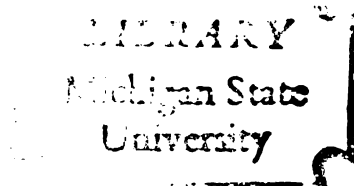




114
777
THS



This is to certify that the
thesis entitled
ISOLATION AND PRELIMINARY CHARACTERIZATION
OF THE PHEROMONE OF A GREENHOUSE FUNGUS GNAT,
BRADYSIA IMPATIENS (JOHANNSEN) (DIPTERA:SCIARIDAE)
presented by

Richard Andrew Kurnot

has been accepted towards fulfillment
of the requirements for

M.S. degree in Chemistry


Major professor

Date December 10, 1981



RETURNING MATERIALS:
Place in book drop to
remove this checkout from
your record. FINES will
be charged if book is
returned after the date
stamped below.

--	--	--

ISOLATION AND PRELIMINARY CHARACTERIZATION
OF THE PHEROMONE OF A GREENHOUSE FUNGUS GNAT,
BRADYSIA IMPATIENS (JOHANNSEN) (DIPTERA:SCIARIDAE)

By

Richard Andrew Kurnot

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Chemistry

1981

ABSTRACT

ISOLATION AND PRELIMINARY CHARACTERIZATION
OF THE PHEROMONE OF A GREENHOUSE FUNGUS GNAT,
BRADYSIA IMPATIENS (JOHANNSEN) (DIPTERA:SCIARIDAE)

By

Richard Andrew Kurnot

6/11/75

The pheromone of a greenhouse Fungus gnat, Bradysia impatiens, has been isolated and partially characterized. Behavioral studies indicate that the pheromone appears to be comprised of one component, a C-14 hydrocarbon with three degrees of unsaturation including at least one double bond. Further investigations are needed to determine the exact structure of the active component.

ACKNOWLEDGMENTS

The author would like to express sincere gratitude to Drs. Donald Farnum and Ring Cardé, and to committee member Dr. William Reusch.

A special thanks is also extended to Liga Dindonis, for her help with the culture maintenance.

The author would also like to thank Dr. Matthew Zabik for the use of his GC-MS facility.

TABLE OF CONTENTS

Chapter	Page
LIST OF TABLES.	iv
LIST OF FIGURES	v
INTRODUCTION.	1
METHODS AND MATERIALS	3
Insect Rearing and Culture	
Maintenance	3
Extract Preparation	4
Biological Assays	4
Isolation of Active Component	5
Quantitative Analysis of Active	
Component	7
Characterization of Active	
Component	7
RESULTS	10
Insect Rearing and Culture	
Maintenance	10
Isolation of Active Component	10
Quantitative Analysis of GC	
Isolation	14
Characterization of Active	
Component	14
GC-Mass Spectrometry of Active	
Component	18
DISCUSSION.	21
BIBLIOGRAPHY.	24

LIST OF TABLES

Table		Page
1	Biological Activity of XF-1150	
	Fractions	11
2	Biological Activity of OV-1	
	Fractions	15
3	Biological Activity of Eluent	
	From Reaction Mixtures of	
	Characterization Tests.	17

LIST OF FIGURES

Figure		Page
1	OV-1 Chromatogram of Active Fraction.	12
2	Regions of OV-1 Chromatogram.	13
3	Quantification of Active Component.	16
4	Mass Spectrum of Active Component	19
5	GC-MS Chromatogram of Active Fraction.	20

INTRODUCTION

Relatively few Dipteran pheromones have been identified (Fletcher 1977). Of the ones which have, most appear to be comprised of one or more long-chain hydrocarbons, usually with some unsaturation present. Carlson et al. (1971) reported the isolation of (Z)-9-tricosene as the compound which attracts the male house fly, Musca domestica, to the female. Uebel et al. (1974) have identified the components a pheromone which elicited mating strikes by male face flies (Musca autumnalis) as (Z)-14-nonacosene, (Z)-13-nonacosene, and (Z)-9-heptacosene. Other dipteran pheromones identified by Uebel et al. include (Z)-9-hentriacontene and (Z)-9-tritriacontene as components of a system which induces mating strikes by male stable flies (Stomoxys calcitrans) (1975), and (Z)-11-hentriacontene as the male copulatory stimulant for Fannia pusio, a common poultry pest (1978).

One species of sciarid fly in which the pheromone has been identified is Lycoriella mali. Kostelc et al. (1980) reported n-heptadecane as a male sex-attractant.

Alberts et al. (1981) have established the existence of a sex pheromone in the sciarid fly Bradysia impatiens. This study will involve the isolation and preliminary

characterization of the chemical components of this
pheromone system.

