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ISOLATION AND IDENTIFICATION OF HOUSE FLY, *Musca domestica* L., REPELLENTS IN THE PEPPER TREE, *Schinus molle* L.

by

Priyantha Dharshana Chandanakumara Wimalaratne

B.Sc. (Hons.), University of Kelaniya, Kelaniya, Sri Lanka, 1985

A THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
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Examining Committee:

Chair: Dr. D. Sutton

Dr. K.N. Slessor (Professor)
Senior Supervisor

Dr. J.H. Borden (Professor), Biological Sciences
Committee Member

Dr. S. Holdcroft (Assistant Professor)
Committee Member

Internal Examiner: Dr. A. Bennet (Assistant Professor)

Date Approved: July 27, 1993

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ABSTRACT

Foliage from the "pepper" tree, *Schinus molle* L., is traditionally used in Ethiopia to "repel" house flies, *Musca domestica* L. The volatile extracts of pepper tree leaves and berries had repellent and deterrent activity against house flies in a two-choice laboratory bioassay. Steam distillation and solvent extraction were efficient methods for extracting the active material from leaves and berries, the former providing the volatile oils in a 1% yield. Fractionation of steam-distilled volatiles with two different fractionation schemes monitored by laboratory bioassays demonstrated that activity is associated with two compounds. Mass spectral data indicated that both compounds have the molecular composition $C_{10}H_{18}O$ and were probably alcohols. The two compounds were identified as *cis*-menth-2-en-1-ol(**A**) and *trans*-piperitol(**B**). The absolute configuration of compound **B** was established as (*1S,6S*)-piperitol by comparison of acetyl lactate derivatives. Racemic **A** and **B**, were synthesized from piperitone and bioassayed with house flies. These results indicate that compound **B** is the major active house fly repellent in the pepper tree.

To

my mother

and

in memory of my father

"God in his wisdom made the fly
And then forgot to tell us why..."

- C&EN, April 12, 1993 -

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The skilled technical assistance of Mr. Greg Owen and Mrs. Marcelline Tracey is gratefully acknowledged.

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LIST OF ABBREVIATIONS

AclacCl	acetyl lactyl chloride
Ac_2O	acetic anhydride
Al_2O_3	aluminum oxide
BF_3	boron trifluoride
C	carbon
CH_2Cl_2	dichloromethane
EI-MS	electron impact ionization mass spectra
GC	gas-liquid chromatography
gh	gram-hours
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
IR	infrared
$LiAlH_4$	lithium aluminum hydride
LC	low pressure silica gel column chromatography
MS	mass spectroscopy
$NaBH_4$	sodium borohydride
NaCl	sodium chloride
$NaHCO_3$	sodium hydrogen carbonate
Na_2SO_4	sodium sulfate

NMR	nuclear magnetic resonance
PCC	pyridinium chlorochromate
Pd	palladium
rt	room temperature
UV	ultraviolet

1. INTRODUCTION

Feeding deterrent-based insect pest control is an exciting branch of semiochemical-based pest management. Deterrence is the oldest and, for many centuries, the most widely used method of insect pest control[1,2]. Studies have been carried out to identify, synthesize and apply insect feeding deterrents. A feeding deterrent is defined as a substance that elicits an avoidance reaction for feeding and oviposition of insects[1,3,4,5]. According to the impact of the action on insect, feeding deterrents can be further divided as repellents, suppressants, deterrents, antibiotics and anorexigenics[5]. Repellents are the substances which cause an insect to make oriented movements away from its source[5,6]. It alters the behavior of an insect pest. Safe, natural repellents are the method of choice for control of insect pest populations while maintaining environmental integrity. Effective use of repellents has been demonstrated by ethnic groups in different parts of the world. Most of these methods involve material from naturally-occurring sources[1,2,7,8,9] . Identification of such compounds could provide valuable assistance for refined semiochemical-based pest management programs.

The present study describes the isolation and identification of a house fly repellent from a tree used in traditional fly control in Ethiopia.

1.1. The house fly, *Musca domestica* L.

The house fly, *Musca domestica* L. (Diptera, Muscidae), is a major domestic insect pest, particularly in tropical countries. Since house flies exhibit scavenger type feeding behavior, they act as mechanical vectors of pathogens (viruses, bacteria, protozoa and helminth eggs) to mankind and livestock[8,10]. These pathogenic transmissions from the house fly cause many contagious diseases[8,10,11].

Since ancient times, several methods have been applied to control house fly populations. Before chemical pesticides were developed, people used physical methods: fly swatters, feather dusters, fans and horse tails to control house flies[1,2]; these methods are still used in many tropical and sub-tropical countries. The application of synthetic pesticides to control house flies is a recent development[12,13]. However, the development of resistant fly populations and the poorly managed domestic usage of toxic chemicals has already made this management method unsatisfactory[11,14]. Recently, several methods have been developed to control fly populations, such as pheromone baited traps[15-17], chemosterilization of adult flies[8,18], control of larval development by juvenile hormone analogues[11,19] and application of some repellents[5]. However, these have proven insufficient to control serious house fly infestations. In the search for new control tactics, it is thus appropriate to investigate traditional methodologies and to determine the scientific basis for their action.

1.2. The pepper tree, *Schinus molle* L.

The Pepper tree, *Schinus molle* L. (California or Australian pepper tree, Peruvian or American mastic tree) (Anacardiaceae), is native to the Peruvian Andes[20] and is widely grown in tropical and sub tropical countries[21]. The tree grows 6-10 m high with a 60-90 cm thick trunk. The foliage consists of 12-20 cm long, pinnate, feathery, green leaves. The flowers are yellow-white and develop into red fruits (drupes)[22].

Fruit from this tree yields a volatile oil that has been used as a substitute for black pepper, in flavor compositions, and in pharmaceutical products[24]. In Greece, Mexico and Peru, the fruit serves for the preparation of beverages, and its bark has been used for tanning skins[22].

Foliage of the pepper tree is reported to be a traditional source of repellents for house flies in Ethiopia[23]. Some rural people drape branches and leaves over their heads to repel house flies. Pepper tree leaves are spread on dining tables, in slaughter houses and meat processing areas.

A number of investigators have examined the chemical constituents of pepper tree and reported the complete and tentative identification of several compounds[24-30]. The structures of a wide variety of natural products have been elucidated. None of these compounds has been reported as a repellent for house flies. However, preliminary field experiments in Ethiopia indicated that pepper tree leaves and berries, and extracts these of were effective in deterring landings and sustained contact by house flies on attractive food stuffs (Appendix 1).

1.3. Objective

On the basis of Ethiopian tradition, I hypothesized that volatile chemical house fly repellents are present in the pepper tree and that their identification might possibly lead to a semiochemical-based pest management program for the control of house flies. Therefore, my objective was to isolate, identify and synthesize the active repellents constituents in the pepper tree.

2. EXTRACTION PROCEDURES AND RESULTS

2.1. Source of active material

Fresh pepper tree berries were obtained from the Ethiopian Institute for Agricultural Research, Addis Ababa. Each berry was separated from the bunch, placed in 95% ethanol in a glass jar (400 mL) with a screw cap, and shipped to Canada. The treated berries were stored in a refrigerator at 4°C, until required.

Fresh pepper tree leaves were obtained from 1-2 years-old trees grown in a greenhouse at Simon Fraser University. Shoots with matured leaves were separated intact from the trees, and were stored in sealed polythene bags at -20°C to prevent microbial growth and the thermal loss of volatile compounds.

2.2. Bioassay

A two-choice laboratory bioassay was developed to test the behavioral activity of volatile extracts from the pepper tree against house flies‡. The method consisted of visually observing the behavioral responses of flies to a bait station treated with volatile extracts and comparing it to the responses

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‡ Method developed by Ms. L.J. Chong, Department of Biological Sciences, SFU.

towards a solvent-treated control station.

Wooden cages (16 × 16 × 13 cm) were used as bioassay chambers. The back wall of each cage was a fine plastic screen. The front wall was a transparent plexiglass sheet with central hole (2 cm diameter), through which insects were released into the cage, and which was then closed with a cork. The plastic sheet could also be moved vertically, which allowed the assayist to place baits and water vials in the cage and remove dead flies. A water vial with a cotton wick was kept inside the cage to maintain constant relative humidity, and to serve as a source of drinking water for the flies.

Glass cover slips (2.2 × 2.2 cm) served as bait stations. Prior to bioassays the center of each cover slip was treated with a 0.05 mL drop of sucrose solution (sucrose:water-1:1, w/v); heating to dryness at 60-70°C in an oven left a sugar coating on the glass. The oven was pre-heated at ~150°C for 1 h and the door was left open for a few minutes to exhaust any volatile contaminants before introducing the cover slips. Dried bait stations were stored in plastic boxes until needed.

Adult house flies were obtained from the insectary at SFU‡. For each bioassay replicate, 20 laboratory-reared, randomly selected flies (mixed sex and age) were released into a cage and held without food for 1 h. Two bait stations were treated, one with 25 µL of a test extract, and the other with 25 µL of solvent. After evaporation of the solvent, both bait stations were

---

‡ Cultures maintained by Mr. A. Syed, Insectary, Department of Biological Sciences, SFU.

placed 10 cm apart on the floor of the cage. The number of sustained fly contacts ≥ 10 s on both bait stations were counted for 10 min. Each treatment was assayed with five groups of 20 flies each conducted at room temperature and humidity.

As the fractionation process proceeded, the gradual gain in purity of extracts resulted in loss of bioactivity after 6 min, presumably because active compounds evaporated faster in the absence of the less volatile substances accompanying them in the crude extract. Therefore, the duration of each replicate was shortened to 6 min.

Percent responses (pooled data for the five groups of flies) to experimental stimuli were compared to those to crude extract control stimuli by a z test ($\alpha = 0.05$)[31‡].

2.3. Methods of extraction

Volatiles can be collected from solid biological materials by solvent extraction[32] , vapor entrapment[39-42] or steam distillation[32]. Many plant volatiles are unstable and thus, the isolation procedure and subsequent treatments of the essential oils can influence their composition and may easily generate artifacts[32-34]. Therefore, all three extraction methods were examined.

---

‡ Statistical analyses done by Mr. S.G. Banneheka, Department of Mathematics & Statistics, SFU.

Solvent extraction involved extraction of fresh pepper berries with 95% ethanol which can extract volatiles efficiently[35-38]. Fresh berries (40 g) were crushed to a fine slurry in a mechanical blender and with 95% ethanol (25 mL). After settling for 4 min, the top liquid layer was decanted and filtered through a Hirsch funnel (100 mL) with a glass wool plug, into an Erlenmeyer flask (250 mL). The blending and filtering process was repeated with two further 25 mL portions of ethanol and the three filtrates were combined. The funnel and filter were washed with ethanol (15 mL) and the subsequent rinse was added to the extract which was centrifuged on a Jouan BR 3.11 centrifuge at 630 × g. The final extract volume was adjusted to 100 mL (0.4 g equivalents of berries/mL) and was then stored at 4°C in a clean glass bottle (~125 mL) with a Teflon-lined cap.

Vapor entrapment experiments employed Porapak Q entrapment methodology used for capturing insect pheromones[39-41]. Porapak Q was conditioned by extraction with anhydrous, reagent grade ether in a Soxhlet extractor for 15 h and, after evaporation of residual ether, was stored in a glass stoppered bottle in the dark. Pepper tree leaf volatiles were captured on Porapak Q as described by Verigin[43] and Wong[44]. Pepper tree leaves (186 g) were placed in a two-piece Nalgene aeration chamber (15.5 cm I.D. × 27 cm) fitted with a 1.5 cm wide ground glass flange about 9 cm from the top. A Porapak Q-filled glass tube trap and an activated charcoal-filled glass tube trap (both traps 2.4 cm O.D. × 12 cm) were fitted to the bottom and top of the chamber, respectively. Each trap contained ~ 25 g of adsorbent sealed by a coarse sintered glass disc at the bottom. The charcoal-filled trap served as an

air scrubber. Air was drawn at 2 L/min for 72 h through a water trap and the scrubber, over the leaves and finally through the Porapak Q trap by means of a water aspirator fitted to the Porapak Q outlet. This aeration yielded 1.34×10^4 gram-hours (gh) of volatiles, based on the weight of the leaves used and duration of aeration.

The Porapak Q from aeration was extracted with pentane (fractionally distilled, Caledon) in a Soxhlet extractor for 6 h. Extracts were concentrated to ~ 2 mL by distillation of the pentane through a glass Dufton column (30 cm). Extracts were made up to 5.5 mL and stored at 4°C.

For steam distillation, pepper tree leaf volatiles were extracted into pentane using a Likens-Nickerson simultaneous distillation-solvent extraction device[45], that concentrates volatiles many thousand fold in a one step isolation from aqueous media[32,34]. Pepper tree leaves (400 g) were blended with distilled water (1500 mL) until a pulp was formed. Because the essential oils in plants are located in specialized structures such as glandular hairs on the epidermis, oil tubes in the pericarp or isolated oil cells, macerating tissue often increases the efficiency of extraction[32]. Two batches of macerated pulp were extracted in the Likens-Nickerson device 6 h and the extracts combined.

The pentane extract was dried over anhydrous Na_2SO_4 and filtered. The filtrate was concentrated by distillation of the pentane through a Dufton column, and the residual solvents were removed by aspiration with nitrogen. Crude oils were transferred into a glass vial with a Teflon-lined cap and stored at 4°C until used.

2.4. Bioassay of extracted volatiles

Dose response bioassays were performed for the three different extracts (Table 2.1) to determine the minimum quantity required to elicit repellency for house flies and to indicate the potency of the extracts and efficiency of the extraction.

2.5. Result and discussion

Maximal activity in 10 min bioassays was obtained with 10 mg equivalents of ethanolic berry extracts, 50 gh of Porapak Q-captured pepper tree leaf volatiles, and 0.3 mg equivalents of steam-distilled oils (Table 2.1).

The results presented in Table 2.1 demonstrate that the two-choice bioassay is suitable for evaluation of the repellency of pepper tree compounds against house flies.

Both leaf-derived extracts were significantly repellent to house flies at most concentrations. The threshold concentration of 50 gh for the Porapak Q entrapped volatile extracts indicates that a relatively large amount of leaves is required to elicit a response compared to the steam distilled leaf volatiles. This indicates that steam distillation is more efficient than the Porapak Q entrapment in collecting the active compounds. During aeration active component(s) were apparently only partially collected, but the Porapak Q extracts were free of non-volatile waxes and lipids, which complicate analysis and which may retard vaporization of the active component(s) during the

Table 2.1: Dose response relationships for house flies in laboratory bioassays to pepper tree volatiles extracted by solvent extraction, vapor entrapment and steam distillation.

Ethanolic extracts of pepper tree berries				Pentane extracts of pepper tree leaf volatiles trapped on Porapak Q				Pentane solution of pepper tree leaf volatile oils extracted by steam distillation			
Dose mg/25µL	Bait	Number of house fly contacts during 10 min	% Response to control station	Dose gh/25µL	Bait	Number of house fly contacts during 10 min	% Response to control station	Dose mg/25µL	Bait	Number of house fly contacts during 10 min	% Response to control station
0.001mg	C	12	36 ***	5 gh	C	33	76 ***	0.05 mg	C	57	58 ***
	S	21			S	11			S	41	
0.01 mg	C	33	54 ***	12.5 gh	C	46	88 *	0.1 mg	C	51	84 **
	S	28			S	6			S	10	
0.1 mg	C	26	76 ***	25 gh	C	42	89	0.2 mg	C	69	82 ***
	S	8			S	5			S	15	
1.0 mg	C	33	92 *	37.5 gh	C	47	94	0.3 mg	C	50	100
	S	3			S	3			S	0	
10 mg	C	52	100	50 gh	C	43	98	1 mg	C	8	100
	S	0			S	1			S	0	

C - Control
S - Bait, treated with extract
% Response to control station = [Total C/(Total C + Total S)] × 100

Percentages different from those to the crude extract control stimulus (those to the highest dose stimulus) are indicated by *, z test, $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$

bioassay. However, steam distillation of leaves provided the volatile oils in 1% yield as a colorless liquid with good repellent activity, and provided better information about the amounts of repelling components present in the source than did vapor entrapment. Therefore, steam distillation was used for the repellent isolation project. Solvent extraction of berries was discontinued because of a lack of berries.

3. ISOLATION OF REPELLENTS

3.1. Analytical gas-liquid chromatography

Steam-distilled volatile extracts of pepper tree leaves were analyzed directly by Gas-Liquid Chromatography (GC) (Table 3.1, Figure 3.1). Analysis was performed with a DB-1 fused-silica capillary column. Comparison of retention times for isolated active components and authentic samples was performed by using both DB-1 and DB-23 fused-silica capillary columns. Three compounds dominated the total extracts, and comprised about 75% of the total oil content. Two regions with large numbers of compounds corresponded to regions for mono- and sesquiterpenes, with monoterpenes being the most abundant.

3.2. Fractionation of repellent extracts

Fractionation of the steam-distilled volatile extracts of pepper tree leaves was conducted using both low pressure silica gel column chromatography (LC)[46] and high performance liquid chromatography (HPLC). In preliminary work active fractions obtained by column chromatography were used for successive HPLC fractionation (Scheme 3.1). Later, crude steam-distilled volatile extracts of pepper leaves were fractionated directly by HPLC (Scheme 3.2).

Table 3.1: Experimental conditions for analytical gas-liquid chromatography.

