selinene. From an applied point of view, the application of the above-mentioned VOCs may help boost the efficiency of biocontrol programs and natural enemies' production techniques.

Keywords**—**Lime fruit, *Citrus aurantifolia*, California red scale, *Aonidiella aurantii*, VOCs, HS-SPME/GC-FID-MS.

I. Introduction

CITRUS is the most widespread arboreal plants in the world and represents one of the most important crops. They are cultivated in over 130 countries in tropics and subtropics area and, extending over 4 million ha [1], [2]. Orange is the most cultivated fruit, followed by the mandarin, tangerine, clementine and satsuma group, then by lemon and lime, and grapefruit and pomelo. Lime (*Citrus aurantifolia* Swingle) is an attractive fruit that consumers are eager to buy in many countries for their unique flavor and acidity, and also serve as a source of industrial and added-value food products [3]. In 2013, the total world production of limes and lemons was 15.4 million tonnes, with India leading the production of 2.5 million tonnes [4]. Lemons and limes are high acid citrus fruit and are grouped in FAO statistics. In 2002/03 Australia imported about 2,500 tonnes of lemon/limes [5]. Citrus are susceptible to a wide range of pests, and California red scale *Aonidiella aurantii* (Maskell) is amongst the most important pests of citrus [5]. *A. aurantii* occurs on a plethora of host plants throughout the world but is fundamentally known as an important pest on citrus [6]. *A. aurantii* is probably the most extended citrus pest in the world [6], [7]. However, the economic importance of *A. aurantii* is mostly related to infestations that reduce the market value of fruit [8], [9].

Herbivorous activity is known to induce a variety of biochemical changes in plants, which are attractive to natural enemies [10]. Previous laboratory studies indicated how the different infestation of fruit shows a variety of chemicals and how the release of VOCs increases or decreases during the infestation process. The efficiency of parasitoid foraging is enhanced through the use of cues that are associated with their hosts. Cues derived directly from the stage of the host used by the parasitoid are the most valuable in that they reduce time spent searching for suitable habitats or host stages [11]. For identifying volatile organic compounds emitted by lime fruit, it is necessary to develop sensitive, non-destructive, rapid and systematic methods that are also cost-effective. There are some studies about the use of HS-SPME for identification of lime juice volatiles [12], and it is successfully used to evaluate potential differences between a healthy and infected fruit [13], or between a healthy or wound-induced plant [14]. The HS-SPME technique is a new, fast, simple, and highly sensitive and solvent-free sample preparation technique for the extraction of volatile compounds [15]-[17]. Many factors affect the optimization of extraction conditions. These include an optimum sealing time and the correct fibre for capturing the VOCs, the temperature during extraction and the fibre absorption from the headspace [18]. This paper will determine the optimal conditions of sealing time, extraction time and

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desorption times for lime fruit volatile isolation by the HS-SPME technique with GC-FID. This study will also evaluate the use of HS-SPME method coupled with GC-MS technique as a potential technology for identifying the effect of California red scale on the VOCs emitted from lime fruit.

II. Materials and Methods

Insect Colonies and Samples

The originated California red scale culture was established rearing from infested citrus orchard located in Western Australia, Australia, 2016. Laboratory cultures of California red scale were reared on lemon fruit (*Citrus limon*) (26 °C, 40–60% RH, L16: D8 photoperiod) in the Murdoch University insect culture room using the method described by [19]. Fresh samples of lime fruit were purchased from different vendors at local shopping centres. The lime fruits' weight was approximately about 100 g each. The fruit was selected and washed well with warm water to remove the wax; limes were stored in a refrigerator, then taken and allowed to become acclimated to room temperature for 24 h before exposure to California red scale colony. For infesting fruits, 20 lime fruit were placed inside four California red scale rearing cages, as described above, for 3 days. These were then removed from the cage and maintained at laboratory temperature until the culture develops to the second instar. Uninfested fruits were similarly removed from the refrigerator and acclimated to laboratory conditions prior to collection and analysis of volatiles.

Optimization of Sealing Time, Extraction Time and Desorption Time (Fibre Desorption)

To determine the best sealing and extraction time; lime fruits individually sealed for 2, 4, 8, 12, 16 and 20 hours in 500 ml glass jars, while each fibre was exposed to the HS of the same glass jars chamber containing individual lime fruit for 3 different time periods (1, 2 and 4 hours). The efficiency of the six different sealing time and three different extraction time was determined by comparing the normalized peak area of the ten compounds from lime fruit under the same extraction time for the sealing time experiments, desorption time, SPME fibre, and GC conditions. After exposure, the fibres were retrieved and injected into the heated injection port (250 ˚C) of a GC-FID and desorbed for 10 min. Each sample was replicated for three times. To determine the best absorption time; the fibres were exposed to the headspace of the 500 ml glass jar containing individual lime fruit for 4 hours. After exposure, the fibre was retrieved and injected into the heated GC injection port (250 ˚C) for desorption. There were three replicate samples. For optimization of GC injector desorption time, three different desorption time (5, 10 and 15 min) were used at 250 ˚C.

