



E-ISSN: 2320-7078

P-ISSN: 2349-6800

www.entomoljournal.com

JEZS 2020; 8(2): 1441-1447

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Received: 22-01-2020

Accepted: 24-02-2020

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Volatile organic compounds as markers for detection of *Callosobruchus maculatus* (Fabricius) BRIWELL 1929 (Coleoptera: Chrysomelydae), major pest of Bambara Groundnut [*Vigna subterranea* (Linneaus), Fabaceae)] by the hymenopteran parasitoids

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Abstract

Volatile organic compounds (VOCs) contribute significantly to food flavor and can be used as indicators to female parasitoids to locate resources like hosts or preys to lay eggs or to feed. Chemical signals play a key role in trophic relationship. *Callosobruchus maculatus*' parasitoids select specific stage larvae of their host as egg-laying sites. The most useful tools to avoid the loss of post-harvest's grains are chemical pesticides. The main objectives of this work concern a characterization of the odours attracting parasitoids to increase their numbers and to reduce use of pesticides harmful to the environment and human health. Experiments were carried out to find out among VOCs release by *C. maculatus* which attract parasitoids. A set of 100 larvae of *C. maculatus* and 3 g of their feces collected from infested seeds at each stage of larvae were separately kept in 10 ml vials. These samples were exposed at the end of olfactometer for choice of the parasitoids females and analyzed by solid phase micro-extraction (SPME) coupled with gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). A four arms olfactory test revealed that *Dinarmus basalis* females were more attracted to larvae IV of *C. maculatus*; *Anisopteromalus calandreae* females were more attracted to the frass and the nymphs' odours elicited significantly greater response in *Eupelmus vuilleti* females than the others. The GC-MS analysis show particularly that 4-Octen-3-one and 1-Octen-3-ol were emitted by the odors' nymphs; 2-Hexen-1-ol and 5-Methyl-4-Hepten-3-one was produced by the frass and 2-Methylpyrazine was specific to the larvae IV. This provides, for the first time, these specific molecular bases for host could be the location's odors' by the hymenopteran parasitoids.

Keywords: Volatile organic compounds, *Callosobruchus maculatus*, parasitoids, olfactory test, GC-FID/GC-MS

1. Introduction

Callosobruchus maculatus (Coleoptera: Chrysomelidae) is a major pest of stored bambara groundnut capable of destroying the whole stock in 6 months if no preventive measure is taken [1]. The most popular protective tools in post-harvest conservation in rural area in northern Cameroon are the use of chemical pesticides in spite of their hazards and adverse effects on consumers and environment [2]. Developing alternative solutions is therefore an emergency. Obviously, use of natural enemies is the best user friendly way to prevent losses of crop due to pests. Several parasitoid insect species preyed on *C. maculatus* including: *Anisopteromalus calandreae* [3], *Dinarmus basalis* Rondani 1877 [4] and *Eupelmus vuilleti* Crawford (1913) [5]. Recent works pointed out these parasitoids as good biological control agent able to parasitize up to 90% of *C. maculatus* larvae in stored seeds [6]. However, under natural conditions, low numbers of initial parasitoids is not sufficient to allow these parasitoids to achieve a biological control of *C. maculatus* in this foodstuff.

Some parasitoids perceive the egg-laying mark done by the adult hosts to detect the presence of eggs or larvae, especially when they are inside the substrate and are therefore not directly accessible. Females *D. basalis* and *A. calandreae*, for example, use marks of females of the *C. chinensis* at the time of egg-laying to locate the larvae present within seeds [7].

However the age of larva is most important for making difference between the larval stages. Ngam^[8] demonstrated that females of *A. calandreae* select particularly 4th instar *C. maculatus* larvae for their egg-laying sites. To our knowledge, no studies have been done on the identification of semiochemicals allowing the detection by the parasitoids of a specific instar of its host for the laying site on the seeds of bambara groundnut infested by its main pest, *C. maculatus*. The present work aims to determine Volatile organic compounds (VOCs) found in the head spaces of each odors source of *C. maculatus* attracted by the parasitoids' females. These experiments could highlight the importance of using semiochemicals in biological control of bambara groundnut against *C. maculatus*' infestation, in consequence by synthesizing the bio insecticidal product less or no dangerous for human health and environment.