Analysis method	Instrument	COLUMN					Detector	Detector temp: °C	Injector temp: °C	He flow rate (cm/min)	Oven temperature program
		Type	Liquid phase	Length (m)	Diameter (I.D)(mm)	Construction material					
GC-1	Hewlett-Packard 5880A	Capillary	DB-1	30	0.25	Fused-silica	FID	250	225	25 at rt	2 min at 80°C, 10°C/min to 210°C, 5 min at 210°C
GC-2	Hewlett-Packard 5890A	Capillary	DB-23	30	0.32	Fused-silica	FID	240	220	25 at rt	2 min at 80°C, 10°C/min to 210°C, 5 min at 210°C

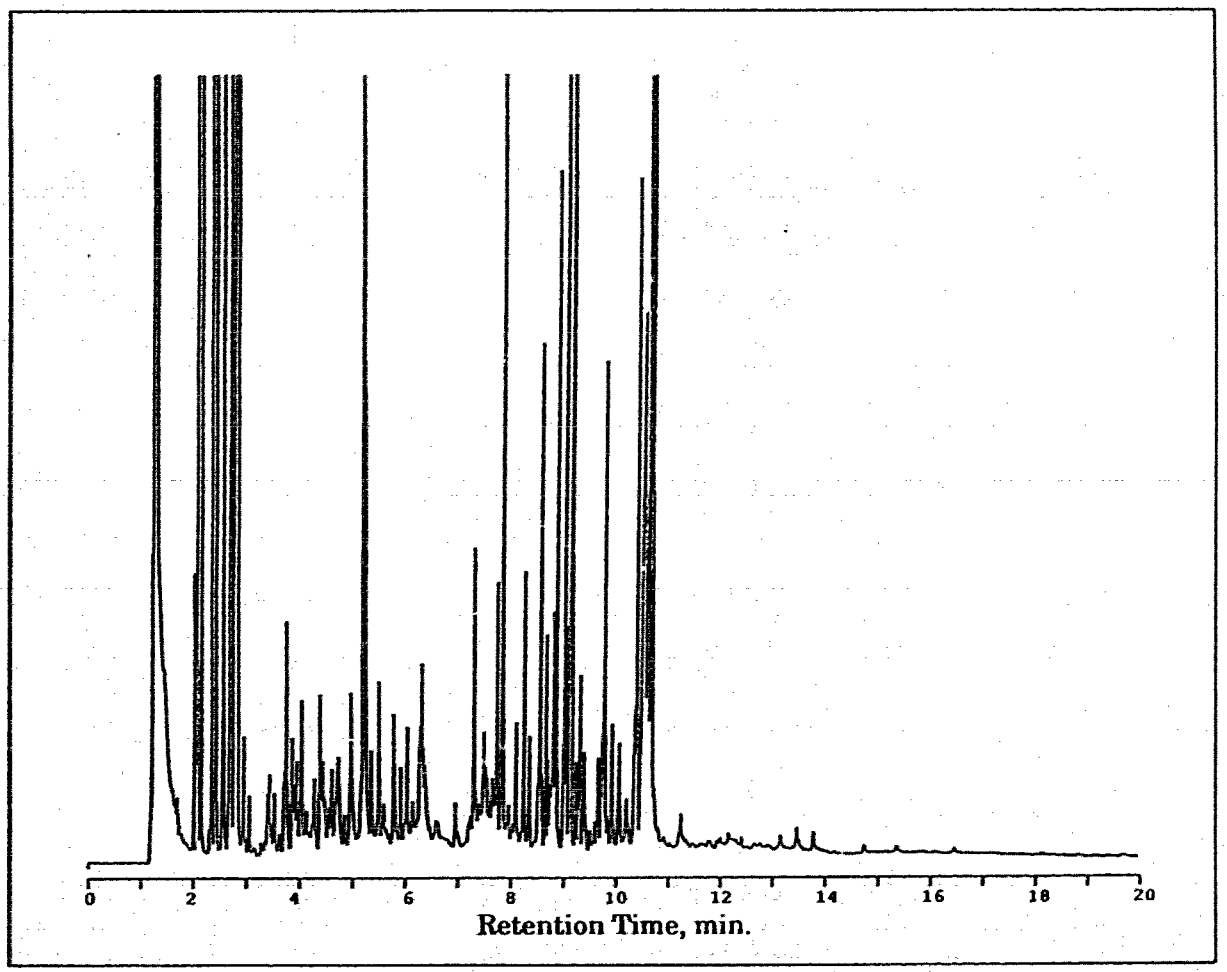
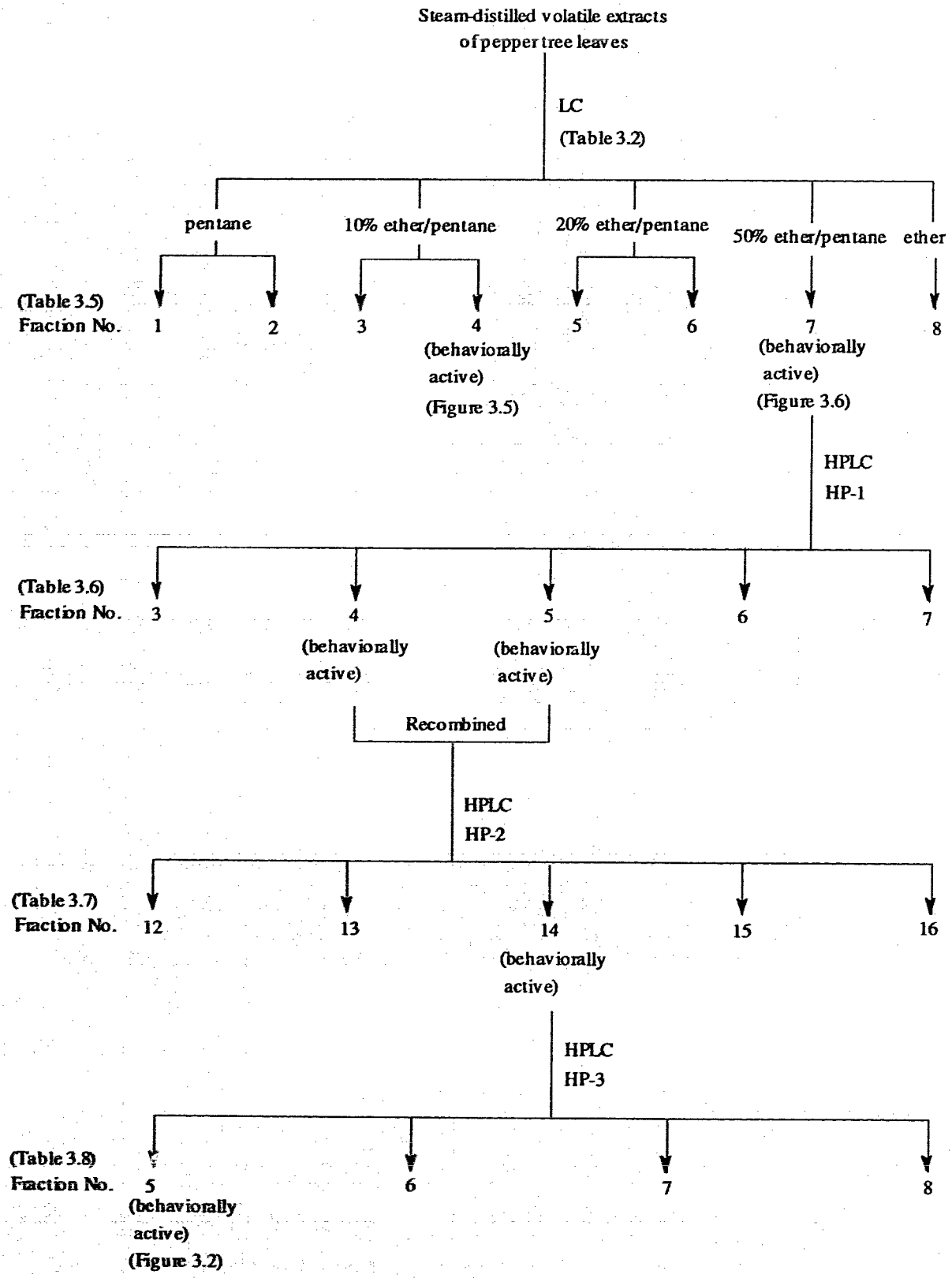
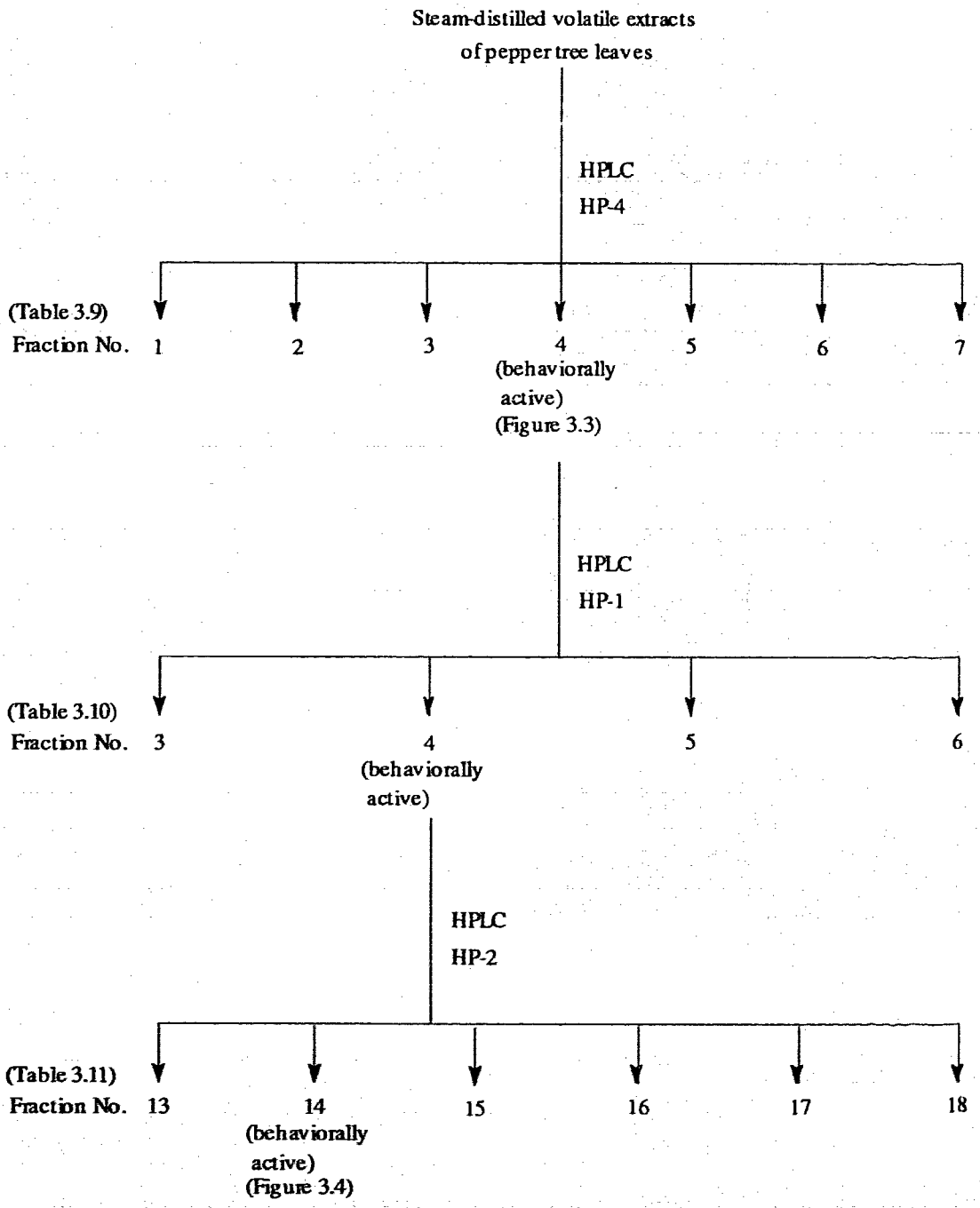


Figure 3.1: GC chromatogram (GC-1) of the pentane extract of steam-distilled volatile extracts of pepper tree leaves.

Scheme 3.1: Preliminary fractionation of steam-distilled volatile extracts of pepper tree leaves.



Scheme 3.2: Direct HPLC fractionation of steam-distilled volatile extracts of pepper tree leaves.



3.2.1. Column chromatography

LC fractionation was performed using a Pyrex glass column (100 cm long, 3.8 cm I.D.) containing silica gel (G-60, 250 g, 0.04-0.063 mm particle size, 230-400 mesh) as the stationary phase. The column was further tightly packed by vibrating with a vortex mixer.

Distilled pentane and diethyl ether were used for the column elution. The column was pre-equilibrated with a measured amount of solvent pentane. When the remaining solvent level reached about 0.5 cm above the silica gel bed column, elution was stopped; the volume difference between the initial solvent volume and the volume eluted was about 375 mL and this volume was taken as the column bed volume.

The sample (2.5 g of crude oil) was applied with the column running. Several portions of pentane were applied to the column to complete the application.

Fractionation of steam-distilled leaf volatiles was performed in five steps with different solvent compositions of pentane and diethyl ether (Table 3.2 and Scheme 3.1). Before starting to collect the fractions, a column bed volume equivalent volume of eluent was discarded. Fractions were collected and analyzed by GC (GC-1 in Table 3.1) as well as being bioassayed on house flies.

Table 3.2: LC fractionation of steam-distilled volatile extracts of pepper tree leaves.

Step No.	Solvent composition pentane : Et_2O	Volume of solvent used (mL)	Fraction No.	Volume of fractions produced (mL)
1	100 : 0	1000	1	500
			2	500
2	90 : 10	1000	3	500
			4	500
3	80 : 20	1000	5	500
			6	500
4	50 : 50	500	7	500
5	0 : 100	500	8	500

3.2.2. High performance liquid chromatography

HPLC was performed on a Waters LC625 liquid chromatograph with a Waters 486 variable wavelength UV-Visible Detector system. Fractions were collected according to run time, and fraction volumes were determined by comparison of solvent flow rate with run time throughout the analysis.

HPLC grade solvents were used and diethyl ether and water were laboratory distilled. All solvents were filtered by 0.45 μm sieve filters under vacuum, in order to ensure that they were particle free, and operating solvent reservoirs were purged with helium.

Neat volatile samples were prepared by weighing and dilution. Amounts of volatiles in diluted samples were estimated as a relative weight by correlating their concentrations and volumes with the original samples. Solvents used for the sample dilution were similar to the initial solvent composition in HPLC analysis program. The diluted samples were concentrated by evaporation under a stream of N_2 with cooling, prior to analysis.

Sample injection and collection of fractions were done manually. Glass vials with Teflon-lined screw caps were used to collect fractions. When fractionation of the same sample was repeated several times, fractions that eluted at the same run time were collected in the same vial.

Fractionation of extracts was performed with three reverse and normal phase columns (Table 3.3). The void volume of each column was measured prior to each experiment. Void volume of the column is expressed as the

Table 3.3: Columns used for HPLC fractionation of steam-distilled volatile extracts of pepper tree leaves.

Column	Construction material	Type	Stationary phase	Length (mm)	Diameter (I.D.)(mm)	Void volume (mL)
Nova pak™silica	Steel	Normal phase	Amorphous silica, 4μm	150	3.9	~1.15
Nova pak™C_{18}	Steel	Reverse phase	Dimethyloctadecylsilyl bonded amorphous silica, 4μm	300	3.9	~2.23
Nova pak™CN-HP	Steel	Reverse and Normal phase	Dimethylcyanopropylsilyl bonded amorphous silica, 4μm	75	3.9	~0.56

amount of eluent required to elute a sample component which does not interact with the stationary phase.

The behaviorally active fractions obtained from silica gel column chromatography were fractionated by HPLC (Table 3.4) on a reverse phase Nova pak™C_{18} column (HP-1, Scheme 3.1). Fractions were extracted into pentane, analyzed by GC (GC-1) and bioassayed on house flies. Behaviorally active fractions were recombined (Scheme 3.1) and re-fractionated by HPLC on a Nova pak™ CN-HP column according to HP-2. Fractions were analyzed directly by GC and bioassayed. The active fraction was re-fractionated by HPLC (HP-3) and bioassayed.

The crude steam-distilled extract was then subjected to a slightly different fractionation (Scheme 3.2) to verify the results obtained in the first fractionation. Initially, the crude extract of pepper tree leaves was fractionated directly by HPLC using a Nova pak™ silica column (HP-4), a non-linear step-gradient program. Prepared fractions were subjected to GC analysis and bioassayed on house flies. The active material was re-fractionated (Scheme 3.2) by HP-1 and HP-2, respectively. Again, each fraction was analyzed by GC and bioassayed on house flies.

Table 3.4: Experimental conditions for HPLC fractionation of steam-distilled volatile extracts of pepper tree leaves.

Analysis method	Column	Solvent	Flow rate (mL/min)	Detector wave length (nm)	Program	Volume of each fraction (mL)	Number of fractions collected
HP-1	Nova pak™ -C_{18}	H_2O/MeCN	1	280	15% for 30 min (isocratic)	1	10
HP-2	Nova pak™ -CN-HP	Et_2O/hexane	0.5	220	1% at 2 min, 1% to 11% from 2-20 min, 11% to 31 min, 11% to 1% from 31-36 min, 1% from 36-46 min	0.5	20
HP-3	Nova pak™ -CN-HP	Et_2O/hexane	0.5	220	Identical to HP-2	0.125	(Fraction collection started at 11 min) 15

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Table 3.4: (Cont'd)

Analysis method	Column	Solvent	Flow rate (mL/min)	Detector wave length (nm)	Program	Volume of each fraction (mL)	Number of fractions collected
HP-4	Nova pak™ -silica	Et_2O/hexane	1	280	1% at 5 min, 5% from 5-10 min, 10% from 10-15 min, 15% from 15-20 min, 20% from 20-25 min, 30% from 25-30 min, 100% from 30-35 min, 100% to 1% from 35-55 min, 1% from 55-65 min.	5	7
HP-5	Nova pak™ -silica	Et_2O/hexane	1	220	5% for 10 min (isocratic)	0.5	15

3.2.3. Results

Both fractions 4 and 7 (Scheme 1) were repellent and yet eluted with solvents of different polarities (Table 3.5), indicating two or more behaviorally active compounds. Fraction 1, the pentane eluent, contained most of the other compounds, indicating their hydrocarbon, or non-polar, nature. Fractions 2 to 7 had fewer and smaller peaks, indicating that the number and relative amount of polar compounds in the extract was less than the non-polar materials. Fractions 4 and 7 that gave the highest repellency were eluted with 10% and 50% ether/hexane, indicating that the active repellents were of medium to high polarity. Re-fractionation of behaviorally active fraction 7 by HPLC (HP-1), produced five fractions which contained GC detectable compounds; fractions 3 and 4 were behaviorally active (Table 3.6). In addition, both fractions yielded similar GC chromatograms. Both active fractions showed repellence for short period as they were fractionated further, possibly because substances removed in the fractionation had prevented the active components from evaporating. However, in the initial two minute period both fractions were strongly repellent (Table 3.6).