Collection of Volatile Compounds

The analysis of volatiles was focused on whole lime fruit. Lime fruit was placed individually into 500 ml jars. One fruit was analyzed in each jar. In each glass jar, a 5 mm port drilled into one side, into which septa (20633 Thermogreen® LB-2 Septa, plug) placed and used for collection of lime fruit VOCs. Aluminum foil 100 m × 44 cm (Vital Packaging Company) was used to cover the glass jar opening and extract volatile organic compounds emitted from fruit. Three experimental replicates were taken for lime fruit. VOCs were collected by solid phase microextraction (SPME) fibre with 50/30 µm Carboxen/DVB/PDMS (2 cm) (Sigma-Aldrich, Bellefonte, USA) coating. The volatiles were collected by inserting the fibre into the jar and exposing it to the headspace. After sealing jars for 16 hours at room temperature (25±1˚C), the SPME fibre was exposed for 4 hours to glass jar headspace to extract the VOCs which were represented the optimum extraction time. The desorption time of SPME fibre was 5 min in the injection port.

Analysis of Volatile Compounds

The separation was performed using a GC system 7829A (serial number CN14272038) fitted with an HP-5MS non-polar column (30 m × 0.25 mm, film thickness 0.25 µm, RESTEK, catalogue number 13423), with a FID. Volatile compounds were analyzed by using (GC Agilent GCMS 7820A equipped with MS detector 5977E (Agilent Technologies, USA) and a DB-35ms column (30 m × 250 µm × 0.25 µm) (Santa Clara, CA 95051, USA). GC-MS operation conditions were as follows: Injector port temperature was 270 °C. The initial oven temperature was 50 °C with increase to 250 °C (increment of 5 ◦C/min). The flow rate of the column was 1:1 ml/min, while the splitless was 20 ml/min at 1.5 min. The run time of GC-MS was 45 min. The glass jar port was drilled from one side with a 5 mm, into which septa (20633 Thermogreen® LB-2 Septa, plug) was placed and used for collection of lime fruit VOCs by SPME. Aluminum foil 100 m × 44 cm (Vital Packaging Company) was used to cover the glass jar opening and extract volatile organic compounds emitted from fruit. Three experimental replicates were taken for lime fruit. Volatile peaks were analysed by AMDIS version 2.72 and identified by using the US National Institute of Standards and Technology 2014 MS database with retention index confirmation [12]. Three replications were analyzed, and the experiment was repeated two times to confirm the chemicals.

Statistical Analysis

For the comparison of volatile compounds between healthy and infested lime fruit, the peak area was analysed by software using the two-way ANOVA test. Differences in the results were compared by using the least significant difference test (LSD $P \leq 0.05$) for determining the means between infested with healthy fruit. The peak area was divided by 100000 for each single compound in optimization experiments and by 10000 in identification experiment.

III. Results and Discussion

Analysis of VOCs in Lime Fruit with Different Sealing Time

Total peak areas from lime fruit sealed for 2, 4, 8, 12, 16 and 20 hours are compared in Fig. 1. The amount of VOCs was significantly different between those collected at different

sealing times. This result showed that 16 hours sealing period achieved higher efficiency for VOCs extraction from lime samples. Therefore, the 16 hours sealing time for lime fruit was selected for subsequent studies. Optimization and extraction studies on aroma volatiles in fresh lime fruit have not been reported. Optimization of sealing conditions was carried out using a 500 ml chamber with two factors: the fiber exposure time and period needed to reach equilibrium in the headspace. Samples were analysed using GC-FID. The criteria were total area of the chromatogram and higher number of peaks. The determination of the optimum time of sealing is essential to obtain maximum efficiency of the SPME fibers for particular VOCs. Normally if there is no significant difference between sealing time, less sealing time is preferred, which agrees with [20] who isolated a number of high-quality chemicals from the headspace using 15 min for banana pulp compared with 140 min sealing time of whole banana. In this study, significant difference was detected between different sealing times so, 16 hours sealing time for fresh lime fruit was selected to isolate high-quality volatiles.

