



Preliminary identification of the sex pheromone of the phytophagous pest *Epilachna vigintioctopunctata* inhabiting the brinjal crop

C Sundareswari^{1*}, D N P Sudarmani¹, P Ponmanickam¹, S Jaya Durkga¹, J John Wilson²

¹ Post-Graduate and Research Department of Zoology, Ayya Nadar Janaki Ammal College, Sivakasi, Affiliated to Madurai Kamaraj University, Madurai, Tamil Nadu, India

² Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi, Affiliated to Madurai Kamaraj University, Madurai, Tamil Nadu, India

Abstract

Sex pheromones are pheromones that organisms emit to entice members of their own species, promote mating, or carry out other tasks that are closely related to sexual reproduction. Species, age, sex, and genetic information are all sent through sex pheromones, which are also designed to attract the opposing sex and identify females for breeding. Sex attractant pheromones are highly sensitive and selective tools for detecting and monitoring populations of insects, yet there has been only one reported case of pheromones being used to monitor protected species. Here, we report the preliminary identification of the sex pheromone of a phytophagous pest, *Epilachna vigintioctopunctata*. The data identified from the results of GC-MS analysis of pheromone extracts of larva and adult *E. vigintioctopunctata* showed 30,40 peaks respectively. Of the 30 and 40 identified components, there are 3 components were commonly seen in both the extract namely, n-Hexadecanoic acid, Octadecanoic acid, and 9,12,15-Octadecatrienol.

Keywords: Pheremone, attractant, field test, *E. vigintioctopunctata*

Introduction

Historically, Epilachninae was referred to as a distinct subfamily of the Coccinellidae, but more recently, it has been noted as a tribe of the subfamily Coccinellinae by Seago *et al.*, (2007) ^[17]. There are roughly 1050 species of phytophagous Epilachnini Mulsant, 1846, spread across 23 genera (Tomaszewska and Szawaryn, 2013) ^[24]. Epilachninae have also been merged under Coccinellinae as Epilachnini by Slipinski and Tomaszewska (2010) ^[23] and Seago *et al.*, (2011) ^[18] based on morphology (Slipinski, 2007) and initial molecular analysis by Giorgi *et al.*, (2009) ^[11].

The Coccinellidae beetle genus *Epilachna* contains a number of pest species, including the mexican bean beetle (*Epilachna varivestis*) (Arroyo, 1997) ^[6]. *Epilachna vigintioctopunctata* (F) is one of the most serious pests of Solanaceous crops in India, especially *Solanum melongena* and *S. tuberosum*. *E. vigintioctopunctata* (Coleoptera: Coccinellidae) or *Henosepilachna vigintioctopunctata*, one of the most destructive pests, also locally known as the hadda beetle, is widely distributed throughout India and other countries (Anam *et al.*, 2006) ^[3].

In Jammu and Kashmir (Jamwal *et al.*, 2013) ^[12] and other parts of India, (Ahmad *et al.*, 2001) ^[1], the 28-spotted hadda beetle, *H. vigintioctopunctata* (Fab.) (also known as *Epilachna vigintioctopunctata* L.), is a polyphagous pest and is regarded as a voracious foliage feeder of many cultivated and wild plants primarily from the families Solanaceae and Cucurbitaceae (Sharma *et al.*, 2012) ^[19].

Sex pheromones are pheromones that organisms emit to entice members of their own species, promote mating, or carry out other tasks that are closely related to sexual reproduction. Species, age, sex, and genetic information are all sent through sex pheromones, which are also designed to attract the opposing sex and identify females for breeding. Because they are typically picked up by direct touch with

chemoreceptors on the insect's antennae or feet, nonvolatile pheromones, also known as epidermal contact pheromones, are more directly associated to social insects. Pests are tracked and captured with the help of insect sex pheromones.

Pheromones are particular cell signals that are employed for conversation among members of the same species (Jeong, 2005) ^[13]. Both Ando and Yamakawa claim that Pheromone was categorised as type 3 (Ando and Yamakawa, 2011) ^[5]: Type I, II, and additional Type. nearly all pheromone on Type I and Type II ^[8]. The majority of Type I molecules are primary alcohols with straight 10- to 18-carbon chains (C10-C18), which include acetate and aldehyde derivatives. It is the most widely used kind (Ando *et al.*, 2004) ^[4]. Type II includes polyenyl hydrocarbons with functional groups for epoxy and ketone and 17–23 carbons.