Further HPLC fractionation (HP-2) of recombined fractions 3 and 4 (Table 3.6, Scheme 3.1), produced five fractions, 12-16, with compounds detectable by GC. Fraction 14 showed strong behavioral activity in the initial two minute period (Table 3.7).

Table 3.5: Response of house flies in laboratory bioassays to LC fractions of steam-distilled volatile extracts of pepper tree leaves.

Fraction No.	Solvent composition ether:pentane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
crude	0:100	0.8	C	6	12	10	28	97
			S	0	0	1	1	
1	0:100	0.8	C	13	11	5	29	62 *
			S	3	8	7	18	
2	0:100	0.8	C	21	6	9	36	47 *
			S	27	5	9	41	
3	10:90	0.8	C	5	12	6	33	44 *
			S	23	8	11	42	
4	10:90	0.8	C	22	12	15	49	100
			S	0	0	0	0	

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Table 3.5: (Cont'd)

Fraction No.	Solvent composition ether:pentane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
5	20:80	0.8	C	18	9	8	35	69 *
			S	8	4	4	16	
6	20:80	0.8	C	22	20	19	61	84 *
			S	0	4	8	12	
7	50:50	0.8	C	26	14	12	52	100
			S	0	0	0	0	
8	100:0	0.8	C	14	16	6	36	48 *
			S	17	10	12	39	

C - Control
S - Bait, treated with test sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

Table 3.6: Response of house flies in laboratory bioassays to HPLC fractions obtained from the re-fractionation of behaviorally active LC fraction No 7 in Table 3.5 using HP-1 of Table 3.4.

Fraction No.	Solvent composition Pentane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
crude	100%	0.8	C	6	7	6	19	86
			S	0	0	3	3	
3	100%	0.8	C	7	7	7	21	75
			S	0	2	5	7	
4	100%	0.8	C	14	4	5	23	85
			S	0	2	2	4	
5	100%	0.8	C	14	3	6	23	56 *
			S	9	4	5	18	

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Table 3.6: (Cont'd)

Fraction No.	Solvent composition Pentane	Dose (mg/25 μL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
6	100%	0.8	C	18	5	5	28	48 *
			S	12	10	8	30	
7	100%	0.8	C	12	5	10	27	44 *
			S	11	12	11	34	

C - Control
S - Bait, treated with test sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

Table 3.7: Response of house flies in laboratory bioassays to HPLC fractions obtained from the re-fractionation of recombined behaviorally active HPLC fractions No 3 and 4 in Table 3.6 using HP-2 of Table 3.4.

Fraction No.	Solvent composition ether:hexane	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min						Total contacts	% Response to control station
				0-2 min		2-4 min		4-6 min			
				Number	% Response to control station	Number	% Response to control station	Number	% Response to control station		
crude	0:100	0.8	C	14	100	7	100	1	100	22	100
			S	0		0		0		0	
12	5.75:94.25	0.8	C	15	56 *	4	44 *	2	33 *	21	50 *
			S	12		5		4		21	
13	6.25:93.75	0.8	C	24	52 *	5	45 *	1	20 *	30	48 *
			S	22		6		4		32	
14	6.75:93.25	0.8	C	36	97	12	86	1	14 *	49	84 *
			S	1		2		6		9	

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Table 3.7: (Cont'd)

Fraction No.	Solvent composition ether:hexane	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min						Total contacts	% Response to control station
				0-2 min		2-4 min		4-6 min			
				Number	% Response to control station	Number	% Response to control station	Number	% Response to control station		
15	7.25:92.75	0.8	C	38	74 *	3	75 *	5	50 *	46	71 *
			S	13		1		5		19	
16	7.75:92.25	0.8	C	6	25 *	1	11 *	5	100 *	52	67 *
			S	18		8		0		26	

C - Control
S - Bait, treated with test sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

Finally, re-fractionation (HP-3) of this behaviorally active fraction, provided four fractions, 5-8, with GC detectable peaks (Table 3.8), fraction 5 being behaviorally active. Two major compounds appeared in the GC chromatogram of fraction 5 (Figure 3.2).

In Scheme 3.2, the steam-distilled volatiles of pepper tree leaves were fractionated (HP-4) and the fractions bioassayed. Only the 4th fraction, eluted with 15% ether/hexane, elicited strong repellence against house flies. This observation indicated that the behaviorally active compounds eluted with two different solvents in LC, have eluted in the same fraction in the direct HPLC fractionation. The behaviorally active fraction showed GC detector responses (Figure 3.3) with identical retention times to the major compounds, **A** and **B,** that appeared in the GC traces of the ultimate behaviorally active fraction (Figure 3.2) produced in Scheme 3.1. That neither of them appeared in the GC chromatograms of behaviorally inactive fractions suggested that these were active compounds. Further HPLC fractionation (HP-1) of fraction 4 (Table 3.9; Scheme 3.2) produced four fractions with GC detectable peaks of which the 4th fraction (Table 3.10) had behavioral activity on house flies. These fractionation results are again in agreement with the results from the first fractionation. Further HPLC fractionation of the active fraction 4 (HP-2), yielded six fractions, 13-18, with GC detectable compounds (Table 3.11). Fraction 14 was behaviorally active on house flies and its GC trace showed the same two major compounds, **A** and **B** (Figure 3.4).

Table 3.8: Response of house flies in laboratory bioassays to HPLC fractions obtained from the re-fractionation of behaviorally active HPLC fraction No 14 in Table 3.7 using HP-3 of Table 3.4.

Fraction No.	Solvent composition ether:hexane	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min						Total contacts	% Response to control station
				0-2 min		2-4 min		4-6 min			
				Number	% Response to control station	Number	% Response to control station	Number	% Response to control station		
crude	0:100	0.8	C	6	100	4	100	9	100	19	100
			S	0		0		0		0	
5	6.75:93.25	0.8	C	15	100	13	68	8	57 *	36	75 *
			S	0		6		6		12	
6	6.75:93.25	0.8	C	7	27 *	14	70 *	7	50 *	28	47 *
			S	19		6		7		32	
7	6.75:93.25	0.8	C	16	48 *	8	47 *	10	66 *	34	52 *
			S	17		9		5		31	

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Table 3.8: (Cont'd)

Fraction No.	Solvent composition ether:hexane	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min						Total contacts	% Response to control station
				0-2 min		2-4 min		4-6 min			
				Number	% Response to control station	Number	% Response to control station	Number	% Response to control station		
8	6.75:93.25	0.8	C	17	51 *	12	44 *	11	58 *	40	51 *
			S	16		15		8		39	

C - Control
S - Bait, treated with sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

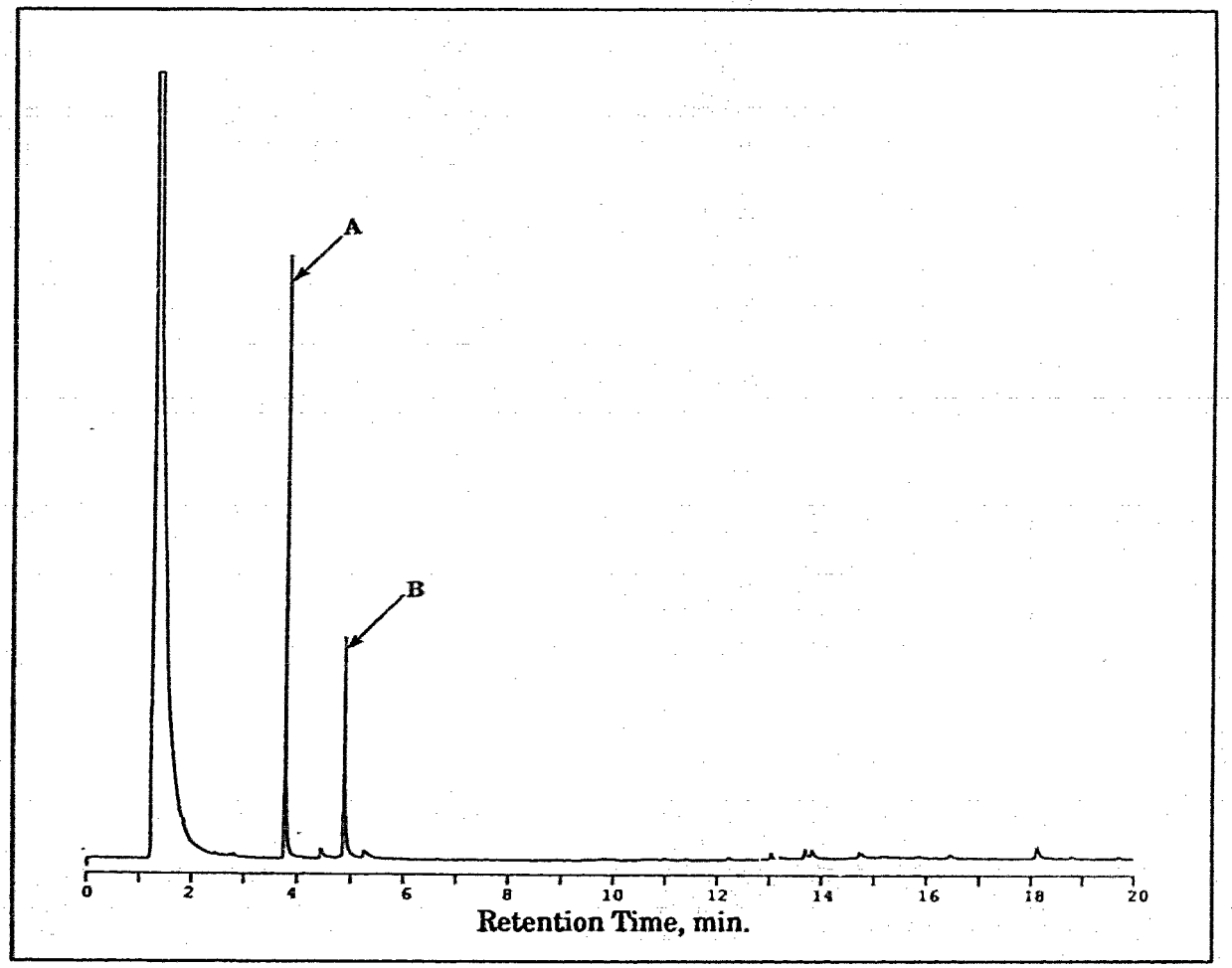

Figure 3.2: GC chromatogram (GC-1) of the final behaviorally active fraction 5 (Table 3.8) obtained from the repeated HPLC fractionation (HP-1, 2 and 3) of active principle in fraction 7 (Table 3.5) that eluted with 50% ether/pentane in LC fractionation of steam-distilled volatile extracts of pepper tree leaves. Compounds **A** and **B** are the major constituents.

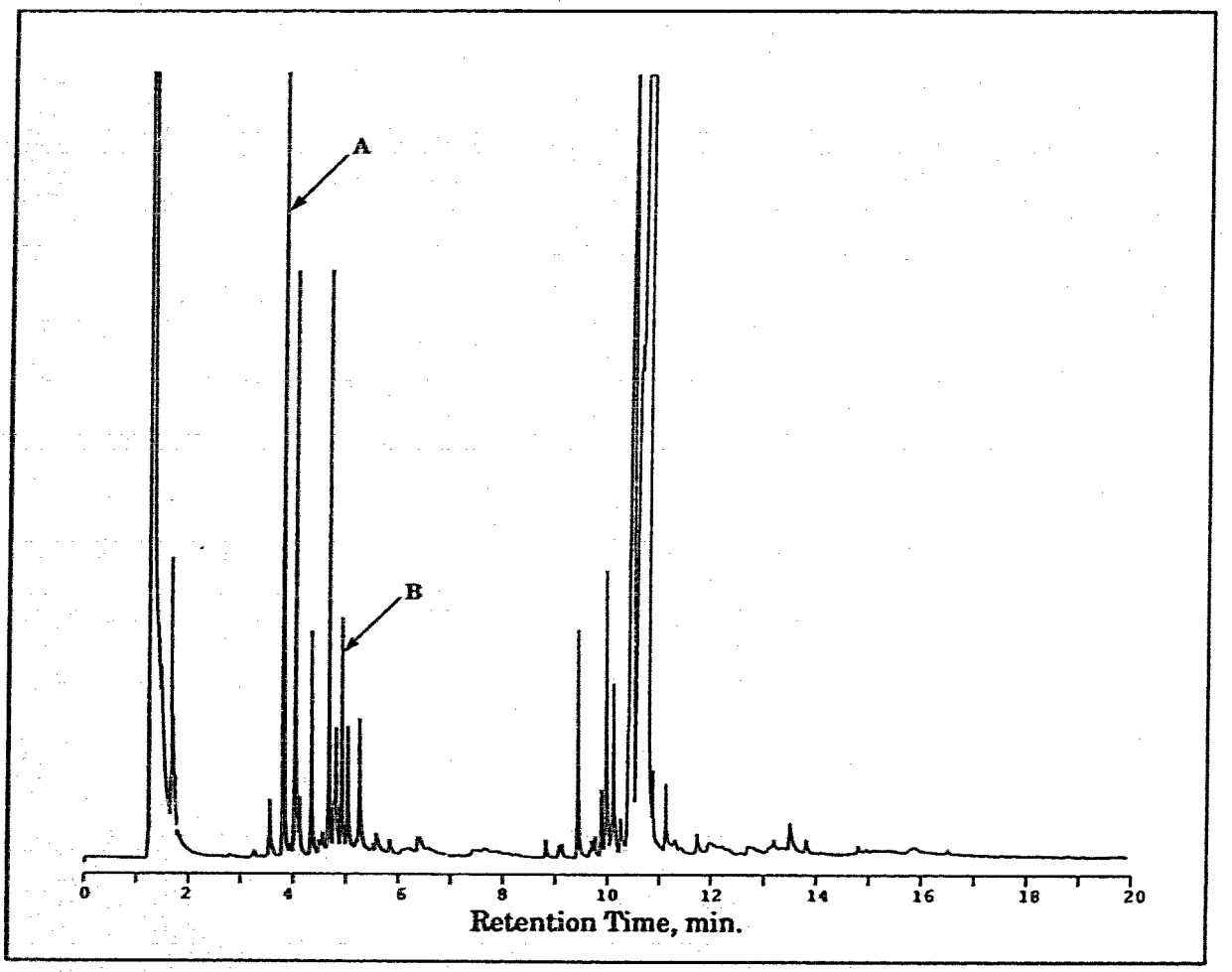

Figure 3.3: GC chromatogram (GC-1) of the behaviorally active fraction 4 (Table 3.9) eluted with 15% ether/hexane in direct HPLC fractionation (HP-4) of steam-distilled volatile extracts of pepper tree leaves. Compounds **A** and **B** correspond to the same compounds in Figure 3.2.

Table 3.9: Response of house flies in laboratory bioassays to HPLC fractions of steam-distilled volatile extracts of pepper tree leaves using HP-4 of Table 3.4 during the second part of fractionation process.

Fraction No.	Solvent composition ether:hexane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
crude	0:100	0.4	C	11	6	5	22	100
			S	0	0	0	0	
1	1:99	0.4	C	8	2	1	11	60 *
			S	2	3	3	8	
2	5:95	0.4	C	11	4	4	19	43 *
			S	10	6	9	25	
3	10:90	0.4	C	10	5	4	19	66 *
			S	3	3	4	10	
4	15:85	0.4	C	6	11	7	24	96
			S	0	0	1	1	

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Table 3.9: (Cont'd)

Fraction No.	Solvent composition ether:hexane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
5	20:80	0.4	C	18	10	4	32	78 *
			S	1	2	6	9	
6	30:70	0.4	C	9	5	2	16	73 *
			S	3	3	0	6	
7	100:0	0.4	C	13	7	10	30	52 *
			S	15	7	6	28	

C - Control
S - Bait, treated with test sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

Table 3.10: Response of house flies in laboratory bioassays to HPLC fractions obtained from the re-fractionation of behaviorally active HPLC fraction No 4 in Table 3.9 using HP-1 of Table 3.4.

Fraction No.	Solvent composition pentane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
crude	100%	0.8	C	13	14	9	36	100
			S	0	0	0	0	
3	100%	0.8	C	14	10	4	28	52 *
			S	9	10	7	26	
4	100%	0.8	C	23	12	11	46	96
			S	0	0	2	2	
5	100%	0.8	C	19	7	9	35	56 *
			S	9	9	9	27	

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Table 3.10: (Cont'd)

Fraction No.	Solvent composition pentane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
6	100%	0.8	C	17	11	7	35	71 *
			S	5	7	2	14	

C - Control
S - Bait, treated with sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

Table 3.11: Response of house flies in laboratory bioassays to HPLC fractions obtained from the re-fractionation of behaviorally active HPLC fraction No 4 in Table 3.10 using HP-2 of Table 3.4.