MATERIALS AND METHODS

Insect Rearing and Culture Maintenance

B. impatiens were reared using a modified version of Kennedy's procedure (1973). Glass shell vials, 25 x 95 mm, were filled with 15 ml of 2% non-nutritive agar solution. Vials were slanted while cooling to maximize surface area of solidified agar. Vials were immediately plugged with foam stoppers to prevent contamination from molds. The vials were allowed to desiccate for one day, at which time granular agar was sprinkled over the agar slant to absorb any remaining moisture which might entrap insects.

Active insects could be placed in each vial for mating and oviposition with an aspirator. This transfer method was more convenient than the "cooling" method used by Alberts et al. (1981), and precluded any adverse behavioral effects which can accompany a transfer which involves anesthetization (Fletcher) (1977). Two males and one female were placed in each vial. Hatched larvae were fed a 1:4 mixture of Brewer's yeast and finely chopped, autoclaved alfalfa. The alfalfa was used as a support for the nutrient Brewer's yeast. The culture was maintained at $25 \pm 1^\circ\text{C}$ with a 16:8 L:D cycle in a controlled environment chamber.

Extract Preparation

Pheromone extracts were prepared by placing whole anesthetized females in 10-15 ml of hexane for ca. 1 hr. at 20-25°C. Extracts were filtered, dried over anhydrous sodium sulfate, and stored at -10°C.

Biological Assays

Biological assays were conducted in glass orientation tubes, 1 m x 3 cm diam. Each glass tube was connected to a 3-way, 105° connecting tube. Prior to reaching the observation tubes, air was forced through a water trap (to prevent insect desiccation) at 1.9 m/sec.

The biological assay procedure involved placing 5-10 active male insects in each observation tube via aspiration. Insects were allowed to acclimate to air flow for 15 min. prior to testing. Test samples were pipetted on to 1.5 cm diam. filter papers, which were suspended in the 105° connecting tubes from cork stoppers by metal wire. Male insect wing-fanning (excitation response) and orientation were monitored at 15, 30, and 60 sec. After sample introduction. A positive orientation occurred when an insect moved into the upwind 10 cm of the tube. Percent response was calculated using the formula

$$\text{percent response} = \frac{\text{positive responses} - \text{background responses}}{n - \text{background responses}}$$

where n equals the number of insects tested. Background responses were determined by monitoring insect activity for 60 sec. prior to introduction of test samples.

The wing-fanning response, which occurred only in the presence of pheromone, was used to determine biological activity.

All biological assays were conducted under optimal conditions of response with regard to time, temperature, insect age, and extract concentration (Alberts et al.) (1981). Experiments were conducted at 15-1600 hr. after the onset of photophase in a 16:8 L:D cycle. Only 1-day-old males were tested, and no females younger than 1-day-old were extracted. Extract concentrations were greater than 50 female equivalents (FE) in all cases, and the controlled environment chamber in which bio-assays were conducted was kept at 25±1°C.

Bio-assay experiments were conducted using a randomized complete block design.

Isolation of Active Component

The active component was isolated using gas chromatography (GC). Extracts were initially separated into

fractions using a polar column (10% XF-1150 on 100-120 mesh Gas Chrom Q, 1.8 m x 2 mm ID at 92°C). Fractions were tested for biological activity through the assay method described earlier. The active fraction was then re-injected on to a nonpolar column (3% OV-1 on 100-120 mesh Gas Chrom Q, 1.8 x 2 mm ID at 100°C) and its chromatogram recorded. The chromatogram was then divided into regions before peaks, regions of peaks, and regions after peaks, and biological activity determined for each region.

Extracts containing 100 FE were prepared, dried over anhydrous sodium sulfate, and concentrated under dry N₂ to 10 µl prior to injection. The eluent from the XF-1150 column was trapped at 5 min. intervals in 20 cm x 1 mm ID capillaries cooled with dry ice. Fractions were rinsed out with 20 µl of hexane and tested for biological activity.

Additional extracts containing 500 FE were prepared and the active fraction collected from the XF-1150 column. The fraction was rinsed out with 10 µl of CS₂, which was concentrated to 2 µl. This concentrate was then injected on to the OV-1 column, its chromatogram recorded, and divided as described earlier. Fractions corresponding to these divisions were collected from additional 100 FE extracts, and tested for biological activity.

Quantitative Analysis of Active Component

The amount of active component in an extract can be determined using gas chromatography by comparing active peak area with that of an appropriate external standard. Tridecane was chosen as this standard based on its similar retention time ($T_R = 15$ min on OV-1 at 100°C) with that of the active peak ($T_R = 14.6$ min. on OV-1 at 100°C).