2. Methods and Materials

2.1. Sampling and Rearing of Insects

Bruchids and Parasitoids were extracted from seeds collected in smallholder granaries during the 2016 sampling campaign in the Far-North region of Cameroon. *Callosobruchus maculatus*, once in the laboratory were reared on sterilized seeds. A check-up was made every two weeks to follow up the infestation. The infested seeds by *C. maculatus* attract the following parasitoids which search the eggs laying sites: *Anisopteromalus calandreae* *Dinarmus basalis* and *Eupelmus vuilleti*.

The larvae used for observations were obtained after putting fifty couples of Bruchids on 20 g of Bambara groundnut seeds in a test tube of 25 ml capacity for 48 hours. For obtaining of different stages of development used for bioassays, the collection of three stages of larvae, the nymphs and the feces has been done.

The old day age of adults of parasitoid were obtained after removing of all adults in the pots and the new emerged adults obtained the next day. The removing of all parasitoids' adults was done one day before experience.

The 9, 13, 18 and 25 days old infested seeds containing respectively the 2nd, 3rd, 4th instars larvae and nymphs were observed separately. To remove larvae from seeds, they were firstly soaked for 5 hours in warm water to soften them and facilitate dissection. Softened seeds are broken manually with a penknife; the larvae presents are removed and collected in Petri dishes. The larvae of same stage and same age are grouped and introduced into the same 10ml glass test tube and hermetically sealed. 100 larvae are collected per stage.

After dissection of the seeds and removal of larvae and nymphs, the collection of feces was carried out using a needle that allowed scraping the inside of the larvae's tunnel and extracting the flours and metabolic wastes. 3 g of this extract was weighed and introduced into a test tube of 10 ml of capacity, sealed hermetically.

2.2. Olfactory analysis of parasitoids females to the different stages of larvae, nymphs and frass

A four-choice olfactometer bioassay was used to test the behavioural response of female parasitoids to the three most biologically stages (larvae, nymphs and frass) of *C. maculatus*. The four arms olfactometer and procedures were as previously described by Kavita *et al.*^[9] with minor modifications. Briefly, it was constituted of a central chamber (10 cm high and 5 cm diameter) connected (at 10 cm length) to four cylindrical plastics jars or "arms" (10 cm high and 5

cm diameter). In a four-branched of olfactometer, three odors sources per test were exposed at the end of each branch of the olfactometer to the female parasitoids' choice. The other end of the olfactometer contains the control (unaffected seeds). For each stage (Larvae II, III and IV and Nymphs) and frass, 5 replications were done. 5 parasitoids females per specie (1-day old) were released at the top of the central chamber. The parasitoids were observed continuously for 2 hours, and those found in each arm were counted and removed. Parasitoids that did not walk into any of the arms within 2 hours were not included in the analysis. After each test, the olfactometer was cleaned with hexane and the arms were rotated (90 °C) to minimize positional effect. All tests were conducted at 30 °C and 60–65% r.h.

2.3. Qualitative Analysis of volatile organic compounds by Chromatographic Gas analysis (GC-MS)

The qualitative analysis is carried out by chromatography gas coupled to the SHIMADZU GC-14B flame ionization detector (CPG-FID) and gas chromatography–mass spectrometry (GC-MS) with Computer-linked Peak Simple Chromatography Data System (Model 333) interface and DB-5 column type. For a heating rate of 7 °C /min, the temperature program used ranges from 30 °C to 250 °C and is then maintained at 250 °C during 12 minutes, a run time was 47.71min. Using a micro-syringe, 1µl of the extract was injected into the column; the injector was in split less mode. The detector and injector temperatures were 250 °C and 280 °C, respectively.

A solid phase micro-extraction (SPME) fiber is introduced into the headspace for three eggs' laying sites chosen by the parasitoids' females of the each specie during precedent tests for 24 hours. This time is required for the adsorption of the odor compounds. The whole is kept in an oven maintained at 30 °C.