Fraction No.	Solvent composition ether:hexane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
crude	0:100	1	C	4	10	5	19	100
			S	0	0	0	0	
13	6.25:93.75	1	C	8	5	3	16	37 *
			S	14	6	7	27	
14	6.75:93.25	1	C	7	11	3	21	91
			S	0	1	1	2	
15	7.25:92.75	1	C	9	5	6	20	49 *
			S	12	5	4	21	
16	7.75:92.25	1	C	8	6	5	19	53 *
			S	7	6	4	17	

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Table 3.11: (Cont'd)

Fraction No.	Solvent composition ether:hexane	Dose (mg/25 µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
				0-2 min	2-4 min	4-6 min		
17	8.25:91.75	1	C	8	4	3	15	52 *
			S	6	5	3	14	
18	8.75:91.25	1	C	5	6	6	17	52 *
			S	6	6	4	16	

C - Control
S - Bait, treated with sample
% Response to control station = [Total C/(Total C + Total S)] × 100

* % Responses labelled by asterisk are significantly different from the crude extract control, z test, $p < 0.05$

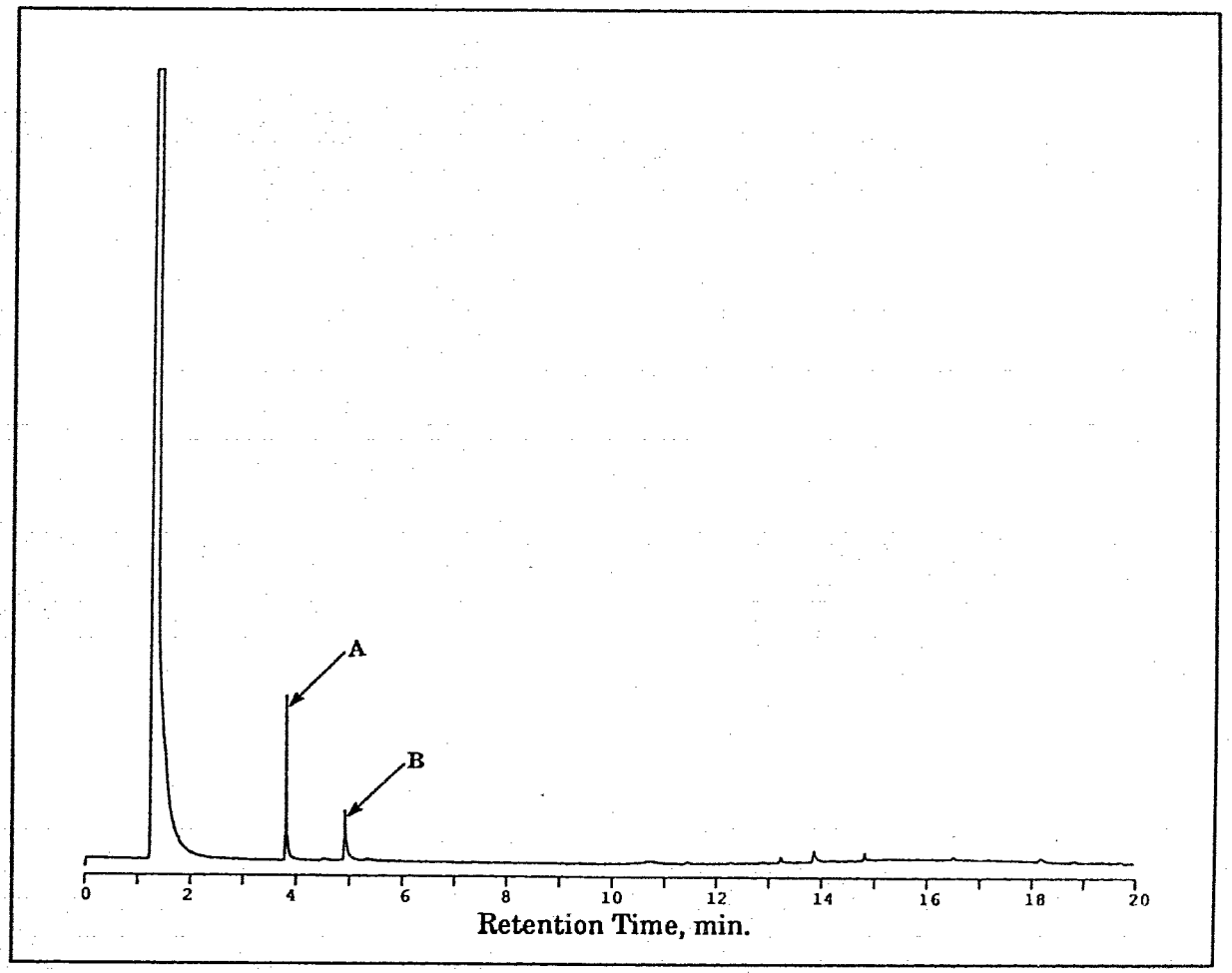


Figure 3.4: GC chromatogram (GC-1) of the final behaviorally active fraction 14 (Table 3.11) obtained from the repeated fractionation of active principle in fraction 4 in Table 3.9 that eluted with 15% ether/hexane by HPLC fractionation (HP-1 and 2) of steam-distilled volatile extracts of pepper tree leaves. Compounds **A** and **B** correspond to the same compounds in Figure 3.2.

Both fractionation processes led to a final active fraction with two major components, **A** and **B**. Furthermore, fractions 4 and 7 (Table 3.5; Scheme 3.1) from the LC separation show a peak in the GC chromatograms (Figure 3.5 and Figure 3.6) at the same retention time as that of compound **A**. In addition, fraction 7 (Figure 3.6) shows a peak at a retention time identical to that of compound **B**. Compounds **A** and **B** have been observed in all fractions exhibiting house fly repellent activity. Compounds **A** and **B** demonstrate considerable ratio differences throughout the fractionations, and **A** is present in two different fractions in the initial column fractionation. Such behaviour is suggestive of compound lability and potential interconversion.

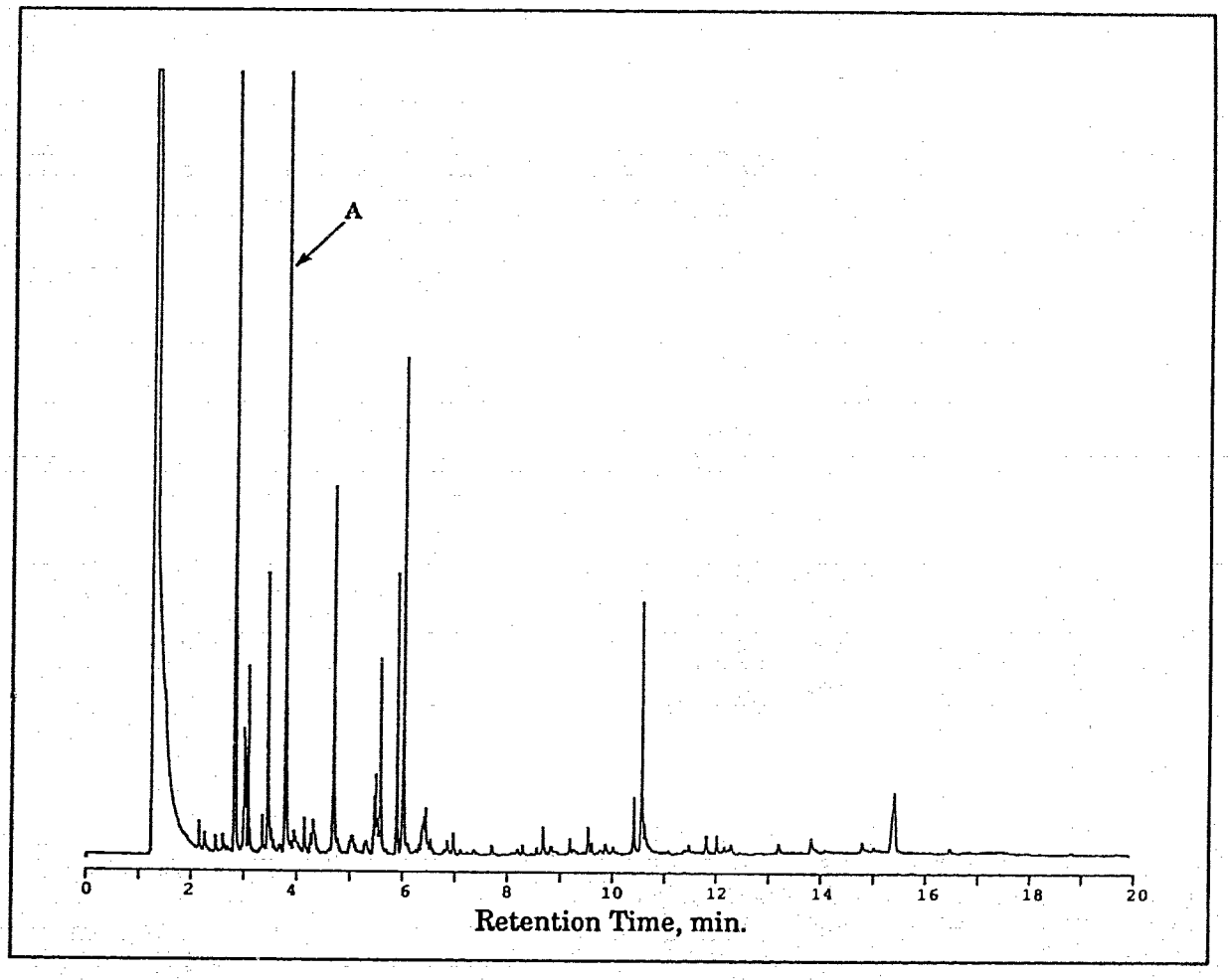


Figure 3.5: GC chromatogram (GC-1) of the behaviorally active fraction 4 (Table 3.5) eluted with 10% ether/pentane in LC fractionation of steam-distilled volatile extracts of pepper tree leaves. Compound **A** corresponds to the same compound in Figure 3.2.

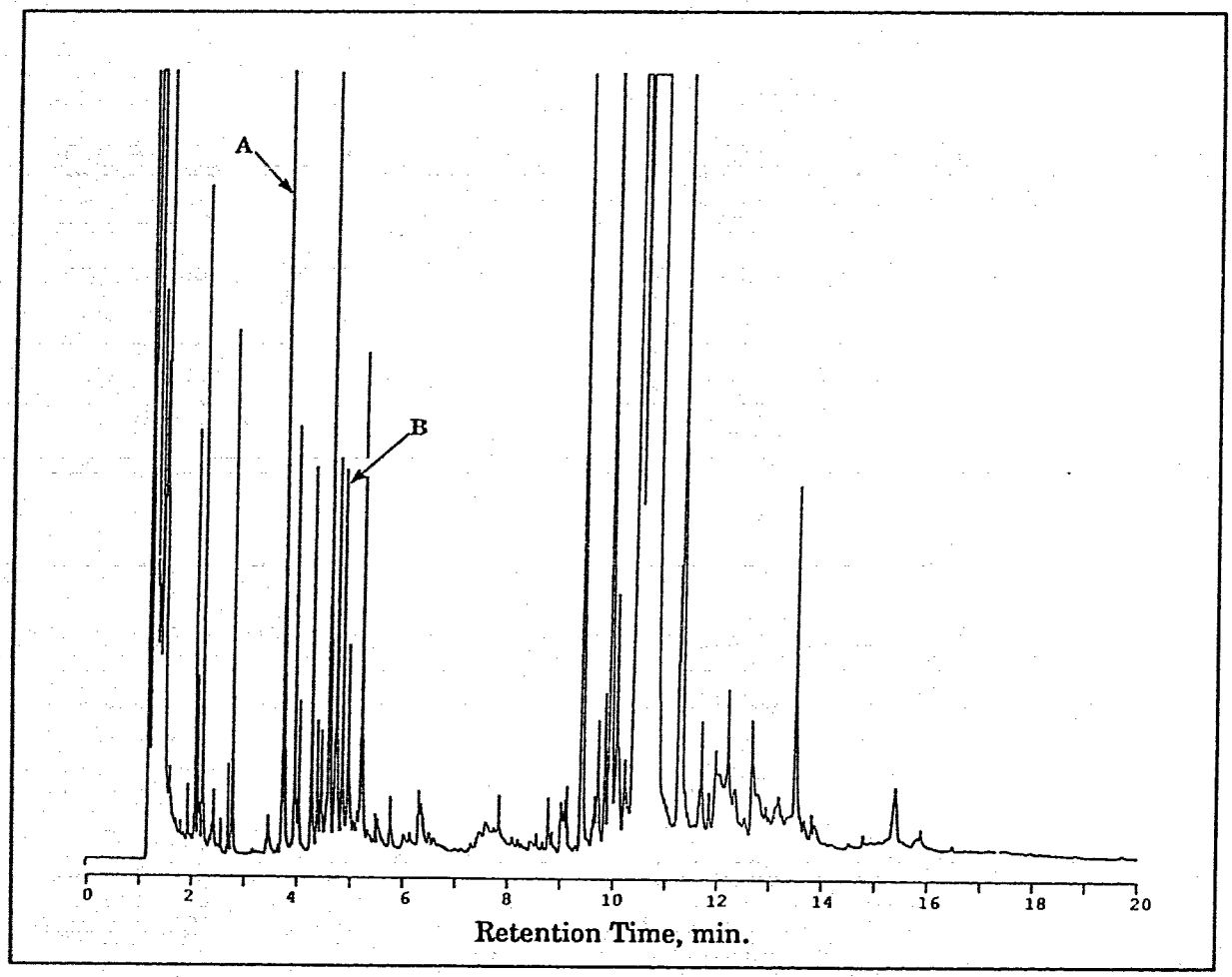


Figure 3.6: GC chromatogram (GC-1) of the behaviorally active fraction 7 (Table 3.5) eluted with 50% ether/pentane in LC fractionation of steam-distilled volatile extracts of pepper tree leaves. Compounds **A** and **B** correspond to the same compounds in Figure 3.2.

4. ANALYSIS OF HOUSE FLY REPELLENTS

4.1. Experimental

4.1.1. Gas-liquid chromatography and gas chromatography-mass spectrometry

Quantitative GC analysis of the active repellents in steam-distilled volatile extracts of pepper tree leaves was performed on a Hewlett-Packard 5880A gas chromatograph equipped with a Hewlett-Packard 5880A integrator. The analytical condition was the same as that used previously for GC-1 in Table 3.1. Qualitative GC analyses for isolated active repellents and synthetic authentic samples were performed according to GC-1 and 2 (Table 3.1).

Electron impact ionization mass spectra (EI-MS) were obtained on a Hewlett-Packard 5985B GC-mass spectrometer.

4.1.2. Acetylation

Acetylation experiments were performed with the recombined fraction of behaviorally active fraction 5 (Table 3.8) and fraction 14 (Table 3.11). The quantity of each compound in the sample was estimated by GC using decane as an internal standard.

The recombined fraction was partially concentrated, transferred into a glass vial (4 mL) with Teflon-lined cap and treated with excess of acetic anhydride (0.3 mL), pyridine (0.3 mL) and 4-dimethylaminopyridine (~5 mg). The solution was stirred overnight at room temperature. The reaction was quenched by adding water and the mixture, extracted into hexane (0.5 mL × 4). The hexane extract was washed successively with 10% HCl (0.5 mL × 5), water (0.5 mL × 2), saturated NaCl (0.5 mL), and dried over anhydrous Na_2SO_4, filtered, and concentrated by aspiration. Products were analyzed by matching GC retention times with standards and GC-mass spectroscopic (GC-MS) fragmentation patterns.

4.1.3. Hydrogenation

Hydrogenation of small quantities of final recombined active fraction was carried out in a thick-walled Reacti-vial™ (1 mL) equipped with a tight-fitting Teflon-lined septum. The vial was loaded with 10% palladium on carbon (~2 mg). A hexane/ether solution of the behaviorally active sample (0.1 mL) and hexane (0.3 mL) were added and the vial was then pressurized with hydrogen and the mixture was stirred for 1 h. The product was filtered through glass wool and concentrated. Identification of the products was made as previously described.

Hydrogenation of acetylated products of behaviorally active fractions, acetyl lactyl derivatives of compound **B** of *trans*-piperitol [*trans*-3-methyl-6-(1-methylethyl)-2-cyclohexene-1-ol] were conducted according to the same procedure.

4.1.4. Oxidation

Oxidation of primary and secondary alcohols with pyridinium chlorochromate (PCC) converts them into their corresponding carbonyl compounds[47] and signals the presence of primary and secondary alcohol groups in compounds.

Behaviorally active sample (0.05 mL) was added to a glass vial (1 mL) with a Teflon-lined cap in dichloromethane (0.4 mL). A small amount of PCC (~50 mg) was then added to the mixture, and stirred for 2 h at rt. The mixture was diluted with anhydrous ether (0.25 mL), and filtered through Florisil and concentrated by aspiration. Identification of products was made as previously described.

4.1.5. Chiral determination using acetyl *S*-lactyl chloride

Acetyl lactyl chloride reagent was prepared as reported by Slessor et. al[48]. Derivatization of compound **B** in the concentrated final behavioral active fraction was carried out in a glass vial (4 mL) with a Teflon-lined cap.

The sample (0.025 mL) was diluted with dichloromethane (0.5 mL) and pyridine (0.25 mL). 4-Dimethylaminopyridine (~0.5 mg) and acetyl lactyl chloride reagent (0.05 mL) were added. The solution was stirred and then kept in a refrigerator (4 °C) overnight. Water (0.5 mL) was added dropwise with stirring. The aqueous phase was removed and the organic phase was further washed with 2% HCl (0.5 mL × 3), saturated $NaHSO_3$ solution (0.5 mL), water (0.5 mL) and finally with saturated NaCl solution (0.5 mL). The organic solution was diluted with hexane and dried over anhydrous Na_2SO_4. The mixture was filtered and concentrated by aspiration. Identification of products was made as previously described.

Similar methods were used to prepare the acetyl lactyl derivatives of authentic compounds of racemic *trans*-piperitol (synthetic), (*1R,2S,5R*)-(-)-menthol, (*1S,2R,5R*)-(+)-isomenthol and (*1S,2R,5S*)-(+)-menthol (all from Aldrich Chem. Co., Milwaukee, WI).

HPLC separation of prepared acetyl lactyl derivatives of *trans*-piperitols was performed according to method HP-5 in Table 3.4.