Characterization of Active Component

A partial characterization can be performed on the active component by conducting reactions on the extracts which alter specific functional groups. If the specific functional group were present, a successful reaction which altered that functionality would modify the GC retention time of the active component and probably eliminate its biological activity. Therefore, elimination of the biological activity of the eluent at the retention time of the active component is evidence that a specific functional group is present, and has been altered in the reaction.

A 400 FE extract was prepared and divided into 4 equivalent portions. The following reactions were performed on each of three portions:

Acetylation

The extract was stirred with 100 μ l of acetyl chloride for 3 hr, after which 1 ml of H_2O was added. The mixture was extracted with 10 ml of ether, which was subsequently dried and concentrated to 10 μ l for injection.

LAH Reduction

The extract was added with stirring to 10 ml ether and 0.1 g $LiAlH_4$. The mixture was refluxed for 4 hr, following which $Na_2SO_4 \cdot 10H_2O$ was added to deactivate any remaining $LiAlH_4$. The reaction mixture was filtered through Celite and concentrated to 10 μ l for injection.

Microhydrogenation

The extract was concentrated and dissolved in 2 ml ethanol. Addition of 0.1 g Pd/C was followed by bubbling H_2 through the solution for the duration of the reaction. The mixture was stirred and refluxed for 4 hr., filtered through Celite and concentrated to 10 μ l for injection.

The fourth portion of the extract was used as the control. This was similarly concentrated for injection.

The four concentrates were then injected on to OV-1 and the eluent from each trapped at the retention time of the active component. Each eluent was then tested for

biological activity, a lack of which would suggest functional group alterations.

GC-Mass Spectroscopy of Active Components

A mass spectrum of the active component was made by performing GC-MS on the active fraction trapped from the XF-1150 column. An OV-1 column was coupled with a DuPont 321 GC-MS, EI source, 60 eV, so that the active component could be separated as in the isolation procedure. The OV-1 column was programmed from 125 to 150°C, at 4°C/min. Injection size was 5 µl. The solvent used was CS₂. Sample size, as determined by comparison with external standard tridecane, was 20 ng.

RESULTS

Insect Rearing and Culture Maintenance

Bradysia impatiens is a monogenic species, i.e., flies emerging from each vial are of the same sex. At $25 \pm 1^\circ\text{C}$, male insects emerged approximately 14 days after mating, while females emerged in about 17 days. Twenty-two percent of mated vials emerged as males, while twenty-one percent emerged as females for an overall success rate of forty-three percent. Considerably more males emerged per successful vial than females.

Isolation of Active Component

The biological activity of each fraction of the XF-1150 eluent is recorded in Table 1. The active fraction appears to be no. 4.

Injection of the active fraction from a 500 FE extract on to OV-1 gave the chromatogram in Figure 1. The chromatogram was divided into Regions I, II, and III as described earlier (Figure 2), and eluent collected from additional 100 FE extracts according to the retention time of each region. Biological activity for each region is

Table 1. Biological Activity of XF-1150 Fractions.

Fraction	Retention Time	Percent Wing-Fanning	Percent Orientation	N
1	0-10 min	0	12.5	21
2	10-15	0	4.5	20
3	15-20	0	7.7	60
4	20-25	65	12.7	60
5	25-30	0	0	61
6	30-60	0	9.1	22

N = number tested.

Column Temperature = 92°C.