The compounds present in the extracts are represented by the simple peaks clearly isolated on a chromatogram as a function of the retention time in abscissa axis. The retention index (Kovats index) was calculated according to the retention times of the compounds to be identified. The VOC analyses were identified using the NIST and WILEY database library (reserves fit hits and Kovat's indices, NIST, 2002) and by the comparison of known standard retention times by comparing their Kovats Index to those stored in pherobase site (<https://www.pherobase.com>) of synthetic standards database of the VOCs and using the basic data of pherobase reported by Robert^[10]. The large possible compounds were confirmed as genuine compound by checking some provided in bibliography data that are properly from insect's species.

2.4. Statistical analysis

Data on the mean number was analysed by using analysis of variance (ANOVA) followed by the Duncan's multiple range test used to group together the mean values that are not significantly different for each stage of development. This analysis was done by using STATGRAPHIC plus 5.0.

3. Results and Discussions

3.1. Behavioral responses of parasitoids females to the different egg laying sites

3.1.1. *Anisopteromalus calandreae* females to the different egg laying sites

In first experiment, among several odors sources, fourth stage larvae, third stage, second stage, nymph and frass, A.

calandrae females were most attracted to frass and fourth stage larvae.

In four arms olfactometer, *A. calandrae* females were more attracted to the frass than second stage, third stage larvae and nymph. And then, *A. calandrae* females were also more attracted to fourth stage larvae than third and second stage

(Figure 1). Therefore, *A. calandrae* female's choices means numbers to the third and second stage were less than one choice (< 1). Statistical treatments reveal a significant difference ($P < 0.05$) between responses of *A. calandrae* females to different odours ($n = 10$; $F = 3.27$; $P < 0.05$).

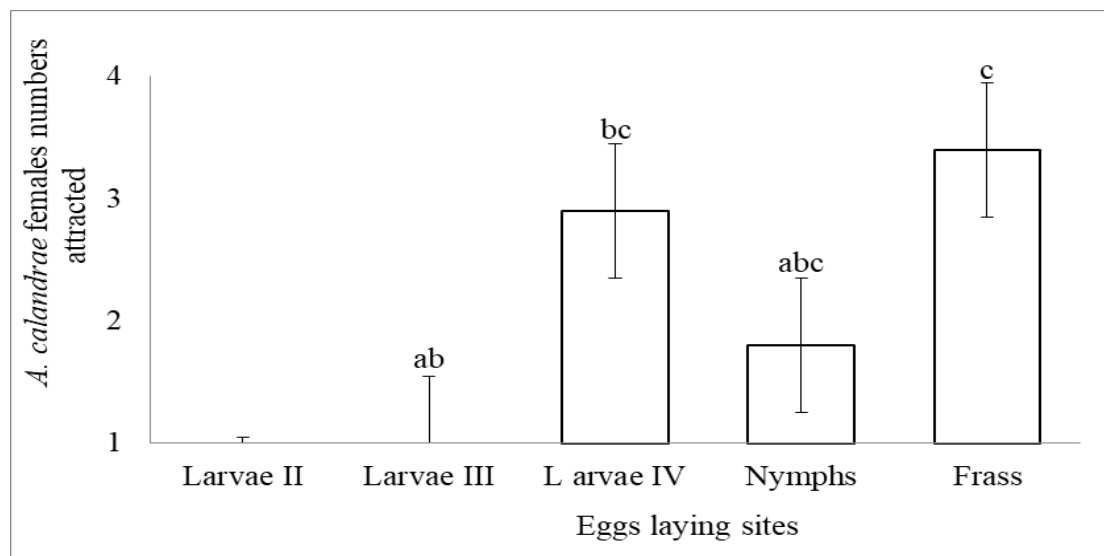


Fig 1: Response of *A. calandrae* females in a four-choice olfactometer bioassay to the eggs laying sites of *C. maculatus*. Values followed by the same letter do not differ significantly, $n = 10$; $F = 3.27$; $P < 0.05$

3.1.2. *Dinarmus basalis* females to the different egg laying sites

In second experiment, *D. basalis* females were more attracted to fourth instar larvae of *C. maculatus*. Differences were recorded to the all five odor's sources. However, fourth stage larvae was the most attracted for all tested odors followed by

the frass odors, third and second stage larvae and nymph odor was the less chosen odors (Figure 2). Statistical treatments reveal no significant difference ($p > 0.05$) between responses of *D. basalis* females to different odours ($n = 10$; $F = 0.9$; $P > 0.05$).