4.2. Results

4.2.1. Identification of house fly repellents A and B

Compounds **A** and **B** comprised 0.2% and 0.03% respectively of the total volatile extracts (Figure 3.1), assuming that both compounds have similar detector responses.

The EI-MS spectra of compounds **A** (Figure 4.1) and **B** (Figure 4.2) suggested that the molecular weight of both compounds is 154, corresponding to a molecular composition of $C_{10}H_{18}O$. This molecular formula corresponds to compounds with two degrees of unsaturation. The fragmentation patterns in both spectra indicate the loss of a methyl (m/e 139) and water (m/e 121), indicating that compounds **A** and **B** have both methyl and hydroxyl functionalities. Analysis of acetylated products of behaviorally active fractions by GC indicated two major detector responses (Figure 4.3), one (**C=A**) at a retention time identical to compound **A** and another (**D**) with a later retention time than compound **B**. The mass spectrum of compound **C(=A)** was identical to that of compound **A** (Figure 4.1), indicating that compound **A** had not been acetylated, suggestive of a tertiary alcohol. The mass spectrum of compound **D** (Figure 4.4) exhibited an enhanced fragment peak at m/e 43, typical of an acetate group. Acetylation of compound **B** indicated the presence of a primary or secondary alcohol.

The GC chromatogram of the hydrogenated products of compounds **A** and **B** showed three major peaks (**E, F** and **G** in Figure 4.5), as did the hydrogenation of previously acetylated products (Figure 4.6). The GC retention and MS fragmentation pattern of one of these compounds (**H** in Figure 4.6) were identical to those of the compound **E** (Figures 4.7, 4.8). These results clearly indicate that compound **E=H** is the only hydrogenated product derived from compound **A** and must contain the partial structure R'-CH=CH-R".

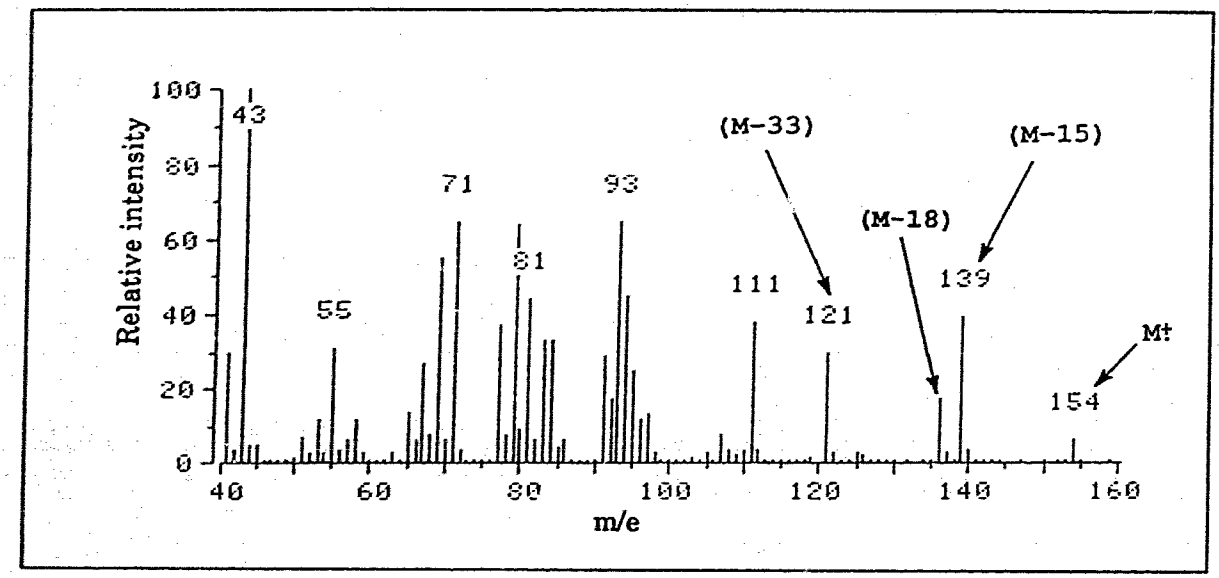


Figure 4.1: EI-MS spectrum of compound **A** in final behaviorally active fraction.

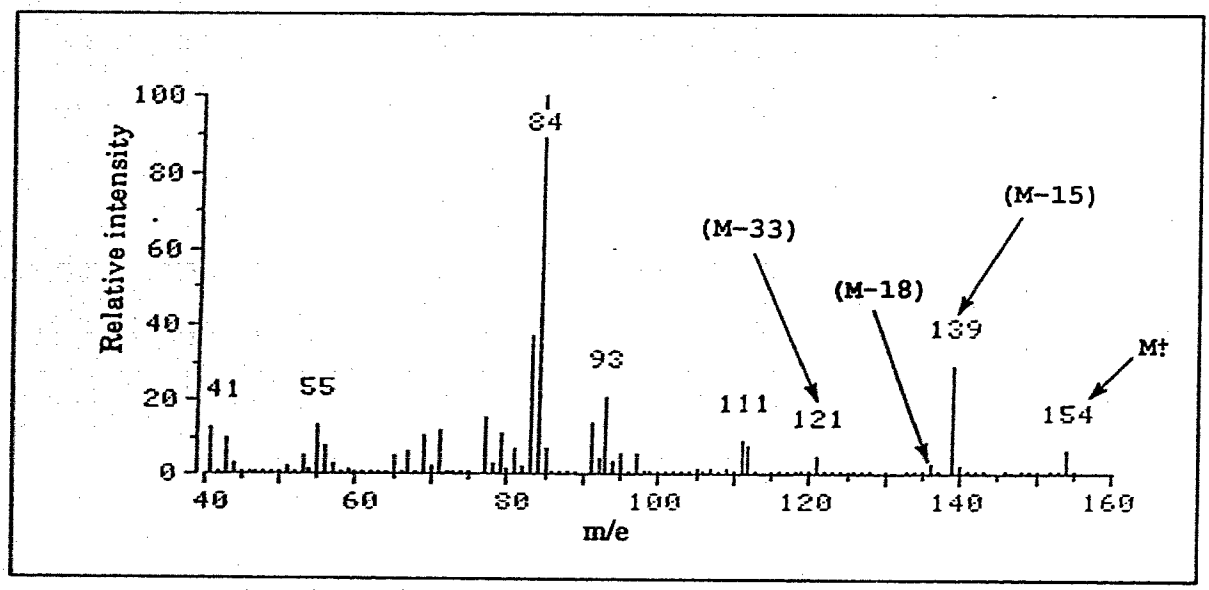


Figure 4.2: EI-MS spectrum of compound **B** in final behaviorally active fraction.

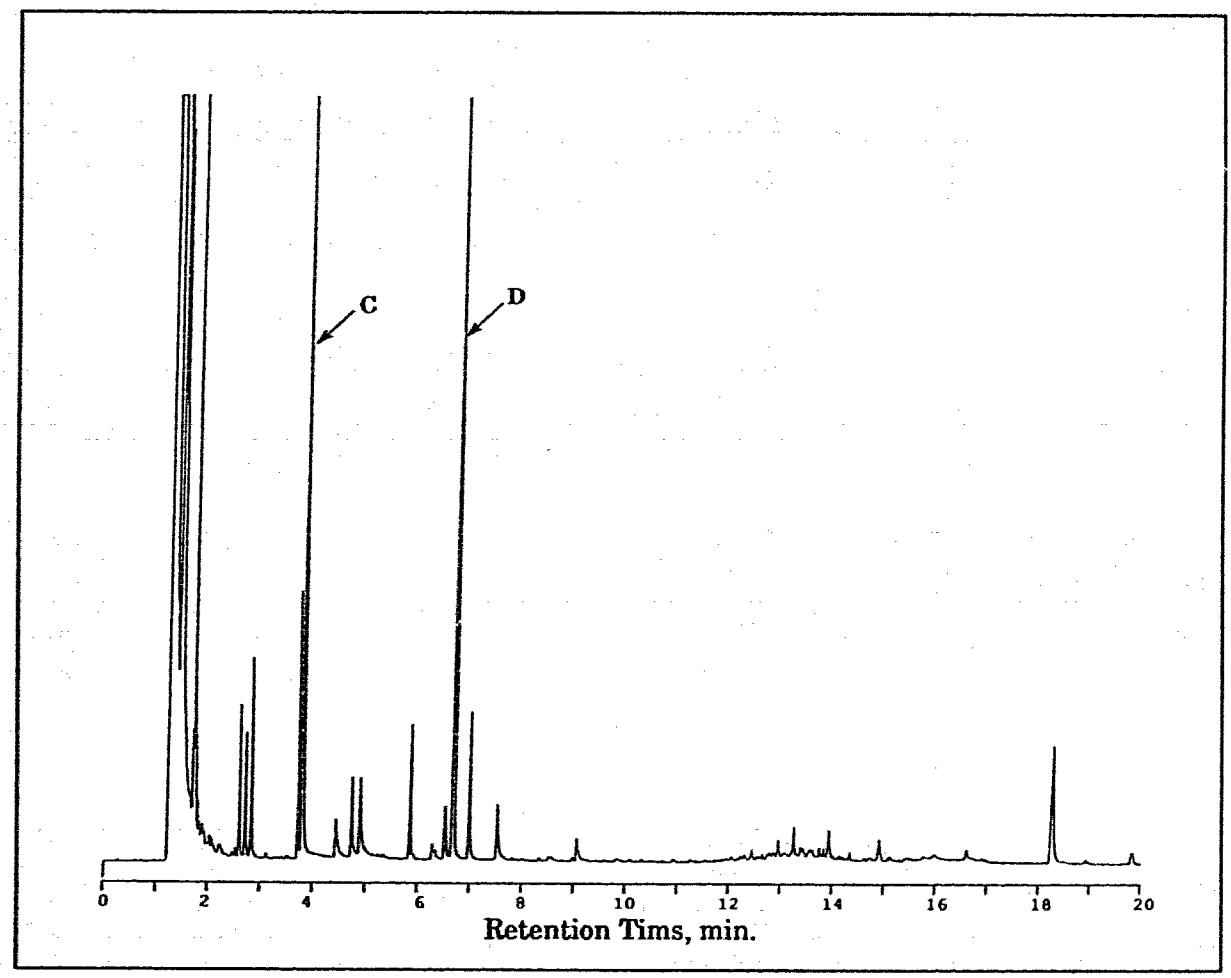


Figure 4.3: GC chromatogram (GC-1) of the acetylated final behaviorally active fraction. Compound **C** is identical to compound **A** in Figure 3.2 and compound **D** is the acetylated derivative of compound **B** in Figure 3.2.

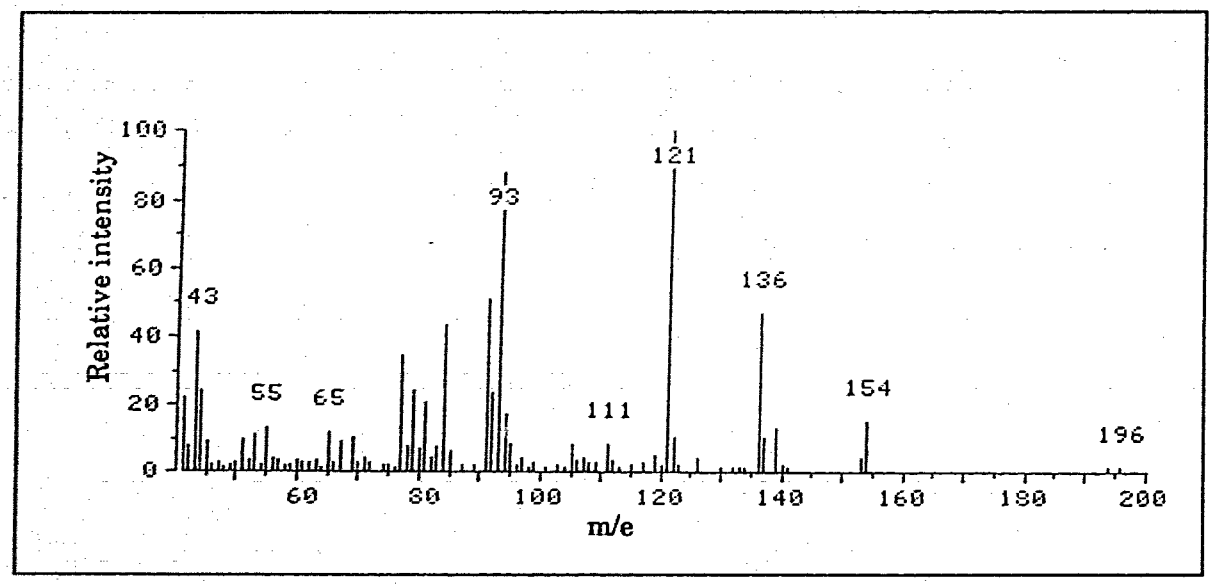


Figure 4.4: EI-MS spectrum of compound **D** (Figure 4.3) in acetylated final behaviorally active fraction.

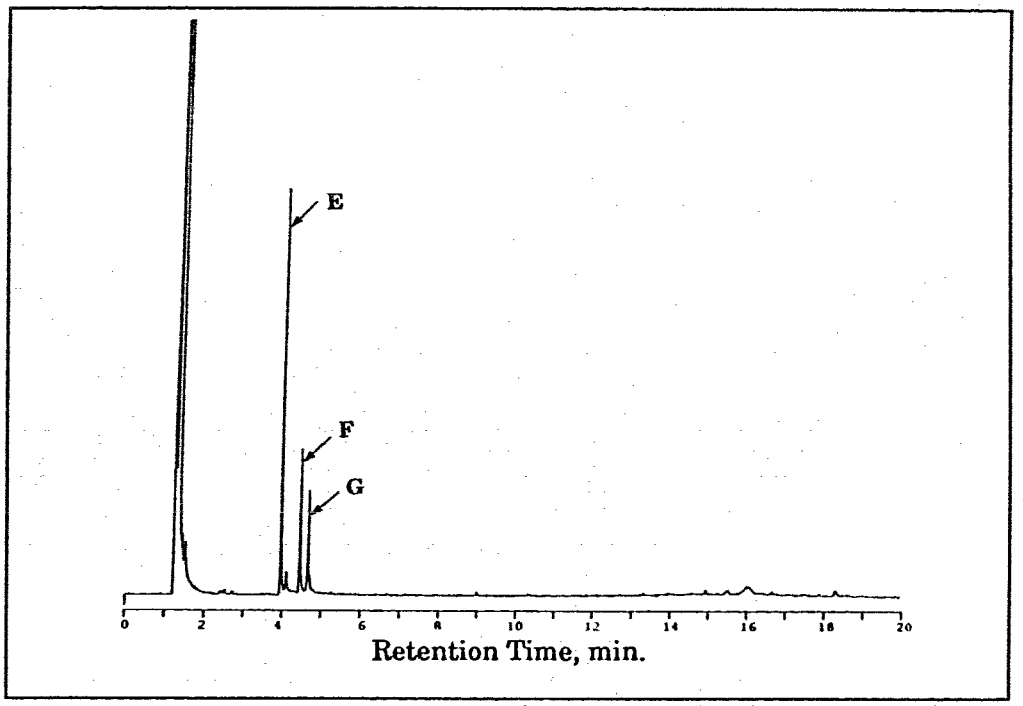


Figure 4.5: GC chromatogram (GC-1) of the hydrogenated final behaviorally active fraction. Compounds **E**, **F** and **G** represent the hydrogenated products of compounds **A** and **B** in Figure 3.2.

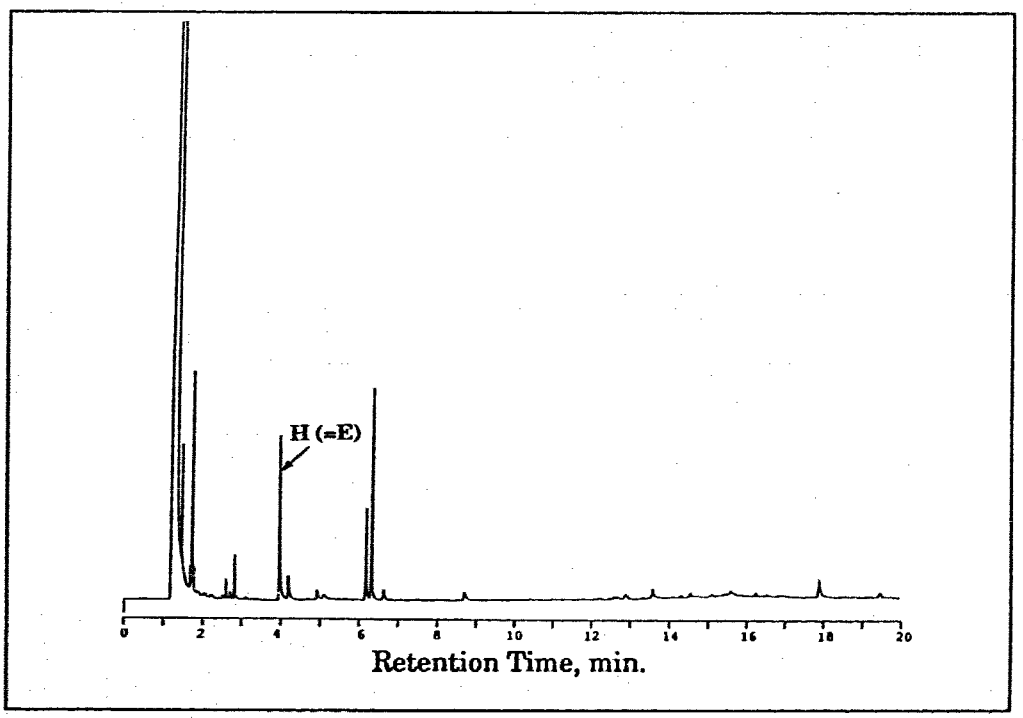


Figure 4.6: GC chromatogram (GC-1) of the products obtained from hydrogenation of acetylated behaviorally active fraction. Compound **H** is identical to compound **E** in Figure 4.5.