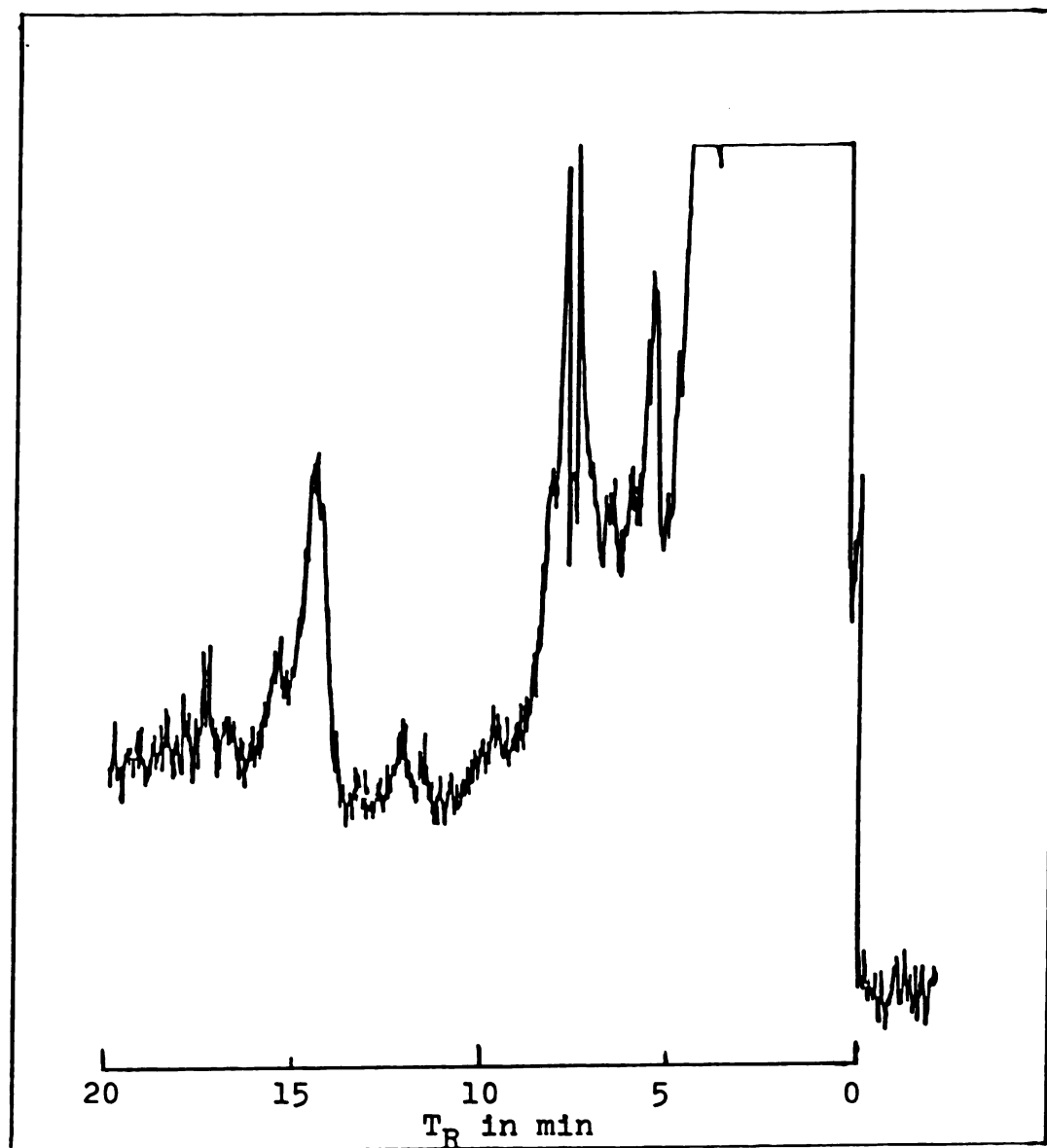


Figure 1. OV-1 chromatogram of active fraction trapped from XF-1150. Column temperature - 100°C ; Injection - 500 FE in $2\ \mu\text{l}\ \text{CS}_2$; Attenuation - 8×10^{-12} .