Additionally, some cases exhibit traits from both Type I and Type II. A few species of Pyraloidae have pheromones that deliver both unsaturated hydrocarbon and epoxy group, according to Yan *et al.*, (2014) ^[25]. The pheromone of *Amyelois transitella* has both Type I (aldehyde, alcohol group) and Type II (epoxy and ketone group), according to Kanno *et al.*, (2010). Sex attractant pheromones are highly sensitive and selective tools for detecting and monitoring populations of insects, yet there has been only one reported case of pheromones being used to monitor protected species. Here, we report the preliminary identification of the sex pheromone of a phytophagous pest, *Epilachna vigintioctopunctata*.

Materials and Method

Insects and pheromone extracts

Source of Insects: Adult beetles were collected from the agricultural fields in and around areas of Sivakasi during March 2021. They were acclimatized and maintained in laboratory for culture. The insects were provided with fresh

brinjal leaves which were grown in animal house, Ayya Nadar Janaki Ammal College, Sivakasi as a feed. The adults were allowed to mate, the eggs laid were maintained in the laboratory in room temperature. When the adults emerged they were sex identified and separated.

Pheromone extraction

In most insect species the pheromone gland is located as the intersegmental cuticle between the eighth and ninth abdominal segments of the ovipositor tip (Ma and Ramaswamy, 2003). Terminal abdominal segments of virgin female were excised to prepare sex pheromone gland extracts (Bergh *et al.*, 1990) [7].

Pheromone glands of the 1-2 old females during calling were removed and extracted in HPLC grade dichloromethane. Aliquots of one female equivalent of gland extract were subjected to gas chromatographic analysis (Agilent GC 7890A / MS5975C). Sex glands from the females were excised and extracted. The excised glands were then placed in dichloromethane then extracted. The extract was then stored at-20° C in microvials with Teflon lined screwcaps until analysis.

Chemical analysis

The pheromone extract was analyzed using a GC-MS method (Agilent GC 7890A / MS5975C). An Agilent DB5MS (30 m × 0.25 mm: film thickness of 0.25 m) was used on the GC-MS, with helium as the carrier gas at a steady flow rate of 1.21 ml/min. The oven temperature was initially set at 60° C, then raised at a rate of 5° C/min to 200° C, accompanied by a final rise to 280° C. The scan was done with a m/z range of 50-650. The mass spectrometer was set to 70ev, and the NIST library was used to identify mass fragmentation trends of pheromone compounds.

Bioassay for pheromone component identification in laboratory

The bioassay procedure used by Borges *et al.*, (1987) [8] was followed with modifications as follows: The 2 days old female beetles which are capable of releasing natural stimulus was provided by five sexually mature virgin females were introduced into one side of the chamber, and the 10 sexually mature adult males were introduced into the other side of the chamber with a cover and placed into the corner of the chamber box and vice versa.

Prior to testing, the insects were allowed to acclimatize for a short period. The chamber was closed. The behavioral responses of males and females were recorded. Females have no response towards the males and males that move towards the female were counted as making a choice; Ten replicates were performed for each sample i.e., virgin female, virgin male, and female abdominal extract and control.

Table 1: List of compounds identified in the larva of *Epilachna vigintioctopunctata*

| Peak no | Retention time (Min) | Name of the compound | Peak area (%) |
|---------|----------------------|------------------------------------|---------------|
| 1 | 4.487 | Methylene chloride | 1.10 |
| 2 | 5.387 | 3-methyl-acetic acid | 1.44 |
| 3 | 5.509 | 2,3-Pyrazinedicarboxylic acid | 4.90 |
| 4 | 6.075 | Pyridazine | 0.64 |
| 5 | 7.053 | Acetophenone | 1.14 |
| 6 | 11.097 | Tetradecane | 0.47 |
| 7 | 11.719 | Tetradecamethyl cycloheptasiloxane | 2.35 |
| 8 | 13.086 | Diethyl phthalate | 1.81 |
| 9 | 13.363 | Alpha 3,4-tris benzeneacetic acid | 3.67 |

Field Test

Field trials of virgin female, crude abdominal extracts or pheromone gland extracts, and a control blank were carried out in farmer's field i.e. brinjal field were the diversity study was carried out in Sivakasi. Water traps were employed throughout the investigations discussed here since preliminary tests revealed them to be the most successful trap design for *E. vigintioctopunctata*. They consisted of a plastic bag containing water with a small amount of castor oil to reduce surface tension, and a supportive pad is placed above the plastic bag with a lure sample in the spin column tube to attract the male beetles. It is a modified method of Youm *et al.*, (1995) [26].