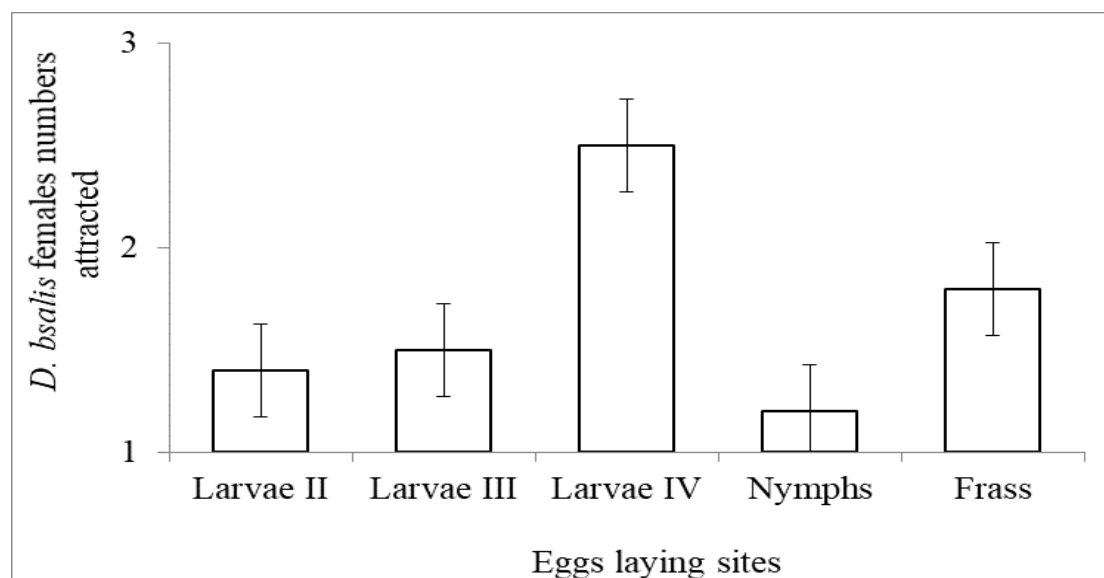


Fig 2: Response of *D. basalis* females in a four-choice olfactometer bioassay to the eggs laying sites of *C. maculatus*.

3.1.3. *Eupelmus vuilleti* females to the different egg laying sites

In third experiment, the nymphs' and frass' odors elicited significantly greater response in *E. vuilleti* females than the others odors' sources but the more chosen odors was recorded to the frass followed by the nymphs and fourth stage

larvae (Figure 3). The third and second stage larvae was the less chosen odors, their means numbers were less than one choice (< 1) Statistical treatments reveal a high significant difference ($P < 0.01$) between responses of *E. vuilleti* females to different odours ($n = 10$; $F = 3.96$; $P < 0.01$).