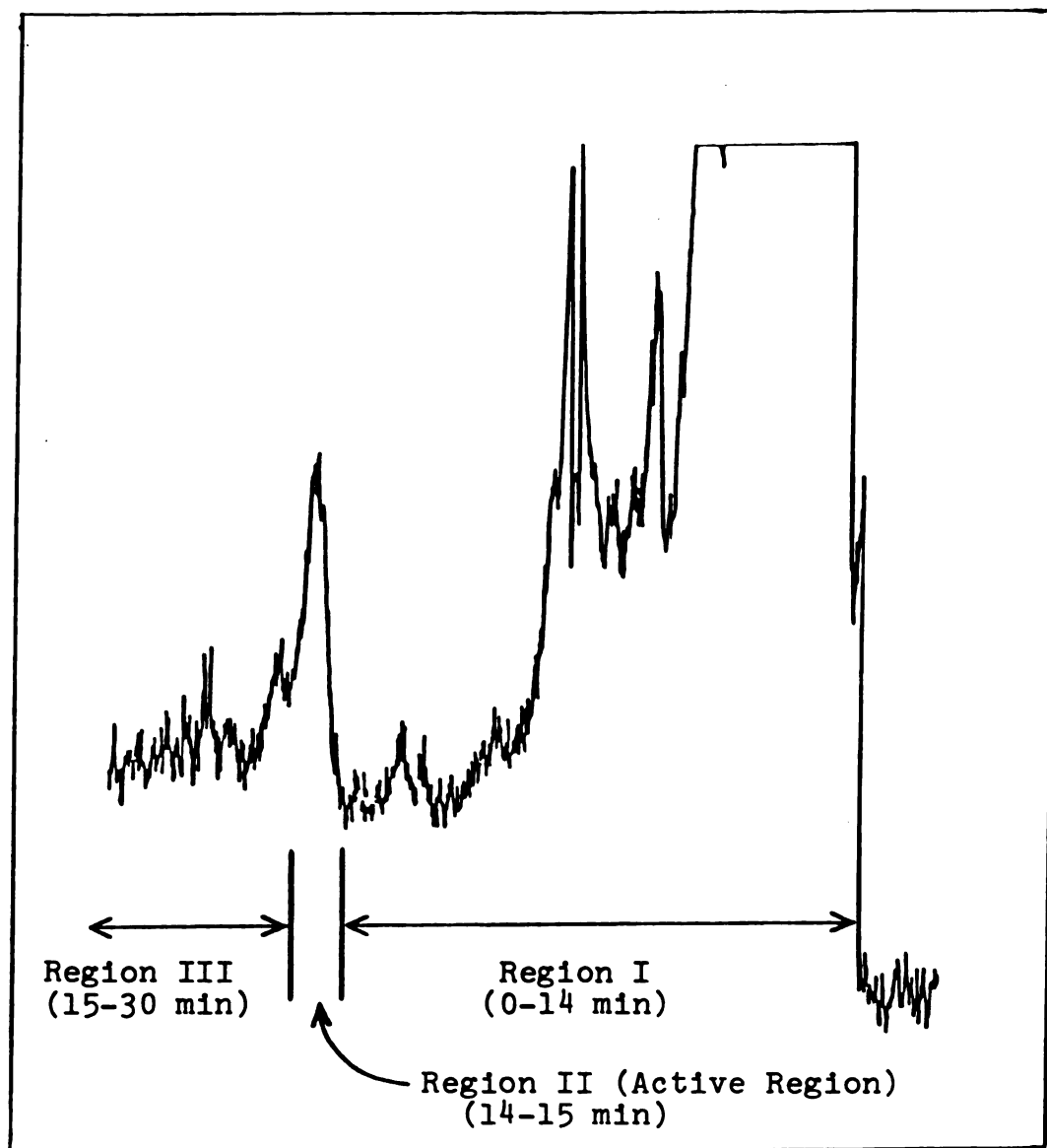


Figure 2. Regions of 3% OV-1 chromatogram; region of activity.

recorded in Table 2. From these results, Region II containing a single peak appears to be active.

Quantitative Analysis of GC Isolation

The amount of active component in the 500 FE extract was determined to be ca. 2 ng by comparison of active peak area with that of the external standard, tridecane. Comparison of the 500 FE extract chromatogram with that of a 2 ng injection of tridecane under similar conditions (Figure 3) shows that peak areas are similar. This information indicates that the isolation procedure yielded about 4 pg of active component per insect extracted.

Characterization of Active Component

Concentrates from the three reaction mixtures and the control were injected on to OV-1, and the eluent from each trapped at the retention time of the active component (T_R = 14-15 min at 100°C). These four samples were then tested for biological activity (Table 3). These tests indicate the presence of unsaturation and no evidence of carbonyl or alcohol functional groups.

Table 2. Biological Activity of OV-1 Fractions.

Fraction	Retention Time	Percent Wing-Fanning	Percent Orientation	N
Region I	0-14 min	0	17.4	29
Region II	14-15	78.1	16.7	32
Region III	15-30	0	0	32

N = number tested.

Column Temperature = 100°C.