Results

Identification of the sex pheromone components of *E. vigintioctopunctata*

The data identified from the results of GC-MS analysis of pheromone extracts of larva and adult *E. vigintioctopunctata* is shown in Fig 1 & 2. The number of peaks obtained from the extracts of larva and adult beetle is 30,40 respectively. The peaks were obtained at from the 30 and 40 peaks obtained at 4-23 minutes of retention time in larval extract and 7-23 minutes of retention time in adult extract (Table 1 & 2). Of the 30 and 40 identified components, there are 3 components were commonly seen in both the extract namely, n-Hexadecanoic acid, Octadecanoic acid, and 9,12,15-Octadecatrienol (Table 3).

Bioassay with fractions of crude extract in laboratory

Bioassays with Live Insects male bugs were attracted to odors of live females (N=80, A.v±SD=8±0.28, SE=0.9). Females were not attracted to live males. In the latter case, most of the females tested did not move towards the septum containing male beetles. So, the female abdominal extracts only extracted and tested. When male *E. vigintioctopunctata* were tested with the crude extract of females, male beetles were attracted to the pheromone extract of females (N=90, A.v±SD=9±0.82, SE=0.9). (Table 4 & Fig 3a)

Field test

Test for examination of the field attraction by crude abdominal extracts and attraction of males towards virgin female were carried out at brinjal field in Sivakasi where the coccinellid beetles were collected. Bioassays with Live Insects male bugs were attracted to odors of live females (N=462, A.v±SD=46.2±5.29, SE=2.29). The crude abdominal extracts of female beetle showed attraction towards male beetles (N=500, A.v±SD=50.2±4.81, SE=2.19). (Table 5 & Fig 3b)

| | | | |
|----|--------|---------------------------------------|------|
| 10 | 14.774 | Unknown | 3.82 |
| 11 | 15.319 | 2-Acetyl-1,3-cyclohexanedione | 0.63 |
| 12 | 15.596 | 1,2-Benzenedicarboxylic acid | 1.13 |
| 13 | 16.041 | Eicosamethyl cyclodecasiloxane | 4.13 |
| 14 | 16.130 | 4-Methyl-alpha-hexanophene | 0.62 |
| 15 | 16.374 | n-Hexadecanoic acid | 1.05 |
| 16 | 17.185 | Tetradecamethyl cycloheptasiloxane | 4.36 |
| 17 | 17.596 | Phytol | 0.53 |
| 18 | 17.963 | Octadecanoic acid | 1.60 |
| 19 | 18.218 | unknown | 5.88 |
| 20 | 18.974 | 3,6-dimethyl benzene | 0.80 |
| 21 | 19.174 | Tetradecamethyl hexasiloxane | 6.28 |
| 22 | 20.085 | N-Benzyl-N-ethyl-p-isopropylbenzamide | 7.75 |
| 23 | 20.240 | 9,12,15-Octadecatrienoic acid | 6.56 |
| 24 | 20.374 | 3-Methyl ester 2-Propenoic acid | 4.90 |
| 25 | 20.507 | Adipic acid | 0.77 |
| 26 | 20.929 | Octadecamethyl cyclononasiloxane, | 8.49 |
| 27 | 21.729 | Eicosamethyl cyclodecasiloxane | 9.48 |
| 28 | 22.518 | Unknown | 6.36 |
| 29 | 22.918 | Eicosane | 0.60 |
| 30 | 23.407 | Unknown | 6.77 |

Table 2: List of compounds identified in the adult of *Epilachna vigintioctopunctata*