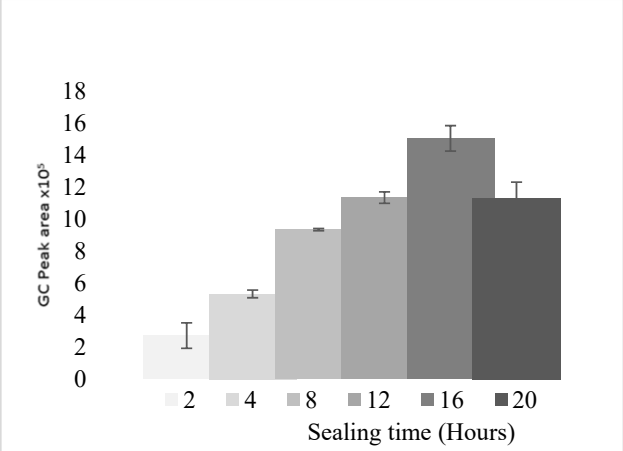

Fig. 1 Peaks of VOCs (units) produced by lime fruit with 2, 4, 8, 12, 16 and 20 hours sealing time. Error bars are LSD at 5% (n = 3)

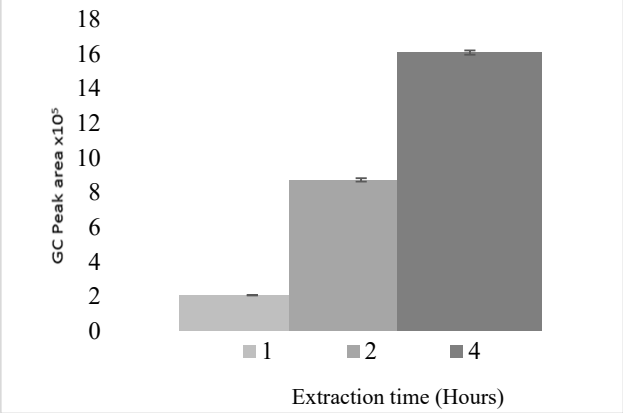

Fig. 2 Effects of extraction time with lime fruit on the peaks area of VOCs at 1, 2 and 4 hours. Error bars were LSD at 5% (n = 3)

Analysis of VOCs in Lime Fruit with Different Fibre Exposure Times

There were significant differences in the amounts of volatile compounds produced at the different extraction times from lime fruit (Fig. 2). Therefore, 4 hours were selected for best extraction time to absorb the VOCs emitted from the lime fruit. Extraction time and temperature are significant parameters in HS-SPME since both have an effect on the equilibrium during extraction of volatile compounds [21]. In this study, optimal extraction time for lime fruit was 4 h which is longer than the time used by [22] who reported 40 min as the best fibre exposure time to extract the volatiles compounds emitted by some species of citrus fruit juice. This difference is most likely due to the difference in fruit extraction part, headspace volume and extraction temperature, since the extraction time depends on the chemical nature of the volatiles present, the fibre polymeric phase, the distribution constant, and the size of the molecular mass. In this study, the results indicated that there were differences between lime fruit extraction time by total peak area. Apparently, more volatiles are emitted from lime fruit to reach the high level from 4 h extraction time (Fig. 2).

Evaluation of Desorption Time (Fiber Desorption)

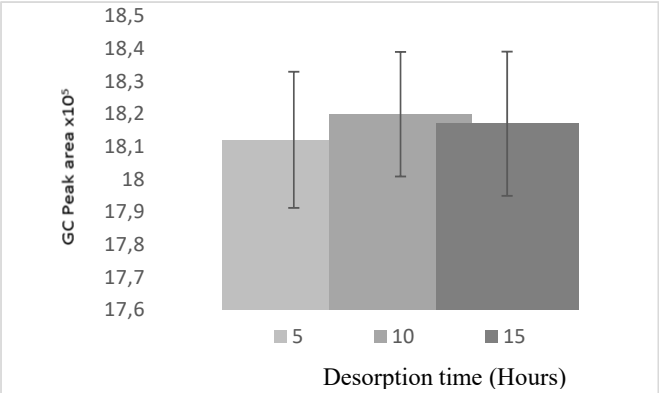

Fig. 3 Effects of different desorption times on desorption of VOCs from lime fruit. Bars represent LSD at 5% (n = 3)