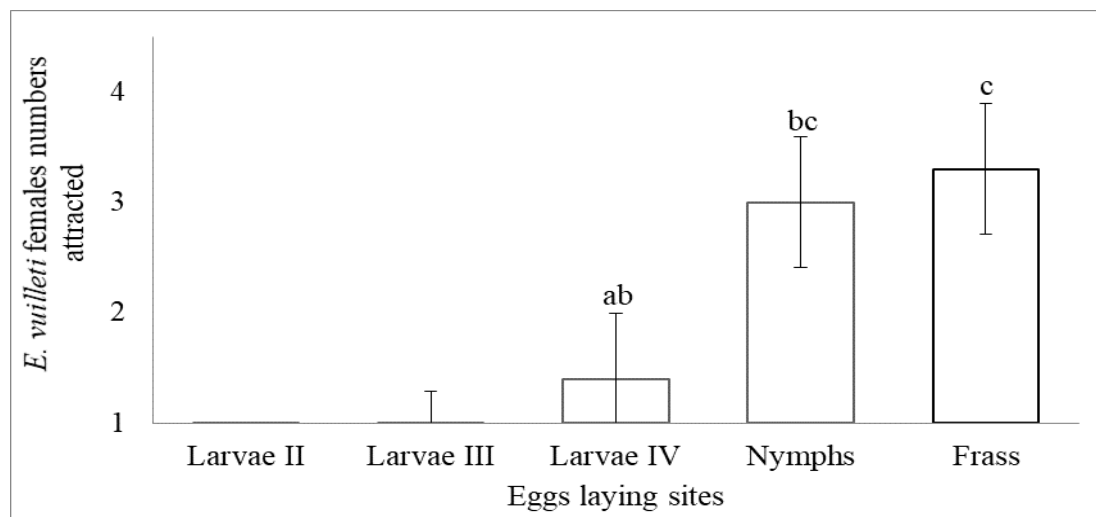


Fig 3: Response of *E. vuilleti* females in a four-choice olfactometer bioassay to the eggs laying sites of *C. maculatus*. Values followed by the same letter do not differ significantly, $n = 10$; $F = 3.96$; $P < 0.01$.

3.2. Volatile organic compounds obtained by Chromatographic Gas

It is of interest that fourth instar larvae, nymph and frass of *C. maculatus* consistently showed the strongest olfactory-activity to their parasitoids. Previously studies have shown typical compounds found in larval stage of bruchids include E-6-ethyl-2-methyl-10-methyl-5,9-undecenal emitted by *C. rhodesianus*^[11] and Cinamaldehyde from *Bruchus rufimanus*^[12], Benzaldehyde and 2,3-Butanediol from *C. chineensis*^[13]. Gas chromatography–mass spectrometry profile has shown twenty five different peaks for some compounds which are specific to each of the three odors' sources odors of fourth larval stage, odors of nymph, and odors of frass. Some compounds are common to all odors' sources.

3.2.1. Major Components of Nymphs' odors

Analysis of the volatiles from nymphs' odours has shown the presence of many components such as: Benzaldehyde also detected in fourth stage larvae, whose peaks appear at the RT

= 9.012 min (Figure 4), this compound has already been cited as consisting of extracts of exuviae of nymphs of eucalyptus bugs, *Thamastocoris peregrinus* (Heteroptera: Thaumastocoridae). This compound is also produced by infested cowpeas by the fourth instar of *C. chineensis* larvae^[13]. The peak at 9.88 (Figure 4) is specific to nymphs and show a major presence of Butan-1, 4-dial. The peak at 11.21 is only to nymph's odors and corresponds to Benzene methanol. The peak at RT = 14.42, specific to nymphs showed the presence of Octadecene-1-ol. A 3-Octen-1-ol is also from the odor of nymphs has a position isomer, a 1-octen-3-ol that had already been identified in insects cells molecular and was found to be produced by males and females of foreign grain beetle, *Ahasverus advena* (walzl) (Coleoptera: Cucujidae), *Oryzaephilus surinamensis* (L.) and *O. mercator* (Fauvel)^[14]. A compound, 4-Octen-3-one (Figure 4) has a position isomer; a 1-octen-3-one is already known to insects especially was identified in the headspace of *Sitophilus granarius* larvae by Abuelnnor *et al.*,^[15].