| Peak no | Retention time (Min) | Name of the Compound | Peak area (%) |
|---------|----------------------|---|---------------|
| 1 | 7.153 | Triethyl phosphate | 2.06 |
| 2 | 12.230 | Dodecanoic acid | 0.45 |
| 3 | 12.497 | Diethyl phthalate | 0.65 |
| 4 | 12.564 | Hexadecane | 0.40 |
| 5 | 14.186 | Octadecamethyl cyclononasiloxane | 14.186 |
| 6 | 15.441 | Eicosamethyl cyclodecasiloxane | 0.35 |
| 7 | 15.808 | n-Hexadecanoic acid | 3.52 |
| 8 | 16.585 | Tetradecamethyl cycloheptasiloxane | 0.59 |
| 9 | 17.252 | 9,12,15-Octadecatrienoic acid | 32.11 |
| 10 | 17.407 | Octadecanoic acid | 9.89 |
| 11 | 17.618 | Heptasiloxane | 1.97 |
| 12 | 17.718 | 9,12-Octadecadienoic acid | 1.09 |
| 13 | 17.841 | Unknown | 0.77 |
| 14 | 18.030 | Unknown | 0.35 |
| 15 | 18.307 | Fumaric acid | 0.39 |
| 16 | 18.507 | 2-Tert-butylcyclohexanone | 0.52 |
| 17 | 18.552 | Biperiden | 1.29 |
| 18 | 18.729 | Decanamide | 0.46 |
| 19 | 19.129 | 9-Eicosene | 0.67 |
| 20 | 19.474 | Unknown | 1.14 |
| 21 | 19.563 | Butyl9,12-octadecadienoate | 1.50 |
| 22 | 19.852 | Oxacyclododecan-2-one Hexadecanoic acid | 0.42 |
| 23 | 20.318 | Unknown | 0.53 |
| 24 | 20.696 | 4-Benzonitrile cyclopropane | 0.74 |
| 25 | 20.863 | UNknown | 0.90 |
| 26 | 21.051 | 9,12,15-Octadecatrienal | 16.20 |
| 27 | 21.151 | Oleoyl chloride | 5.32 |
| 28 | 21.451 | 13-dodecy hexacosane | 1.33 |
| 29 | 21.596 | Methyl 9,12-heptadecadienoate | 0.44 |
| 30 | 21.862 | 2,6-Dimethyl-3,4-bis pyridine | 0.66 |
| 31 | 22.062 | Oleyl alcohol | 0.34 |
| 32 | 22.196 | Eicosane | 1.06 |
| 33 | 22.407 | Ethanol | 0.63 |
| 34 | 22.673 | 10-heptyl-10-octyle-Methoxyacetic acid | 3.30 |
| 35 | 22.840 | 1-chloro-Sulfurous acid | 0.42 |
| 36 | 23.096 | Z-8-Pentadecan-1-ol acetate | 0.32 |
| 37 | 23.273 | Tocopherol | 0.35 |
| 38 | 23.429 | OOctacosyl acetate | 1.22 |
| 39 | 23.596 | Heneicosane | 2.92 |
| 40 | 23.640 | 1-Nonadecene | 2.33 |

Table 3: List of volatile compounds present in both larva and adult *E. vigintioctopunctata*

| S. No | Name of the Compound | Formula | Chemical structure |
|-------|-------------------------|--|--------------------|
| 1 | n-Hexadecanoic acid | C ₁₆ H ₃₂ O ₂ | |
| 2 | Octadecanoic acid | C ₁₈ H ₃₆ O ₂ | |
| 3 | 9,12,15-Octadecatrienol | C ₁₈ H ₃₀ O | |

Table 4: Bioassay for pheromone component identification in laboratory

| No of observations | Control | Number of males attracted towards septum with Virgin female | Number of males attracted towards septum with virgin male | Number of males attracted towards septum with female abdominal extract |
|--------------------|---------|---|---|--|
| 1 | 0 | 8 | 0 | 9 |
| 2 | 0 | 8 | 0 | 8 |
| 3 | 0 | 7 | 0 | 10 |
| 4 | 0 | 8 | 0 | 9 |
| 5 | 0 | 9 | 0 | 10 |
| 6 | 0 | 9 | 0 | 8 |
| 7 | 0 | 8 | 0 | 9 |
| 8 | 0 | 7 | 0 | 10 |
| 9 | 0 | 9 | 0 | 8 |
| 10 | 0 | 7 | 0 | 9 |
| N | - | 80 | - | 90 |
| A.v±SD | - | 8±0.28 | - | 9±0.82 |
| SE | - | 0.9 | - | 0.9 |