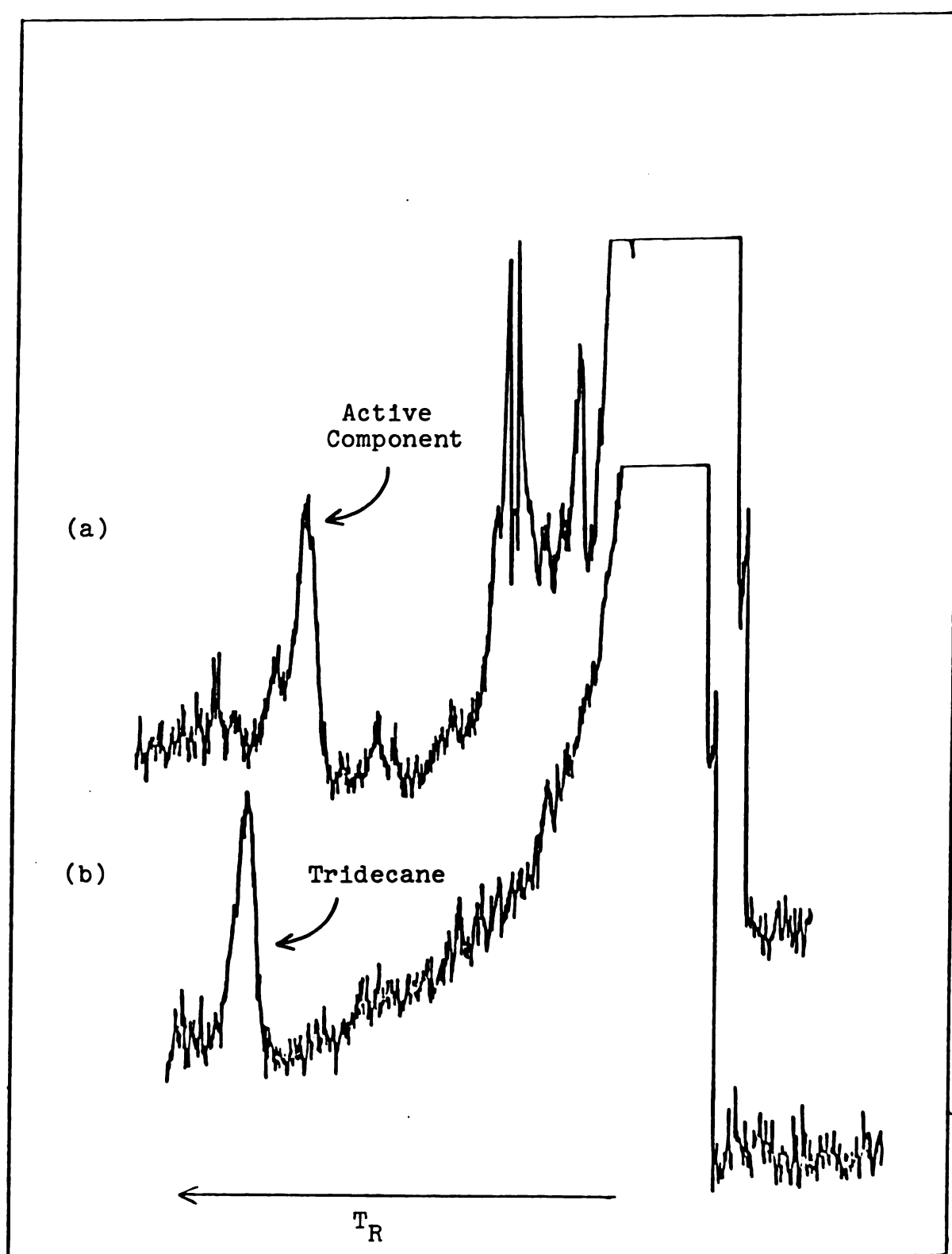


Figure 3. Quantification of active component. Curve (a) 500 FE active fraction from XF-1150 (peak area=139.5); (b) 2 ng tridecane external standard (peak area=138.7).

Table 3. Biological Activity of Eluent from Reaction Mixtures of Characterization Tests.

Test	Percent Wing-Fanning	Percent Orientation	N
Blank	71.4	16.7	21
LAH Reduction	54.5	10.5	22
Acetylation	65.0	7.1	20
Microhydrogenation	0	0	23

N = Number tested.

All eluents were trapped at $T_R = 14-15$ min from OV-1 at 100°C .

GC-Mass Spectrometry of Active Component

GC-MS of a fraction which contained 20 ng of the active component yielded a weak signal at $m/e = 192$, and a moderate signal at $m/e = 177$ (Figure 4) when the active component was ionized. No signals were observed at higher m/e ratios while signals at m/e ratios lower than this range were rendered useless due to background interference. However, the two signals are potentially useful, as signals which differ by 15 mass units are usually due to the molecular ion (M^+) and the ion resulting from the loss of a methyl fragment ($M-15$).

Figure 5 shows the chromatogram from the GC-MS. Points of ionization for background and active component mass spectra are labelled.