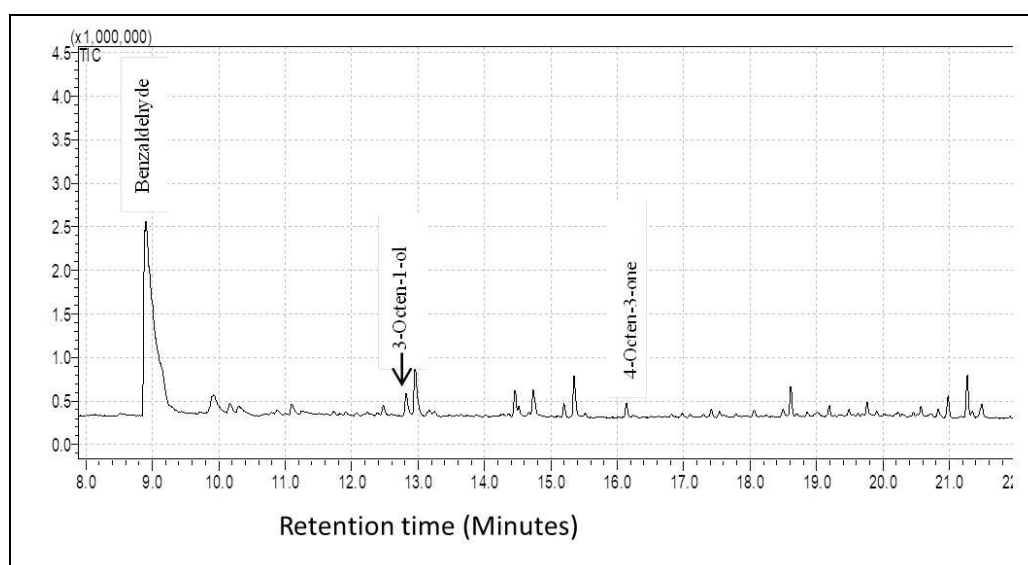


Fig 4: Representative chromatograms of volatile organic compounds from the nymphs' odors

3.2.2. Major Components of Larvae IV' odors

Volatiles extracts of fourth stage larvae from Bambara groundnut's seed revealed the presence of: 2-Methylpyrazine

with retention time at 14.56 (Figure 5) specific of fourth stage, its substitution isomer (2,5-Dimethylpyrazine) is already known to insects especially in *Solenopsis invicta* adult

extracts. Kavita *et al.* [9] showed an attraction of *Pseudacteon tricuspis*, parasitoid of a fly *Solenopsis invicta* (Diptera: Phoridae) by 2,5-Dimethylpyrazine and 2,6-Dimethylpyrazine. Ethyl-1-hexenol; Hexadecane; 1-Iodo-Octatetracontane and 6-Methyltridecane were also found in

heads space of fourth instar larvae.

From RT 27.97 to 37.92 (Table I), 6 compounds are present but have not yet been known in insects' activities: Dioctyl Phthalate, Tetratriacontane, 5-Phenyl-2-Pentalen, Tricosane, 1-Iodo-Octatetracontane, Dihydroxyacetic acid.