A.v=Average;SD=Standard Deviation;SE=Standard error

Table 5: Bioassay for pheromone component identification in Field

| S. No | Control | Number of males attracted towards septum with Virgin female | Number of beetles attracted towards control trap | Number of males attracted towards septum with female abdominal extract |
|--------|---------|---|--|--|
| 1 | 0 | 42 | 0 | 53 |
| 2 | 0 | 43 | 0 | 45 |
| 3 | 0 | 44 | 0 | 50 |
| 4 | 0 | 38 | 0 | 49 |
| 5 | 0 | 43 | 0 | 52 |
| 6 | 0 | 52 | 0 | 43 |
| 7 | 0 | 44 | 0 | 56 |
| 8 | 0 | 53 | 0 | 58 |
| 9 | 0 | 51 | 0 | 48 |
| 10 | 0 | 52 | 0 | 46 |
| N | 0 | 462 | 0 | 500 |
| A.v±SD | - | 46.2 ±5.29 | - | 50±4.81 |
| SE | - | 2.29 | - | 2.19 |

A.v=Average;SD=Standard Deviation;SE=Standard error

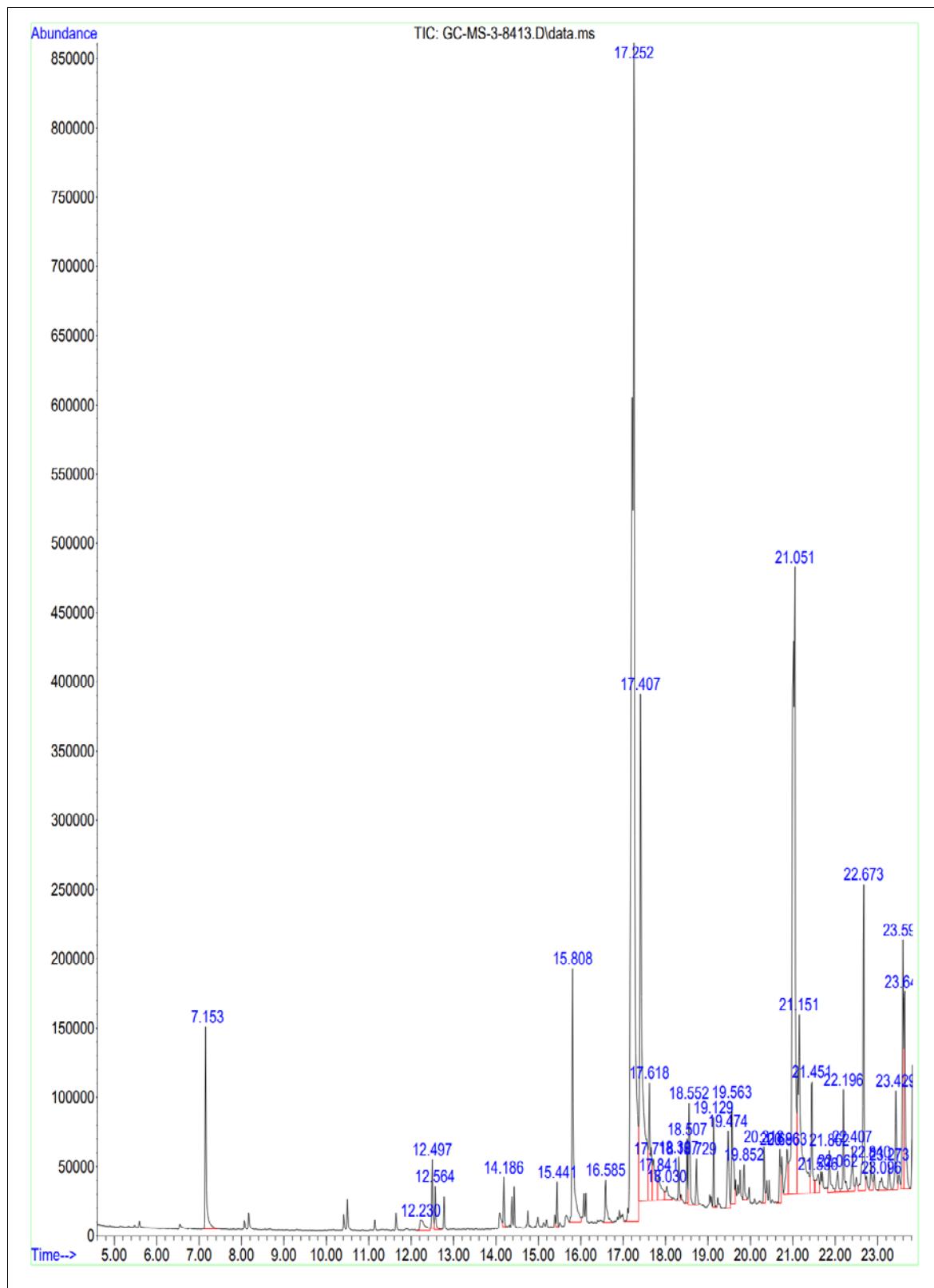


Fig 1: GC-MS analysis of abdominal tip extract of *E. vigintioctopunctata* larva

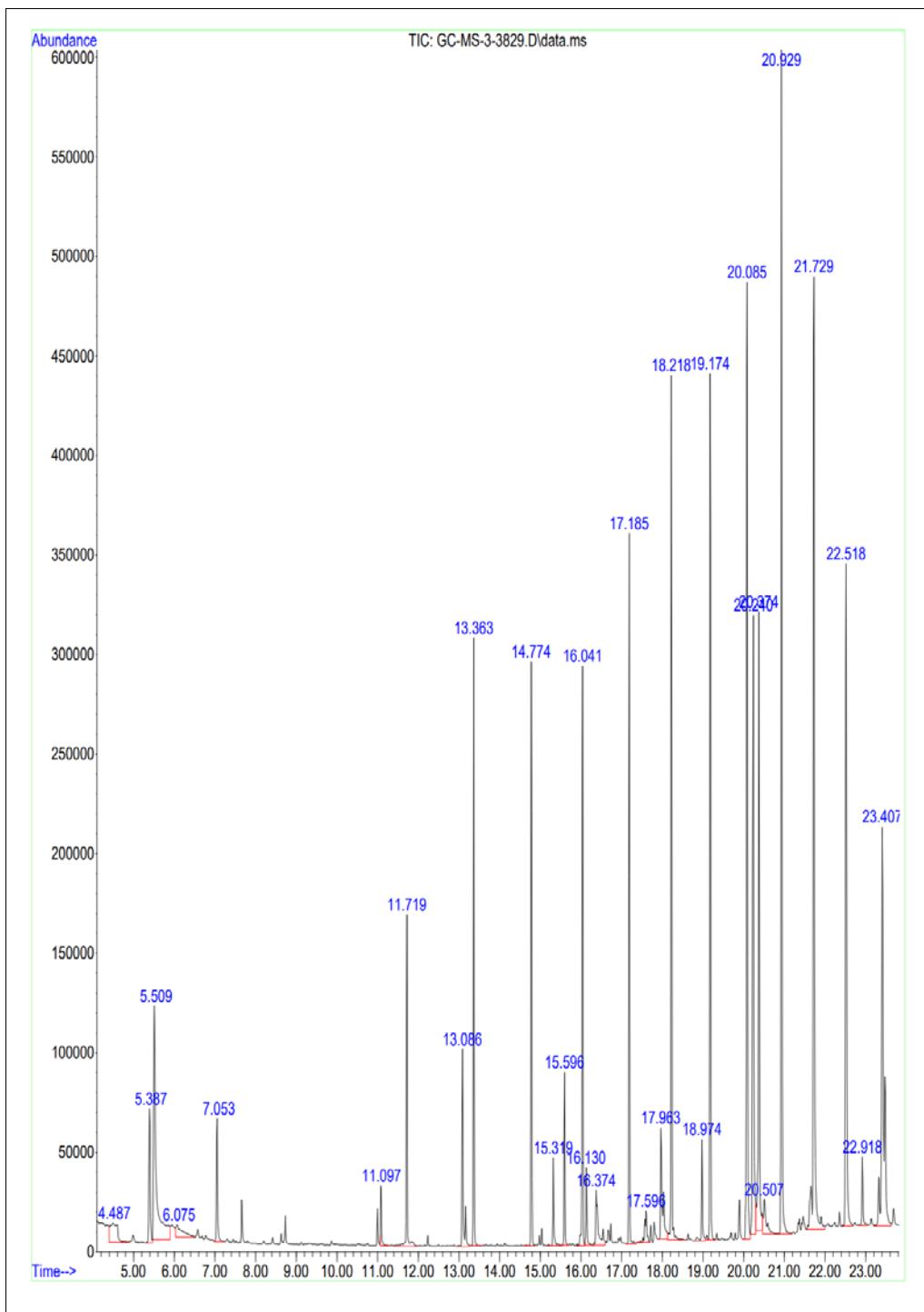


Fig 2: GC-MS analysis of abdominal tip extract of *E. vigintioctopunctata* adult female





Plate 3b: Field test using pheromone trap method

Discussion

The pheromone gland extract or abdominal extracts of *E. vigintioctopunctata* is subjected to GC-MS analysis. From the GC-MS study 30,40 peaks were obtained from the extracts of larva and adult beetle respectively. Of the 30 and 40 peaks obtained, 3 components were commonly seen in both the extract namely, n-Hexadecanoic acid, Octadecanoic acid, and 9,12,15-Octadecatrienol. These three components will be the sex pheromone components of Female *Epilachna* beetle.