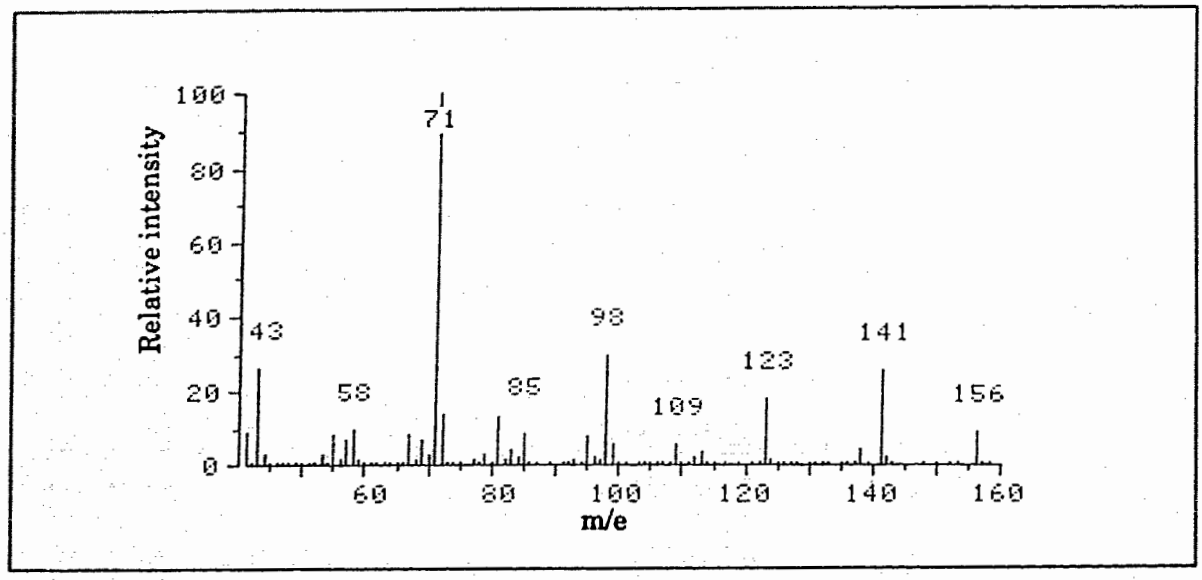


Figure 4.7: EI-MS spectrum of compound **E** (Figure 4.5) in hydrogenated final behaviorally active fraction.

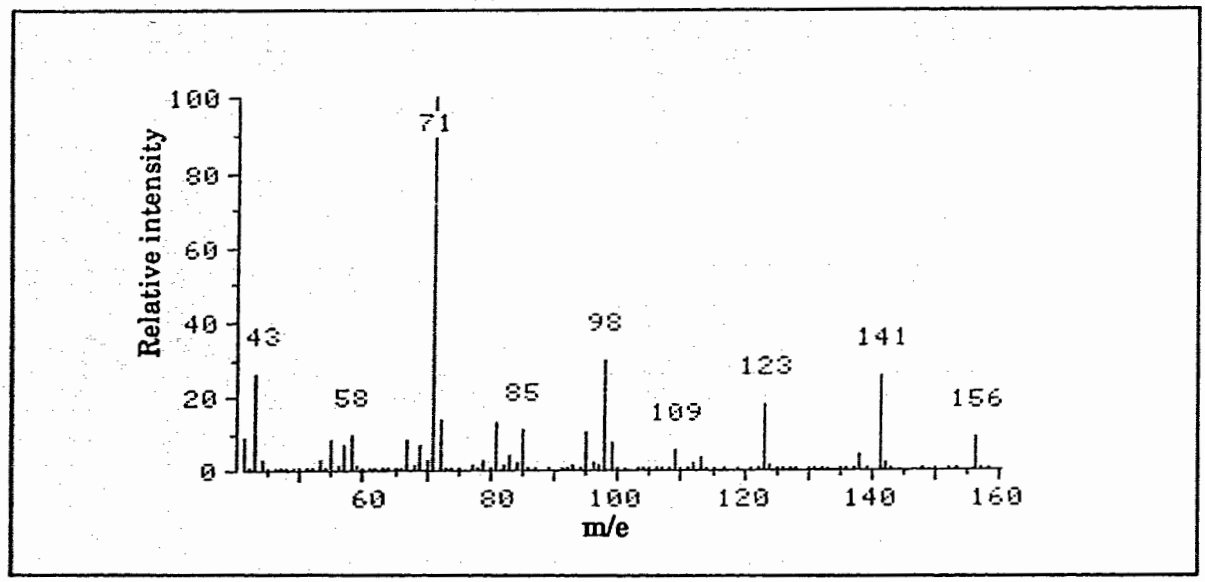
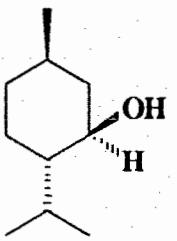


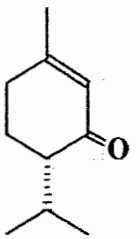
Figure 4.8: EI-MS spectrum of compound **H** (Figure 4.6) in hydrogenated products of acetylated behaviorally active fraction.

Generation of two compounds by hydrogenation indicated the presence of a trisubstituted double bond in compound **B**. One of compound **B**'s hydrogenated products, **F** (Figure 4.5), gave retention and mass spectral data (Figure 4.9) identical to those of (-)-menthol (Figure 4.10), providing the ring size and substitution pattern for compound **B**.



(-)-menthol

Oxidation of both compounds **A** and **B** yielded a single product indicating that both **A** and **B** are structurally related. The GC retention time and MS fragmentation pattern of the oxidized product (Figure 4.11) matched those of piperitone (Figure 4.12).



Piperitone

The substituted double bond in compound **B** must have been in an analogous position to the double bond in piperitone and must correspond to one of the two alcohols. Since one of the two hydrogenation products of **B** was

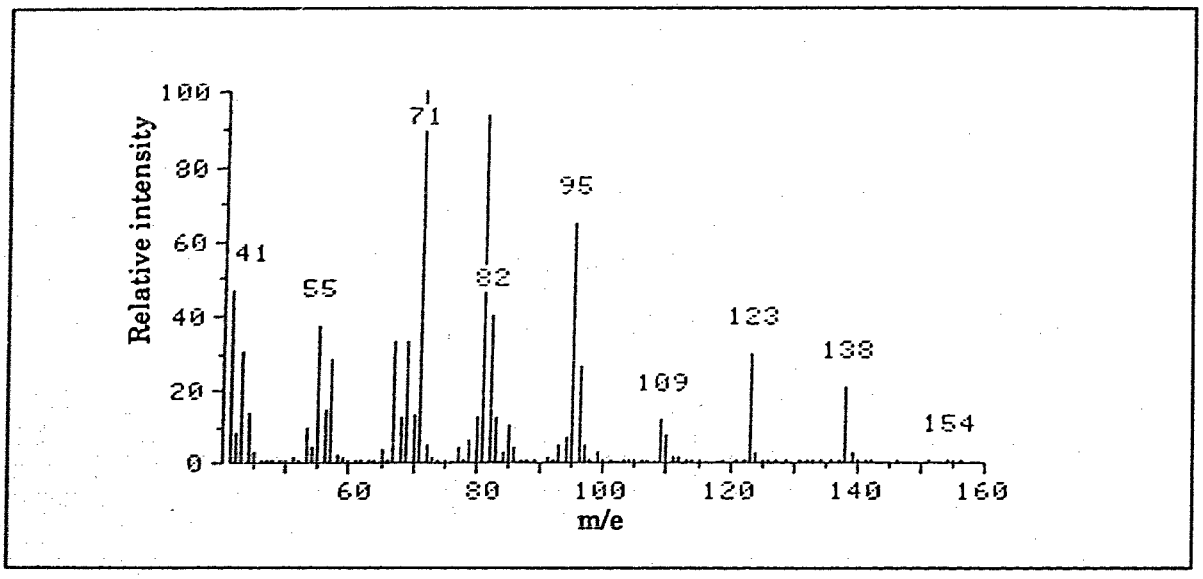


Figure 4.9: EI-MS spectrum of compound **F** (Figure 4.5) in hydrogenated final behaviorally active fraction.

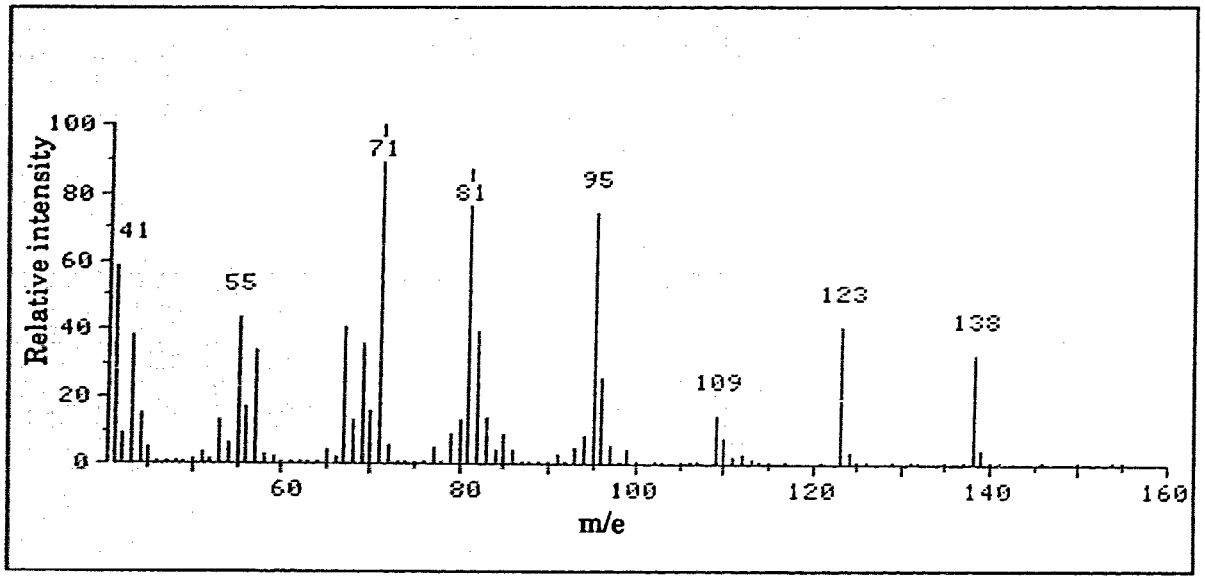


Figure 4.10: EI-MS spectrum of (-)-menthol.

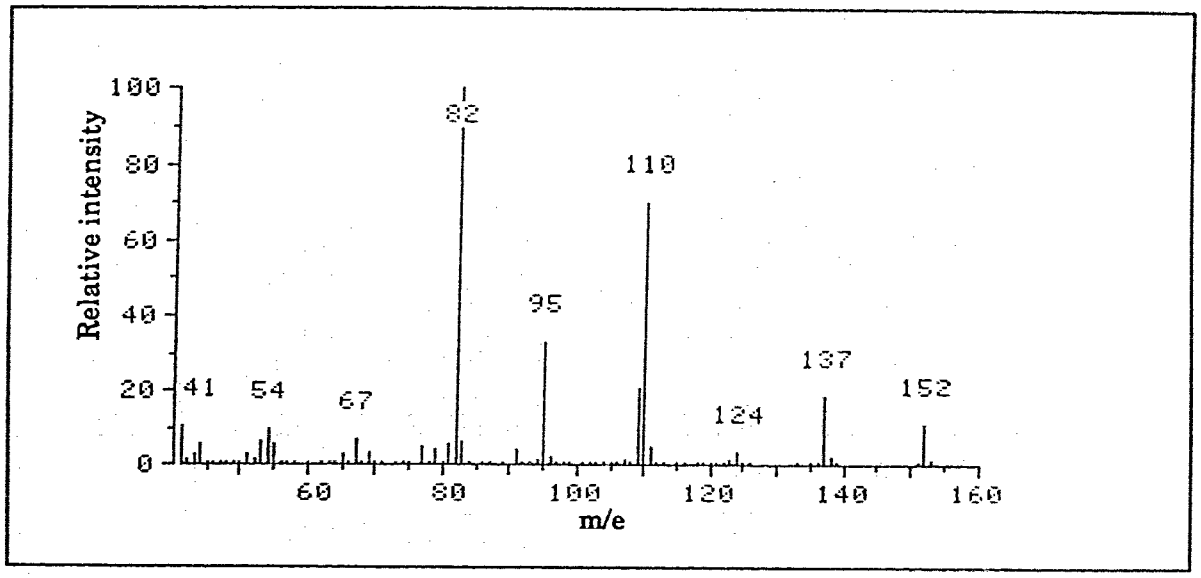


Figure 4.11: EI-MS spectrum of oxidized product of compounds **A** and **B** in final behaviorally active fraction.

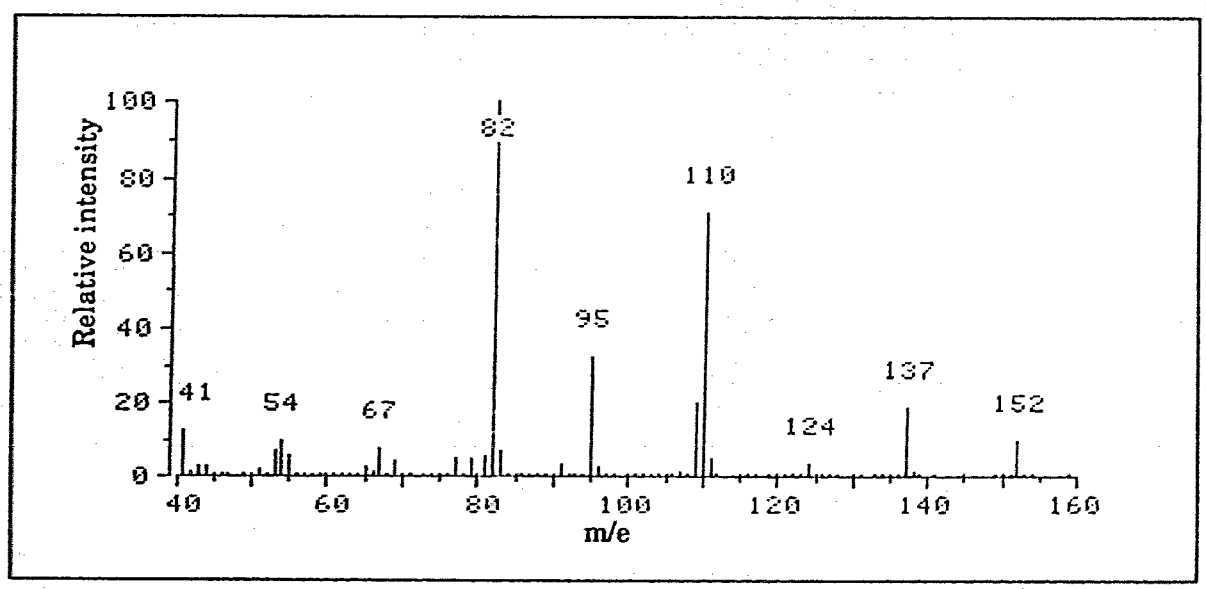
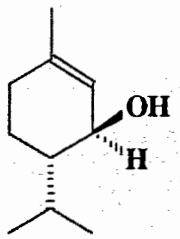


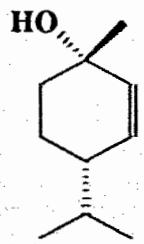
Figure 4.12: EI-MS spectrum of piperitone.

(-)-menthol, in which the hydroxy and isopropyl groups are *trans*, compound **B** must have the same *trans*- orientation, establishing compound **B** as *trans*-piperitol.

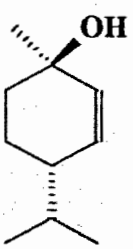


trans-piperitol

Compound **A**, a tertiary alcohol, generated only one product upon hydrogenation, which indicated that the double bond in **A** was not located at a branch, and hence, does not give rise to an additional chiral center and product upon hydrogenation. Formation of piperitone during the PCC oxidation of compound **A** suggests isomerization of **A** to piperitol in the acidic reaction mixture followed by oxidation. Under mildly acidic conditions, the hydroxyl group of piperitol is known to migrate between C-1 and C-3 positions[49]. Therefore, these observations suggest either *cis*- or *trans*-menth-2-en-1-ol as a most likely structure for compound **A**.



cis-menth-2-en-1-ol



trans-menth-2-en-1-ol

Among the GC retention data obtained from synthetic isomers of both *cis*- and *trans*-menth-2-en-1-ol and *trans*-piperitol, only *cis*-menth-2-en-1-ol and *trans*-piperitol matched that of compounds **A** and **B** respectively, on two columns with different retention characteristics (GC-1 and 2 in Table 3.1). Furthermore, the MS fragmentation patterns were identical for *cis*-menth-2-en-1-ol (Figure 4.13) and compound **A** (Figure 4.1) and for *trans*-piperitol (Figure 4.14) and compound **B** (Figure 4.2).