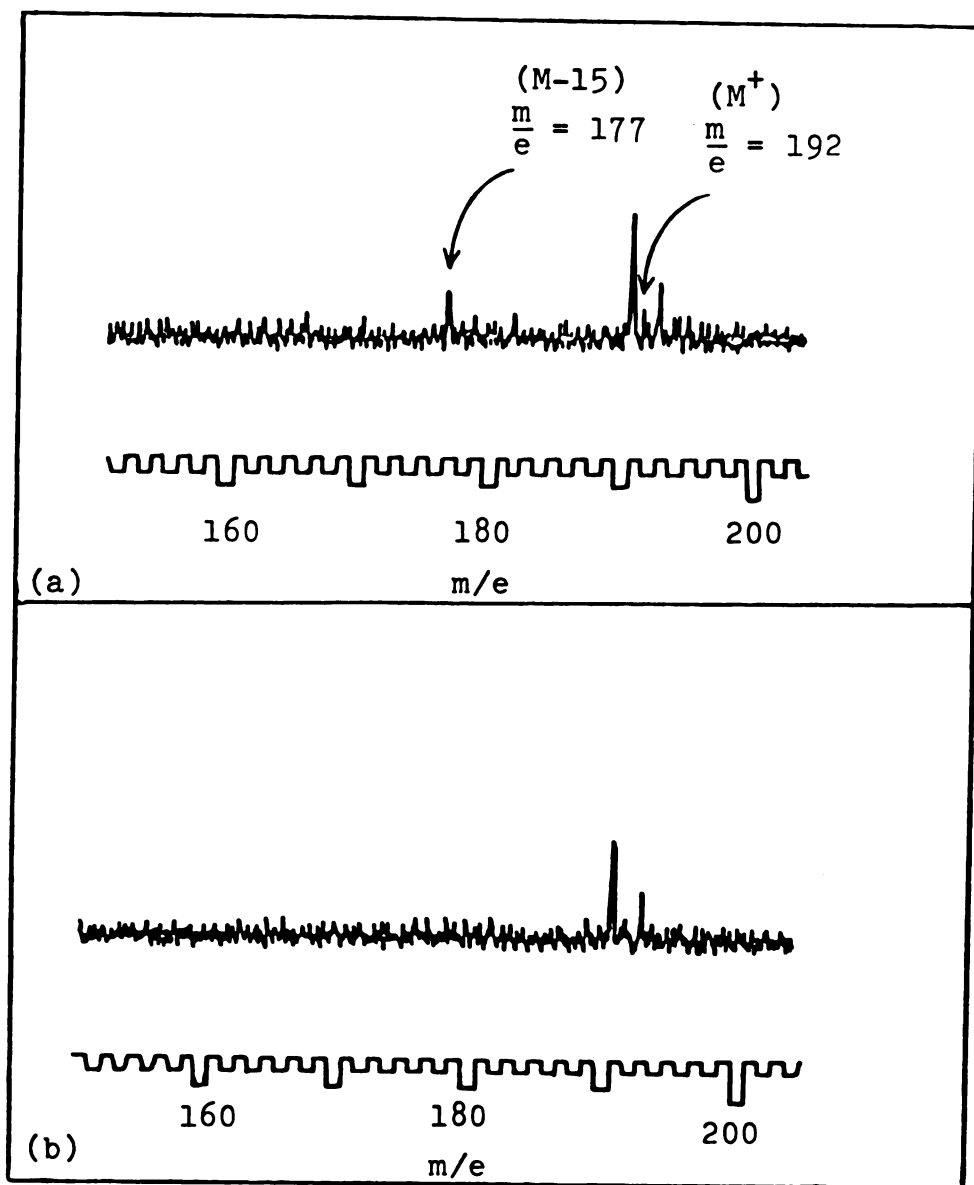


Figure 4. (a) Mass spectrum of active component, 20 ng sample.
(b) Background (mass spectrum of solvent).

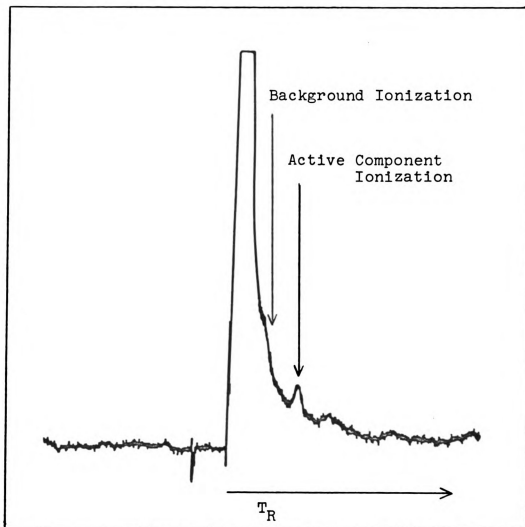


Figure 5. Chromatogram from GC-MS. 5 μ l injection of active fraction from 10% XF-1150, 5000 FE extract, in CS_2 . GC-MS column: 3% OV-1 on 100-120 mesh Gas Chrom Q. Column Temperature: 125°C programmed at 4°C/min to 150°C.