There were no significant differences for the various desorption times (5, 10 and 15 min) of the three-phase fiber. Therefore, as desorption time, 5 min was selected (Fig. 3) at 250 ˚C of desorption temperature. Desorption time is an important process, and it depends on the speed of desorption of the VOCs from the fibre through the injection port. In theory, desorption time should be appropriate enough to release all the absorbed volatile compounds from the fibre. Since we cannot play much with the desorption temperature as every fibre has a temperature stability range based on the fiber coating (e.g., for the three-phase fibre, 270 ˚C is recommended as the limit of temperature tolerance [23]), and also exposure of the fibre to very high temperatures can shorten the fibre's life so in order to achieve the best result for release of volatile components we have to optimize the desorption time. Apart from this, GC sensitivity might be affected by high

temperatures used for the release of the VOCs from the fibre in the injection port; this could be due denaturation, destruction, or decomposition of the chemicals at a higher temperature [24]. Therefore, there is a necessity to optimize the temperatures used to increase the maximum release of VOCs from the fibre without compromising the composition of VOCs released.

Volatiles Compounds' Identification

Volatile organic components were identified by matching their retention index values and mass spectra with standards; all run in the laboratory under the same chromatographic conditions. The identification was also based on comparing an unknown mass spectrum with spectra available on the NIST database, mass spectral data system or from the literature [25], [26]. 18th VOCs were identified emanating from uninfested and infested lime fruits. D-Limonene increased significantly after California red scale infestation. In contrast, three VOCs decreased significantly during infestation and were characteristic of uninfested lime fruits: Undecane, α-Farnesene and 7-epi-α-selinene (Table I).

TABLE I
GC PEAK AREA (ONE UNIT CORRESPONDS TO A 10000 AREA) OF VOLATILE COMPOUNDS IN HEALTHY AND INFESTED LIME WITH *A. AURANTII* DETECTED BY GC-MS

Compounds	RT	RI	Non-infested	Infested
Cyclotrisiloxane, hexamethyl-	3.646	825	35.71	45.90
p-Xylene	5.871	862	7.038	2.534
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	6.565	928	4.085	6.047
Methane, trimethoxy-	9.557	702	1.692	4.599
1,1-Difluoro-2,2,3-trimethyl-cyclopropane	11.956	927	1.043	6.048
β-Pinene	12.464	970	4.856	5.607
o-Cymene	13.602	1025	8.893	9.436
D-Limonene	13.773	1018	164.0*	261.2*
1,3,6-Octatriene, 3,7-dimethyl-, (Z)	14.4	1024	82.79	93.21
γ-Terpinene	14.748	1047	38.74	34.40
Undecane	16.227	1100	72.08*	28.60*
(E)-4,8-Dimethylnona-1,3,7-triene	16.582	1116	48.90	30.42
Octane, 2,7-dimethyl-	24.454	931	4.925	5.574
cis-α-Bergamotene	25.214	1411	7.079	8.709
Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1S-(1α,7α,8aα)]-	26.707	1486	9.621	9.220
α-Farnesene	27.095	1499	470.4*	421.8*
7-epi-α-selinene	27.298	1474	81.14*	18.66*
(3E,7E)-4,8,12-Trimethyltrideca-1,3,7,11-tetraene	28.896	1580	9.434	4.328

*Means there are significant differences between infested and non-infested fruit (LSD mean P ≦ 0.05). (RT) retention time, (RI) retention index.

Semiochemicals released by plants in response to herbivore attack and the subsequent use of these chemical signals by natural enemies to locate these herbivores is a widespread phenomenon observed in various tri-trophic systems. These VOCs may serve as a part of the plant defense system by attracting parasitoids and predators during pest colonization and thereby limiting pest damage on the plant. Among the 18 lime VOCs identified, just D-Limonene increased significantly following California red scale infestation. D-Limonene is a natural cyclic terpene serving as a cue for any behavioral activity in insect species, although it was known to be produced by many plants, particularly by citrus fruits (orange, lemon, mandarin, etc.) (plant family Rutaceae). There is a plethora of considerable information has been acquired on the effects of D-limonene emitted from plants on herbivorous pests and their natural enemies [27]-[29]. There are three volatile compounds decreased following California red scale infestation: Undecane, α-Farnesene and 7-epi-α-selinene. Studies point out that VOCs that reduced following infestation were not attractive to natural enemies [30].

IV. CONCLUSION

The conclusion from this study indicate that HS-SPME coupled with GC and FID could be used to detect VOCs from lime fruit species without cutting or extracting juice and essential oil and the optimum condition for sealing and extraction period was 16 and 4 hours headspace equilibrium respectively. Glass jar with 5 min desorption time showed a good result for extract VOCs from lime fruit. The main compounds found in healthy and infested lime fruit were D-limonene, Undecane, α-Farnesene and 7-epi-α-selinene. This detailed study optimizes the extraction conditions of SPME on the identification of a large number of volatile compounds from lime fruit.

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