4.2.2. Determination of chirality of pepper tree derived, *trans*-piperitol

Two peaks appeared in the gas chromatogram of the acetyl lactate of synthetic *trans*-piperitol (**J**, **P**), which could be separated into individual diastereoisomers by HPLC (HP-5). Hydrogenation of each isolated product generated two compounds (**L** and **M**, **R** and **S**, Scheme 4.1), all of which had distinct GC retention times and appropriate MS fragmentation patterns. Comparison of GC retention times and MS fragmentation of the four with those of acetyl lactates of authentic compounds indicated that products **L**, **R** and **S** corresponded to the acetyl lactates of (*1R,2S,5R*)-(-)-menthol (**T**), (*1S,2R,5S*)-(+)-menthol (**U**) and (*1S,2R,5R*)-(+)-isomenthol (**V**) respectively. Because both compounds **R** and **S** are derived from compound **Q** by hydrogenation, compound **Q** was identified as the acetyl lactate of (*1R,6R*)-piperitol. Of the *trans*-piperitols, only compound **K** generated compound **L** during its hydrogenation, and since compound **L** was the acetyl lactate of

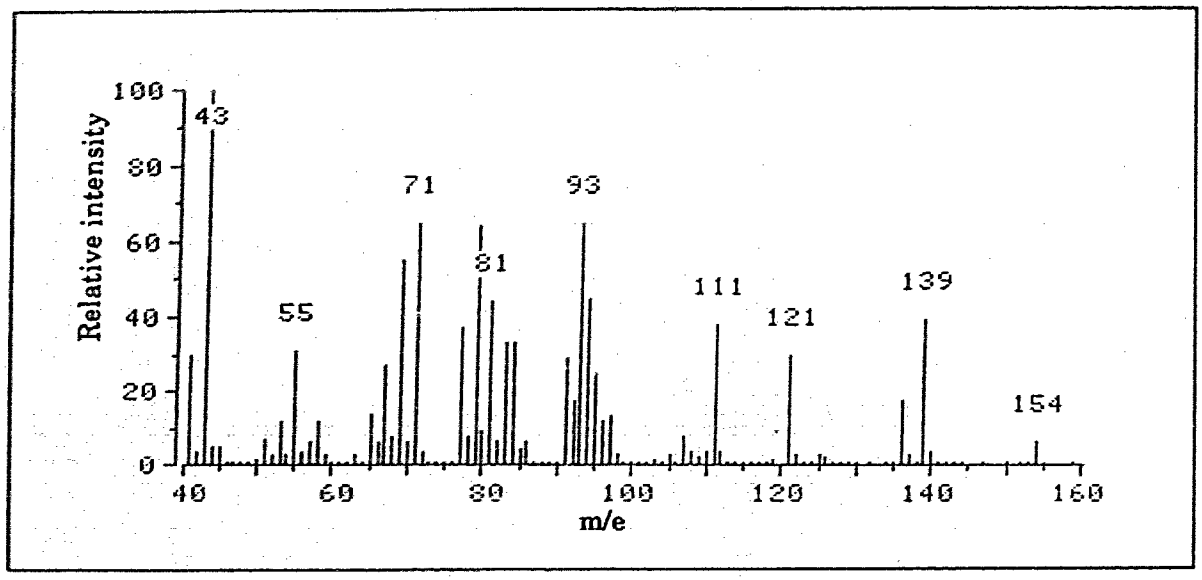


Figure 4.13: EI-MS spectrum of *cis*-menth-2-en-1-ol.

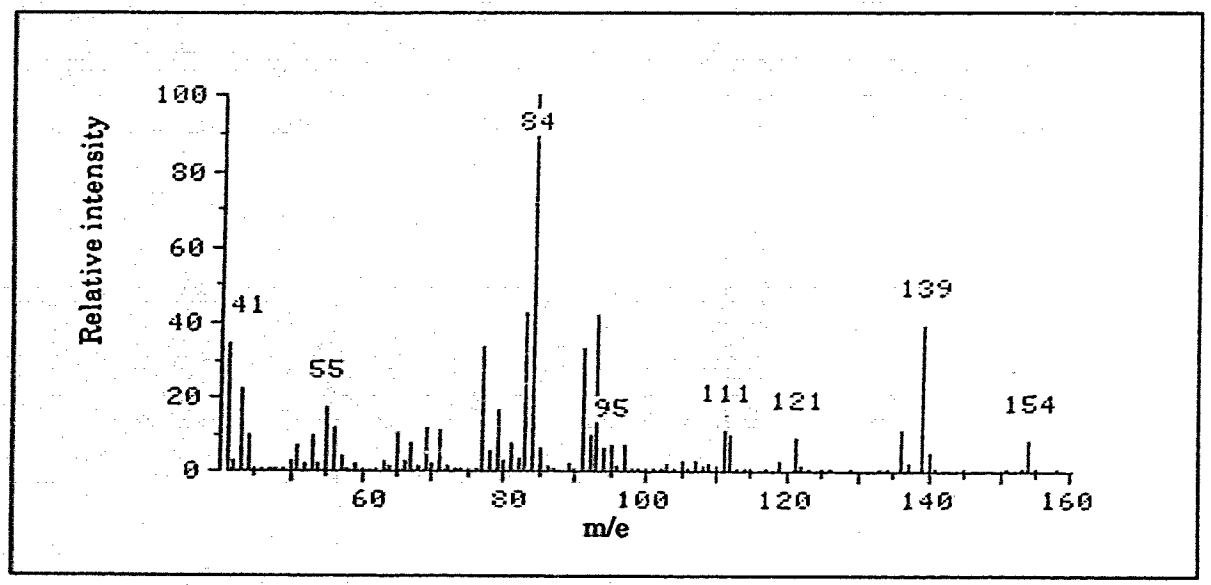
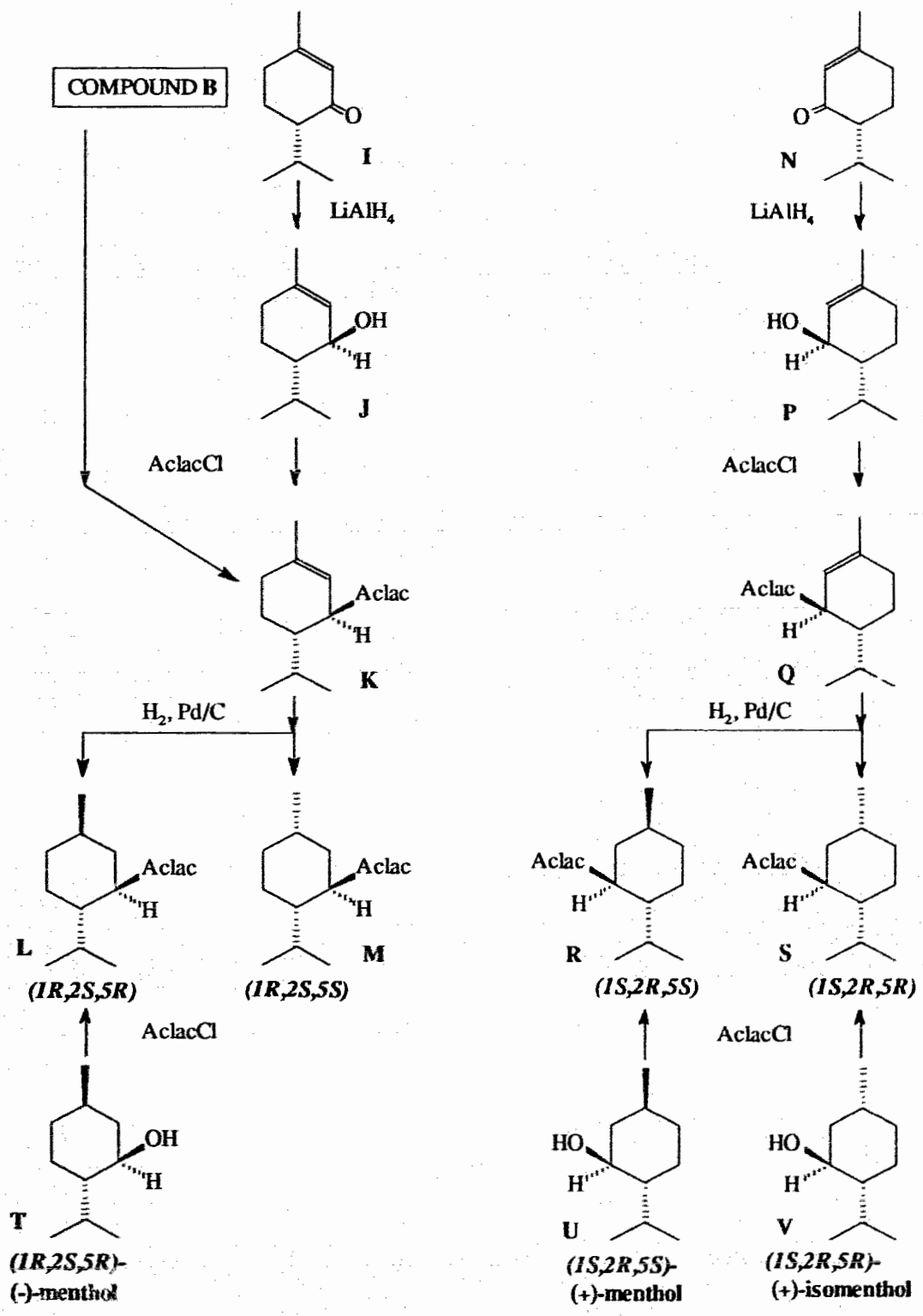


Figure 4.14: EI-MS spectrum of *trans*-piperitol.

Scheme 4.1: Determination of chirality of compound B.



(*1R,2S,5R*)-(-)-menthol, compound **K** must have been the acetyl lactate derivative of (*1S,6S*)-piperitol. Derivatization of the combined final behaviorally active fractions (fraction 5 in Table 3.8 and fraction 14 in Table 3.11) produced a single product (**K**) which was identical in GC retention time and MS fragmentation (Figure 4.15) to that of compound **K** (Figure 4.16). Hydrogenation of the **B**-derived **K** produced two products identical with those of compounds **L** and **M** respectively. These results establish the structure of compound **B** to be (*1S,6S*)-piperitol.

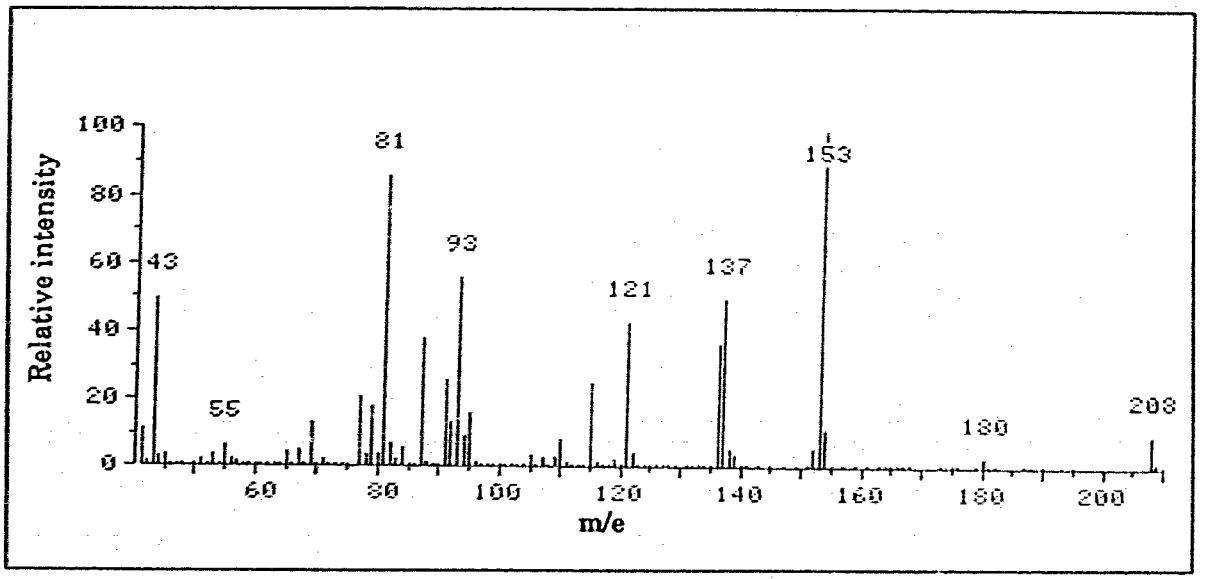


Figure 4.15: EI-MS spectrum of acetyl lactate of compound B.

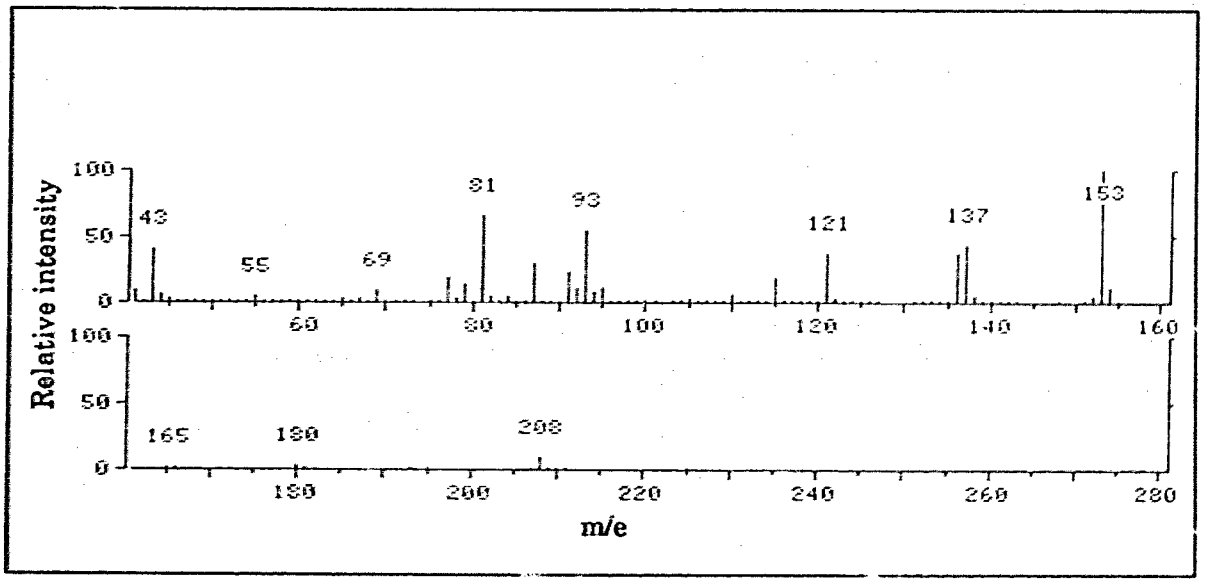


Figure 4.16: EI-MS spectrum of compound K in Scheme 4.1.

5. SYNTHESES AND BIOASSAY OF *CIS*-MENTH-2-EN-1-OL (A) AND *TRANS*-PIPERITOL (B)

5.1. Synthetic approaches

Both compounds *cis*-menth-2-en-1-ol (**A**) and *trans*-piperitol (**B**) have been reported as naturally occurring phytochemical constituents of several plants. Compound **A** was isolated from the oil of *Chamaecyparis obtusa* (Hinoki)[50] and raspberries[51] and compound **B** was isolated from essential oils of eucalyptus as well as andropogon plants[52,53].

Dye-sensitized photo-oxidation of menth-1-ene has been the most common synthetic route to compound **A**[51]. Leffingwell and Shackelford[54] reported the preparation of **A** by pyrolysis of the acetate obtained by ring opening of menth-1-ene epoxide. Grignard reaction of cryptone with methylmagnesium iodide yielded a mixture of both isomers of compound **A**[51]. In the synthesis of **A** using optically active piperitone epoxide by the ring opening method, Klein and Ohloff[55] reported similar results to those previously reported for the Grignard reaction. Moreover, the possibility of preparation of **A** from piperitol has been reported[49].

Most synthetic methods for **B** have been initiated with piperitone. In 1930, Read and Story[53] established the synthesis of piperitols from piperitylamine. Other synthetic methods are directly associated with the reduction of piperitone by metal hydride reagents. Direct reduction of piperitone with $NaBH_4$ into alcohols (piperitols) was found to be unsuccessful[56] because of the generation of 1,4 addition products. However,

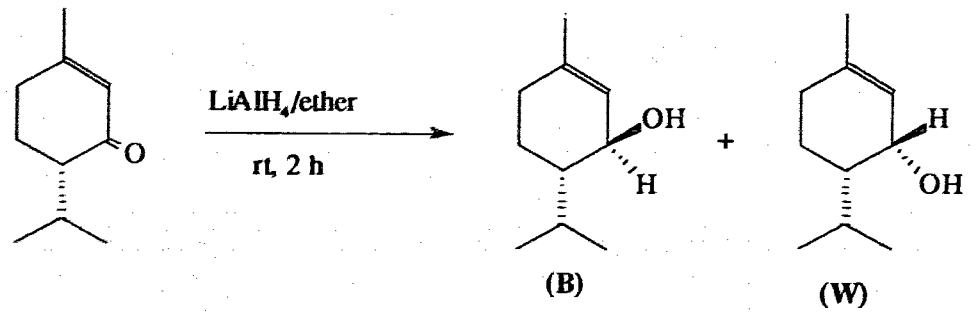
in the presence of cerium trichloride, borohydride reduction of piperitone gives almost exclusively the corresponding allylic alcohols, *cis*- and *trans*-piperitol with the ratio of 65 : 35[56,57]. The complete conversion of piperitone into *cis*- and *trans*-piperitols (35 : 65 ratio) can be accomplished by direct reduction of piperitone with $LiAlH_4$[56,58]. *Cis*-piperitol produced during the reduction provides a suitable source for the synthesis of compound **A**, which can be accomplished by allylic rearrangement[59].