DISCUSSION

The results from Table II and Figure 1 indicate that the Bradysia impatiens sex pheromone has been isolated as a single peak in the OV-1 chromatogram. No evidence was present, such as multiple areas of activity on either column, which would suggest that this peak contains more than one component.

The strategy of injecting whole extracts onto the OV-1 column, and analyzing the resulting active fraction was in effect a "clean-up" procedure. This initial fractionation allows active material to be separated from biologically inactive nonpolar components which elute with the solvent. The reinjection of the remaining active fraction onto the OV-1 column similarly allows separation from inactive polar components.

This gas chromatography clean-up method is preferential to a standard column chromatography clean-up procedure in that minimal amounts of solvent are used. While solvent quantities on the order of milliliters are normally needed to elute the various fractions from a standard packed column, only 10-20 μ l of solvent were required to rinse the trapped eluent from the capillary tubes. The latter method decreases interference from solvent impurities

which become increasingly important when dealing with extremely small amounts of sample.

The use of GC-MS precludes the necessity of actually trapping the isolated active peak in the OV-1 chromatogram of the XF-1150 active fraction. However, if quantities of essentially pure active component were desired, they could be secured by either trapping the eluent at the proper retention time or by employing a GC splitter.

The limited mass spectral information obtained with 20 ng of active component necessitates a larger sample to be used in further GC-MS investigations. However, the signals at $m/e = 192$ and $m/e = 177$ are probably due to M^+ and $M-15$ ions. A molecular weight of 192 is indicative of a C-14 hydrocarbon with three degrees of unsaturation. This information coupled with the evidence of alkene functionality encountered in the characterization tests indicates that the active component is a C-14 hydrocarbon with at least one double bond and two other degrees of unsaturation, such as additional double bonds or rings.

Care must be taken when interpreting the results of the characterization tests, however. While a lack of biological response is evidence for a functionality, a continued response does not necessarily indicate the lack of that functionality. If the latter assumption is falsely made, cases are ignored where reactions are proceeding, but not to completion. One way around this problem would be to dilute the reaction mixture to a threshold concentration

and compare with the threshold concentration of the active starting material. Any significant deviations would indicate a partial reaction.

The quantification of active material was based on the assumption that the detector response would be equivalent for both the C-13 external standard and the active component. This is a valid assumption when using a flame ionization detector if weight rather than mole quantities are compared.

In conclusion, evidence has been presented which indicates that the female produced sex pheromone of Bradysia impatiens has been isolated as a single component. Mass spectroscopy coupled with functional group characterization tests indicate that this component may be a C-14 hydrocarbon with three degrees of unsaturation including at least one double bond. Further investigations using larger quantities of material will be required to identify the exact structure of this compound.

BIBLIOGRAPHY

BIBLIOGRAPHY

- Alberts, S. A., Kennedy, M. K., and Carde, R. T., 1981. Pheromone-Mediated Anemotactic Flight and Mating Behavior of the Sciarid Fly Bradysia impatiens, Environ. Entomol.
- Carlson, D. A., Mayer, M. S., Silhacek, D. L., James, J. D., Beroza, M., and Bierl, B. A., 1971. Sex Attractant Pheromone of the Housefly: Isolation, Identification, and Synthesis. Science 174:76.
- Fletcher, B. S. 1977. Behavioral Responses of Diptera to Pheromones, Allomones, and Kairomones. In: Chemical Control of Insect Behavior, Eds. H. S. Shorey and J. J. McKelvey, Jr. Wiley Interscience.
- Kennedy, M. K. 1973. A Culture Method for Bradysia impatiens (Diptera: Sciaridae). Ann. Entomol. Soc. Am. 66:1163.
- Kostelc, J. G., Girard, J. E., Hendry, L. B. 1980. Isolation and Identification of a Sex Attractant of a Mushroom - Infesting Sciarid Fly. J. Chem. Ecol. 6:1.
- Uebel, C., Sonnet, P. E., Miller, R. W., and Beroza, M., 1974. Sex Pheromone of the Face Fly, Musca autumnalis (DeGeer). J. Chem. Ecol., 1:195.
- Uebel, E. C., Sonnet, P. E., Bierl, B. A., Miller, R. W., 1975. Sex Pheromone of the Stable Fly: Isolation and Preliminary Identification of Compounds That Induce Mating Strike Behavior. J. Chem. Ecol., 1:377.
- Uebel, E. C., Meyer, Schwarz, Menzer, R. E., and Miller, R. W., 1978. Mating Stimulant Pheromone and Cuticular Lipid Constituents of Fannia pusio (Wiedemann), J. Chem. Ecol. 4:73.

MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 02842 0721