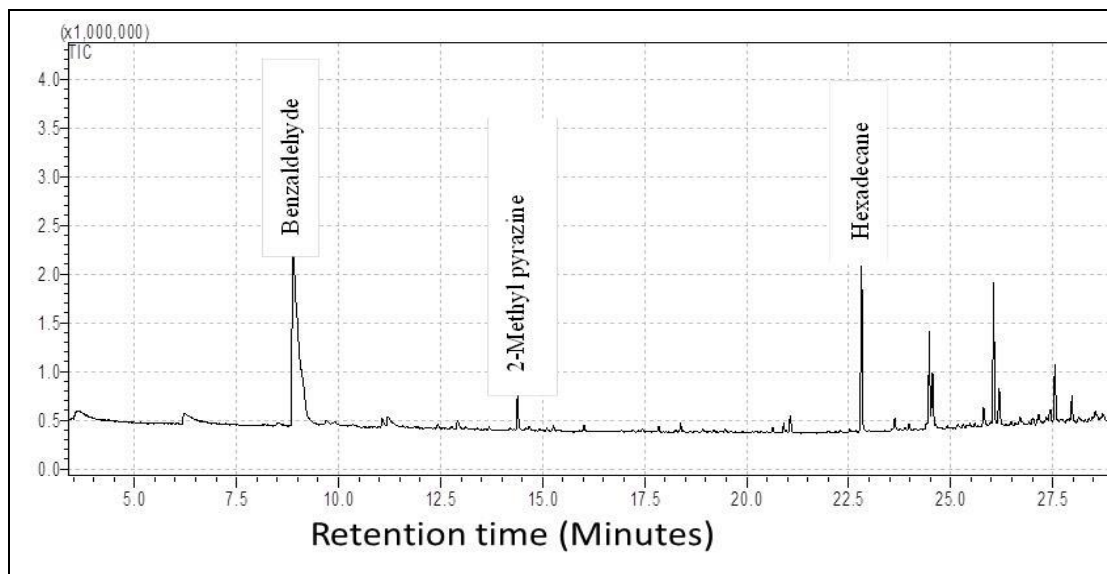


Fig 5: Representative chromatograms of volatile organic compounds from the Larvae IV' odors

3.2.3. Major Components of frass' odors

At RT = 12.88, there is the presence of (E) 2-Hexen-1-ol (Figure 6), specific to the frass' odors. And at the RT of 15.11 to 22.76, 9 compounds are present: one of these is cited as produced by the insects' activities; it is; 5-Methyl-4-hepten-3-one (have not yet been found in the cellular substances of insects). 5-Methyl-4-Hepten-3-one, identified in frass only, with RT = 20.85 and KI = 1489.87 (Table I), has not yet been cited as insect compounds. However, *Sitobion avenae* on cereals have also shown production of 6-methyl-5-hepten-2-one to be associated with aphid feeding [16]; these components

are position-isomers. Compound (E) 2-Hexen-1-ol identified in frass at RT = 12.9 and KI = 1080 as well as 5-Methyl-4-Hepten-3-one have not yet been identified as an insect odor but the compound (Z) 2-Hexen-1-ol had been yet known as a specific molecular basis for host location by an aphid parasitoid [16]. Ethyl-1-Hexanol specific to frass; 4-Methylcyclohexanol, 1,4-Butandial and 4-Ethyl-1-Octyn-3-Ol (Table I) are common to 3 odors' sources; 4-acetylbutyric acid, 2-Cyclohexylcyclohexanone, Oxalic Acid, Cyclohexylmethyl Tridecyl Ester, 6-Methyltridecane.

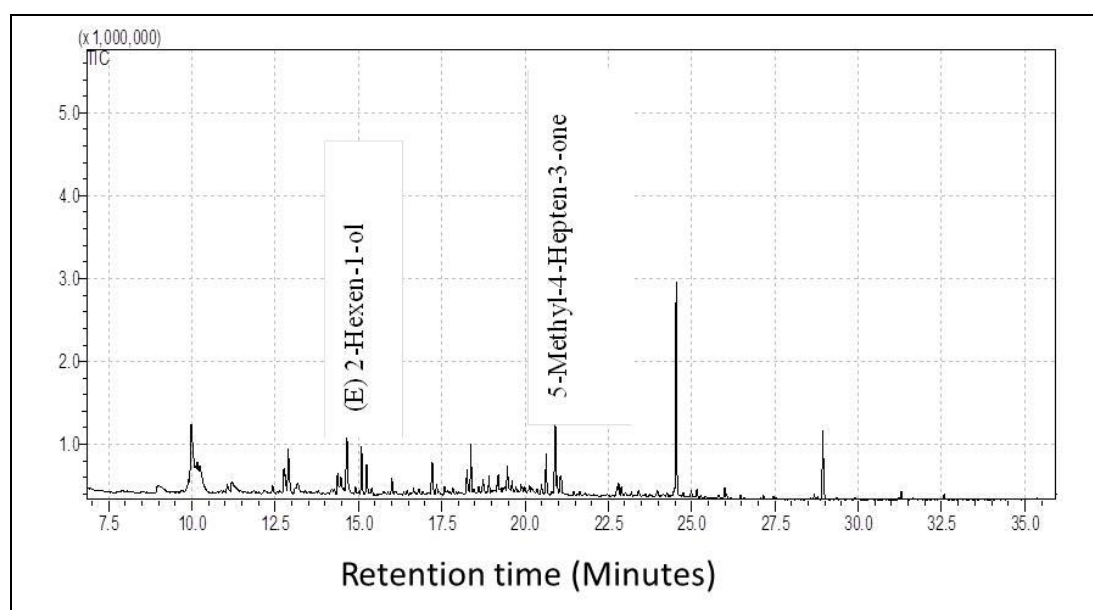


Fig 6: Representative chromatograms of volatiles organics compounds from the frass' odors.