Bioassays with Live Insects male bugs were attracted to odors of live females (X^2 = Females were not attracted to odors of males. In the latter case, most of the females tested did not move towards the septum containing male beetles. When female and male *E. vigintioctopunctata* were tested with the crude extract of males, male beetles were attracted to the extract of female odor. Test for examination of the field attraction, the crude pheromone extracts of female beetle showed attraction towards male beetles. The present study here focuses on finding the volatile pheromone components in the female beetle. Trap lures with these substances attracted a lot of male beetles just as well. This is the first report on preliminary identification of sex pheromone components of *E. vigintioctopunctata*.

Standard methods are typically used to extract and identify sex pheromones, and GC and GC-MS are frequently used. Though head space sampling rather than abdominal tip excision is reported to be more efficient and results in fewer contaminants, and GC-MS rather than GC for identification of pheromone components which are used today, Kuwahara and Casido (1973) ^[15] provided four important aspects for pheromone determination that are still somewhat relevant. i) Because the pheromone is specific to or only occurs in this area of the body, abdominal tips can be used as the source material for extraction, ii) To prevent interferences with analysis, no chlorinated solvents are utilized, iii)(c) The pheromone can be purified by hydrolyzing it to alcohol and then re-esterifying it, iv) Trichloroacetylation of the alcohol and detection of the trichloroacetate derivative by electron-capture gas chromatography satisfy the standards for analytical sensitivity and specificity.

The attraction efficiency of synthetic (E)-10-hexadecenal and (Z)-10-hexadecenol at 9:1 in the cardamom plantations of Arehalli and Goudahalli in Karnataka, India, was studied by Chakravarthy and Thyagaraj (1998) ^[9] using this trap. The moth-collecting polybag (0.28 0.45 m) was placed underneath a white plastic funnel (0.21 m dia.) that was fastened to a yellow plastic plate. Below the funnel, the pheromone mixture was suspended in the centre.

Similar study was also carried out by Dung *et al.*, (2021) ^[10] in citrus fruit borer, *Citripestis sagittiferella* in order to find the sex pheromone components preliminarily. Programs for biological control and integrated pest management might benefit from a global mapping of the sex pheromones for complexes of commercially significant pentatomids. Tachinid flies are significant biocontrol agents and frequently use heteropteran pheromones as host-finding kairomones (Aldrich, 1988; Todd, 1989) ^[2, 22].

The study by Tatsuki and Sugie, (1992) ^[21] coincide with the present study, they investigated the raw extracts of *Chilo suppressalis* (Walker) in GC, which revealed the chemical makeup of the pheromone components via the mass spectrum (Tatsuki and Sugie 1992) ^[21].

Using an early version of a Y-tube olfactometer and a wind tunnel equipment, Konno *et al.*, (1980) ^[14] were the first to discover that *C. punctiferalis* female pheromone was used to draw in males. For mate searching and grouping, insects respond to a variety of olfactory cues, including volatiles from plants (such as phytophagous insects), hosts (such as parasitoids and predators), and pheromones. To see how insects react to various scent signals, Y-tube olfactometers and wind tunnels are frequently used (Ranjith, 2007) ^[16].

Further chemical and behavioral research must be carried out, including testing individual components that are now being identified.

Conclusion

The data identified from the results of GC-MS analysis of pheromone extracts of larva and adult *E. vigintioctopunctata* showed 30,40 peaks respectively. Of the 30 and 40 identified components, there are 3 components were commonly seen in both the extract namely, n-Hexadecanoic acid, Octadecanoic acid, and 9,12,15-Octadecatrienol. Bioassays with Live Insects male bugs were attracted to odors of live females. Females were not attracted to odors of males in laboratory. Male *E. vigintioctopunctata* were tested with the crude extract of males, male beetles were attracted to the pheromone extract of females. In the field test, female pheromone extracts were found to have attraction towards male beetles.

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