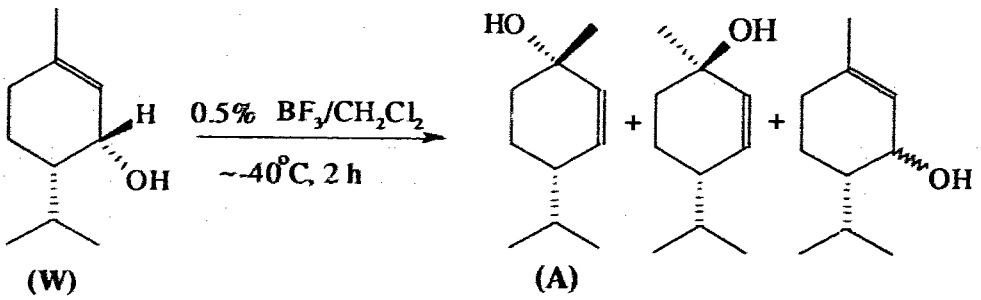
5.2. Synthesis from piperitone

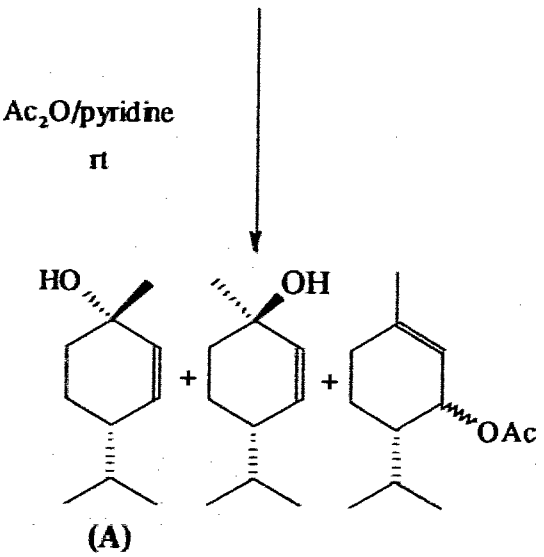
Syntheses of racemic mixture of both compounds **A** and **B** were performed according to the route illustrated in Scheme 5.1. Piperitone was first stereoselectively reduced with $LiAlH_4$ into a mixture of allylic *cis*- and *trans*-piperitols and then separated by column chromatography on Al_2O_3. The resulting *cis*-piperitol was then converted to compound **A** by acid catalyzed rearrangement.

NMR spectra of synthesized compounds were recorded with a Bruker 400 MHz instrument and chemical shifts were reported in parts per million (ppm). Infra-red (IR) spectra were obtained with a Perkin Elmer 1600 FTIR instrument on neat samples (NaCl plates).

Scheme 5.1: Synthesis of compounds **A** and **B**.







5.3. Stereoselective metal hydride reduction

5.3.1. Experimental

A two-necked flask (500 mL) equipped with a magnetic stirring rod, air condenser and 100 mL dropping funnel, was charged with anhydrous ether (200 mL, Caledon) and $LiAlH_4$ (0.45 g, 0.01 mol). The suspension was stirred at room temperature for 20 min and cooled in an ice bath. To this was added, with stirring over a period of 10 min, a solution of racemic piperitone (3.5 g, 0.02 mol, ICN) in anhydrous ether (50 mL). The ice bath was removed and the solution was stirred for 2 h. Saturated NH_4Cl was added until the solid dissolved, the ether layer decanted, and the aqueous layer extracted with ether (25 mL × 3). The combined ether extract was washed with water (50 mL × 3), saturated NaCl (25 mL), and dried over anhydrous Na_2SO_4. The dried extract was filtered and ether was removed on a rotary evaporator to yield *cis*- and *trans*-piperitols (3.2 g). The crude product was separated by column chromatography (Alumina, Fisher,80-200 mesh) with pentane, ether and methanol. The ratio of *cis*- and *trans*- unsaturated alcohols was 33:67, and the former eluted with pentane : ether (1:1) whereas the latter eluted with ether : methanol (6:4).

The first eluted *cis*-alcohol (**W**), 0.9 g of product with 99% purity by GC, exhibited the following spectra:

IR (film) 3373, 2927, 2869, 2724, 1673, 1473, 1448, 1383, 1233, 1159, 1129, 1046, 1022, 956, 902, 847, 802 cm^{-1};

m/e (relative intensity); 154($M^{+\cdot}$, 4), 139(28.5), 121(2.5), 112(9), 111(8), 97(5), 95(5), 94(3), 93(13), 92(2.5), 91(9), 85(7), 84(100), 83(35), 81(6), 79(9), 77(11), 71(9), 69(9), 67(5), 65(4), 56(10), 55(13.5), 53(6), 51(3), 43(13).

^{1}H NMR ($CDCl_3$) ppm 0.955 (3H,-CH_3,d,J = 6.5 Hz), 0.995 (3H,-CH_3,d,J = 6.5 Hz), 1.31 (1H,m), 1.6-1.8 (2H,m), 1.69 (3H,-CH=C(C$\underline{H}_3$)-,s) 1.9-2.1 (3H,m), 4.12 (1H,-C(OH)$\underline{H}$-,m), 5.63 (1H,=C$\underline{H}$-,m).

The *trans*-alcohol (**B**), 1.7 g of product with 99% purity by GC, exhibited the following spectra:

IR (film) 3318, 2930, 2871, 2724, 2341, 1676, 1466, 1434, 1384, 1297, 1232, 1158, 1049, 1027, 985, 899, 840 cm^{-1};

m/e (relative intensity); 154($M^{+\cdot}$, 8.6), 139(39), 136(11), 121(9.5), 112(10), 111(11.6), 97(7), 95(7), 94(6.5), 93(42), 92(10), 91(33), 85(6), 84(100), 83(43), 81(8), 79(16.5), 78(6), 77(34), 71(11.5), 69(12), 67(8), 65(10.6), 56(12), 55(17.4), 53(10), 51(7), 43(22.4).

^{1}H NMR ($CDCl_3$) ppm 0.84 (3H,-C$\underline{H}_3$,d,J = 7 Hz), 0.96 (3H, -C$\underline{H}_3$,d,J = 7 Hz), 1.24 (1H,m), 1.6-1.75 (2H,m), 1.67 (3H,=C(C$\underline{H}_3$)-,s) 1.9-2.05 (3H,m), 4.01 (1H,-C(OH)$\underline{H}$-,d,J = 7.5 Hz), 5.38 (1H,=C$\underline{H}$-,m).

5.4. Acid catalyzed rearrangement of *cis*-piperitol

5.4.1. Experimental

An Erlenmeyer flask (250 mL), equipped with magnetic stirring rod, was charged with a prepared solution of 0.5% BF_3 in CH_2Cl_2 (150 mL), stirred and cooled to -78°C on a dry ice-acetone bath. To this solution was added, with stirring, a solution of *cis*-piperitol (W) (0.5 g, 0.003 mol) (obtained from the previous experiment) in CH_2Cl_2 (5 mL). The mixture was stirred for 2 h at about -40°C. The reaction was monitored by GC every 15 min. The reaction was quenched with water (100 mL) and the organic layer, separated. The aqueous layer was extracted with CH_2Cl_2 (25 mL × 2). The combined CH_2Cl_2 extract was washed with water (25 mL × 4), saturated $NaHSO_4$ (25 mL), saturated NaCl (25 mL) and dried over anhydrous Na_2SO_4. The solvent was partially removed on a rotary evaporator.

The mixture was transferred to an Erlenmeyer flask (100 mL) with a magnetic stirring rod, and treated with excess acetic anhydride (20 mL) and pyridine (15 mL). The mixture was stirred overnight at rt. The reaction was quenched with water (50 mL) and the mixture extracted with hexane (25 mL × 4). The hexane extract was washed with water (25 mL × 6), saturated NaCl and dried over anhydrous Na_2SO_4. The solution was filtered, evaporated and chromatographed on a silica gel column with hexane : ether - (8 : 2), yielding 0.13 g of product with 99% purity by GC. This product exhibited the following spectra:

IR (film) 3353, 2959, 2871, 1744, 1726, 1643, 1464,1384, 1368, 1230, 1206,1170, 1122, 1105, 1084,1063, 1012, 996, 968, 944, 906, 863, 818, 807, 734 cm^{-1};

m/e (relative intensity); 154($M^{\cdot+}$, 6.4), 139(39), 136(17.5), 121(30), 111(38), 107(8), 97(13), 96(12), 95(25), 94(45), 93(65), 92(17), 91(29), 86(6), 84(33), 83(33), 82(7), 81(44.5), 80(9.5), 79(64), 78(8), 77(37), 71(65), 70(7), 69(55), 68(8), 67(27), 66(6.5), 65(14), 58(12), 57(6), 55(31), 53(11.5), 51(7), 45(5), 43(100).

^1H NMR ($CDCl_3$) ppm 0.89 (3H,-C$\underline{H}_3$,d,J = 7 Hz), 0.91 (3H,-C$\underline{H}_3$,d,J = 7 Hz), 1.27 (3H,-C(OH)C$\underline{H}_3$-,s), 1.35-1.55 (2H,m), 1.65 (2H,m), 1.85 (2H,m), 5.65 (2H,m).

5.5. Comparison of synthetic and natural materials

Gas chromatography and mass spectroscopic comparisons of synthetic samples were performed to confirm their identity with respect to the isolated natural compounds **A** and **B**.

GC analysis of synthetic *cis*-menth-2-en-1-ol and *trans*-piperitol on two GC columns with different retention characters (GC-1 and GC-2, Table 3.1) showed identical retention times to those of natural compound **A** and **B**, respectively, and matched those reported[60,61]. Moreover, the mass spectral fragmentation patterns of both natural compounds **A** and **B** matched those of synthetic *cis*-menth-2-en-1-ol and *trans*-piperitol, respectively and were identical with those in the NIST‡ library.

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‡ U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD.

5.6. Bioassay of synthetic repellents

5.6.1. Methods

Laboratory bioassays were conducted to observe the activity of each of the synthetic candidate repellents on house flies as described in section 2.2. Known weights of racemic samples of **A** and **B** diluted in fractionally distilled hexane were assayed on house flies.

Three series of experimental samples were prepared with **A** and **B** individually and a 1 : 1, w/w mixture of **A** and **B.** Dose response experiments were conducted to determine the minimum quantity required from each compound, separately and in combination, to elicit a significant repellence on house flies (Tables 5.1 - 5.3).

5.6.2. Results

Synthetic compounds **A** and **B** were both repellent eliciting maximum response at concentrations of 1 mg (Table 5.1) and 0.1 mg (Table 5.2), respectively. Compound **B** showed maximum response for the full 6 min test period whereas compound **A** gave maximum response for only 4 min at the 0.1 mg level. Although there was no statistically significant difference in the responses to compounds **A** and **B** at 0.1 mg compound **B** appears to be the major repellent in steam-distilled volatile extracts of pepper tree leaves.

Table 5.1: Dose response of house flies in laboratory bioassays to synthetic compound **A** (*cis*-menth-2-en-1-ol).

Stimulus	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
			0-2min	2-4min	4-6min		
crude pepper oils	0.8	C	9	10	8	27	100
		S	0	0	0	0	
compound A	0.001	C	12	8	11	31	62 ***
		S	8	6	5	19	
,,	0.01	C	28	11	10	49	78 **
		S	3	4	7	14	
,,	0.1	C	24	14	4	42	93
		S	0	0	3	3	
,,	1	C	14	12	9	35	100
		S	0	0	0	0	
,,	10	C	19	8	6	33	100
		S	0	0	0	0	

C - Control
S - Bait, treated with sample
% Response to control station = [Total C/(Total C + Total S)] × 100

Percentages different from those to the crude extract control stimulus are indicated by *, z test, $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$

Table 5.2: Dose response of house flies in laboratory bioassays to synthetic compound **B** (*trans*-piperitol).

Stimulus	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
			0-2min	2-4min	4-6min		
crude pepper oils	0.8	C	10	14	6	30	100
		S	0	0	0	0	
compound B	0.001	C	10	9	5	24	60 ***
		S	7	5	4	16	
,,	0.01	C	14	8	5	27	66 ***
		S	1	6	7	14	
,,	0.1	C	17	11	3	31	100
		S	0	0	0	0	
,,	1	C	26	14	6	46	100
		S	0	0	0	0	
,,	10	C	11	6	9	26	100
		S	0	0	0	0	

C - Control
S - Bait, treated with sample
% Response to control station = [Total C/(Total C + Total S)] × 100

Percentages different from those to the crude extract control stimulus are indicated by *, z test, $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$

A higher response was observed for the binary mixture of **A** and **B** than for the individual compounds. The binary mixture elicited maximum response for 4 min at a 0.01 mg concentration (Table 5.3), whereas neither of the compounds tested alone were maximally responsive at this concentration (Table 5.1 and 5.2). Moreover, the 1 : 1 mixture of compounds **A** and **B** presented as 0.01 dose of the mixture contained only half the dose of either compound tested alone. The high volatility and evaporation of both **A** and **B** is the likely cause of the decline in activity observed at low concentrations.

Table 5.3: Dose response of house flies in laboratory bioassays to 1 : 1 ratio mixture of synthetic compounds **A** (*cis*-menth-2-en-1-ol) and **B** (*trans*-piperitol).

Stimulus	Dose (mg/25µL)	Bait	Total number of sustained contacts in five replicates during 6 min			Total contacts	% Response to control station
			0-2min	2-4min	4-6min		
crude pepper oils	0.8	C	15	15	9	39	100
		S	0	0	0	0	
1:1 ratio mixture of compounds A and B	0.001	C	21	11	11	43	60 ***
		S	14	6	9	29	
,,	0.01	C	27	12	9	48	83 **
		S	0	0	10	10	
,,	0.05	C	24	16	10	50	100
		S	0	0	0	0	
,,	0.1	C	21	12	10	43	100
		S	0	0	0	0	

C - Control
S - Bait, treated with sample
% Response to control station = [Total C/(Total C + Total S)] × 100

Percentages different from those to the crude extract control stimulus are indicated by *, z test, $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$

6. SUMMARY

Steam distillation of pepper tree leaves and solvent extraction of pepper tree berries were effective for extracting house fly repellents; steam distillation provided volatile oils in 1% yield. Vapor entrapment was inefficient in capturing house fly repellents from pepper tree leaves. A two-choice laboratory bioassay was used to monitor the repellent isolation process, utilizing three 2-min periods to observe the feeding activity on sugar-coated, glass cover slips treated with chromatographically purified fractions.

Fractionation of steam-distilled volatile extracts with two different schemes led to behaviorally-active fractions containing compounds **A** and **B**, which comprised about 0.23% of the total volatile extracts. Mass spectral analysis of **A** and **B** disclosed a molecular composition of $C_{10}H_{18}O$, with fragmentation indicative of a hydroxyl group. Compounds **A** and **B** were identified as *cis*-menth-2-en-1-ol and *trans*-piperitol, respectively. Using the acetyl lactyl derivative the absolute configuration of **B** was assigned as (*1S,6S*)-piperitol. The absolute configuration of **A** is unknown.

The mass spectra and GC retention times of synthetic racemic **B** and **A**, prepared from piperitone and *cis*-piperitol, respectively, proved to be identical to those of the natural compounds. Both compounds **A** and **B** were repellent to house flies, but **B** was slightly more active than **A**.

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APPENDIX 1

Preliminary studies for the possible source of biological activity

To observe the biological activity of possible sources for repellents against house flies, preliminary studies were conducted on the grounds of a slaughter house in Nazareth, Ethiopia‡. Feeding and landing of house flies on food baits were observed in two experiments, one with pepper leaves and berries, the other with extracts from leaves and berries.

The first, unreplicated experiment (Exp. 1) was performed with fresh pepper leaves and berries. Five plastic pans (yellow color, 20 × 25 × 8 cm) were placed in randomized order 2 m apart in a cement trough. At the center of each pan was an open Petri dish (10 cm diameter) filled with food bait: Injera (Ethiopian bread) soaked with milk and topped with cow manure. In one treatment intact pepper tree leaves lined the pan several layers deep beneath the Petri dish. In a second treatment the leaves were cut with scissors in into approximately 5 cm long pieces. The Petri dishes in the next two treatments were surrounded with intact or crushed fresh pepper tree berries at least one layer deep. The fifth pan contained only a

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‡ Studies were conducted by Dr. T. Abate, Institute for Agricultural Research, Addis Ababa, Ethiopia, and Professors J.H. Borden and K.N. Slessor of Departments of Biological Sciences and Chemistry, respectivly, SFU.

food bait and served as the control. Landings of house flies on baits were recorded during a 45 min period. The placement of the treatments was re-randomized every 15 min.

Exp. 2 tested the repellency of leaf and berry extracts against house flies. Extracts were made by placing 50 g of freshly picked, cut pepper tree leaves or crushed pepper berries in ethanol (100 mL), and leaving the extract preparations in a closed glass jar for approximately 16 h. Six open Petri dishes were filled with a cow manure bait, over which approximately 5 mL of milk was poured. The leaf extract (10 mL, 5 g equivalents) was poured over the bait in each of two treatments, berry extract (10 mL, 5 g equivalents) was added to each of two more dishes, and ethanol (10 mL) was poured over the bait in two solvent control dishes. House fly landings were measured as in first experiment, except that contacts were recorded as brief, lasting < 2 sec or sustained, > 2 sec.

Preliminary study results

Results of the Exp. 1 (Table 1) show that both, cut pepper leaves and crushed berries, repelled house flies while intact leaves and berries did not. These results indicate that the pepper tree berries and leaves contain one or more compounds that repel house flies.

Results of Exp. 2 (Table 1) show that the active repellents in the pepper tree are solvent-extractable and stable in solution. The leaf extract reduced sustained contacts by house flies to half those to the control, and the

berry extract was twice as repellent as the leaf extract (Table 1). In addition, all of the landings on the solvent control were sustained from several sec to several min, while several landings on the extract-treated manure were brief. It is possible that the conspicuously green color of the leaf extract could have attracted the flies, partially offsetting the repellency in comparison to the clear berry extract.

Response of house flies in in preliminary field bioassays to baits treated with pepper tree leaves and berries in different states (Exp. 1) and to baits treated with ethanolic extracts of pepper tree leaves and berries (Exp. 2).

TREATMENT	NUMBER OF HOUSE FLY LANDINGS	
Experiment 1		
untreated control	25	
bait treated with fresh pepper tree leaves	26	
bait treated with fresh pepper tree berries	24	
bait treated with cut pepper tree leaves	7	
bait treated with crushed pepper tree berries	8	
Experiment 2	Sustained contacts	Brief contacts
bait treated with ethanol (control)	42	0
baits treated with ethanolic extracts of pepper tree leaves (5 g equivalent)	20	5
baits treated with ethanolic extracts of pepper tree berries (5 g equivalent)	11	9