Table 1: Gas chromatography–mass spectrometry response for volatiles organics compounds common to the three odors of *Callosobruchus maculatus*.

RT	VOCs	KI	Samples		Refs	
			Nymphs	Frass	Larvae IV	
9.012	Benzaldehyde	9500	12.27%	-	6.7%	+
11.21	Benzene methanol	1041	10.14%	-	-	+
12.88	(E) 2-Hexen-1-Ol	1090	-	10.70%	-	+
14.65	2-Methylpyrazine	1167	11.34%	-	-	+
15.11	Ethyl-1-Hexanol	1200	-	9.46%	-	-
15.20	4-Methylcyclohexanol	1200	11.24%	9.34%	6.14%	-
16.03	1,4-Butandial	1250	7.79%	8.52%	5.57%	-
17.19	4-acetylbutyric acid	1300	8.92%	10.93%	-	-
18.3	4-Ethyl-1-Octyn-3-Ol	1350	7.95%	10.00%	5.64%	-
20.57	2-Cyclohexylcyclohexanone	1470	7.84%	9.40%	-	-
20.9	5-Methyl-4-Hepten-3-One	1480	-	10.52%	-	+
21.26	4-Octen-3-One	1500	12.46%	-	-	+
22.76	Hexadecane	1600	-	-	6.4%	+
24.46	1-Iodo-Octatetracontane	1700	-	-	5.91%	-
24.54	Oxalic Acid, Cyclohexylmethyl Tridecyl Ester	1700	-	10.81%	5.28%	-
26.05	Nonadecane	1850	-	-	6.12%	-
27.55	6-Methyltridecane	1925	-	10.46%	5.06%	-
27.97	Ethanedial	1935	-	-	4.93%	-
28.98	Eicosane	2000	-	-	6.33%	-
29.35	2-Nitroethanol	2333	-	-	5%	-
29.91	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	2335	-	-	4.5%	-
31.67	Dihydroxyacetic acid	2350	-	-	4.22%	-
32.93	1-Iodo-Octatetracontane	2370	-	-	5.42%	-
35.29	Tricosane	2600	-	-	5.7%	-
35.93	Diocetyl Phthalate	2650	-	-	6.26%	-

RT, retention time (min); KI, kovat's index; Rfs + means the VOCs detected by others authors; - means VOCs not detected

4. Conclusion

Chemical signals play an important role in recognizing partners in a trophic relationship. The works cited above clearly illustrate the possibility of using kairomones by the natural enemies of *Callosobruchus maculatus* on bambara groundnut seeds. Because, parasitoids of *C. maculatus* as known as act to be attract by the egg-laying marks by the adults hosts to detect the presence of eggs or larvae, the purpose of work reported here was to identify semiochemicals released from *C. maculatus* stage larvae and their excretory product as its markers. Aldehydes volatiles, alkylpyrazine and acetones are previously unknown from bruchids extracts. A search of the pherobase on VOCs database in site <https://www.pherobase.com> has retrieved only two compounds: E-6-ethyl-2-methyl-10-methyl-5,9-undecenal emitted by *C. rhodesianus* and Cinamaldehyde from *Bruchus rufimanus*. We have recently identified: Benzaldehyde; Benzene methanol; 4-Octen-3-one; (E) 2-Hexen-1-ol; 5-methyl-4-hepten-3-one; 3-octen-1-ol; 2-methylpyrazine. The possible semiochemicals roles of these volatile are being studied. Further study showing the parasitoids' behavior towards these semiochemical molecules, could confirm the use of these compounds in the methods of pests control.

5. Acknowledgment

The authors wish to thank Thomas Heiss of SHIMADZU Austria for assistance with laboratory work.

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