The Olfactory Basis for Attraction of the Bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) to Host-Plant Flowers

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A thesis submitted in partial fulfilment of the requirements of the University of Greenwich for the Degree of Doctor of Philosophy

This research programme was carried out at the Natural Resources Institute, UK and field experiments were in collaboration with the Volcani Center, Israel and Ali Tareen Farm, Pakistan

October 2000

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Abstract

The objective of this work was to investigate whether or not olfactory clues play a role in host plant location by the polyphagous moth, *Helicoverpa armigera*. Volatiles collected from flowers of African marigold, *Tagetes erecta*, and sweet pea, *Lathyrus odouratus*, were found to elicit electroantennographic (EAG) responses from the antennae of female *H. armigera*. Compounds active in GC-EAG analyses of *T. erecta* floral headspace samples, identified by GC-MS and comparison of retention times on polar and non-polar GC columns with authentic standards, were (*E*)-myroxide, benzaldehyde, (±)-linalool, phenylacetaldehyde and (-)-piperitone. EAG-active compounds in *L. odouratus* floral headspace samples were identified as diacetone, (-)-linalool, phenylacetaldehdyde and benzyl alcohol. Increases in upwind flight to air entrained extracts of floral odours indicated that these cues caused attraction when presented to female *H. armigera*. A synthetic *T. erecta* blend comprising benzaldehyde, (±)-linalool, phenylacetaldehyde and (+)-limonene gave significant increases in upwind flight approaches. Limonene (either (+)- or (-)-) was found to be important for the behavioural response despite having low EAG-activity. There was no significant difference in upwind flight response to odours from the live flower and the synthetic floral blend. Significant increases in upwind flight were also obtained when insects were presented with a synthetic *L. odouratus* blend which contained the four EAG-active compounds identified from GC-EAG studies. In field trapping experiments in Israel there was a significant difference in *H. armigera* catches in traps with a standard 4-component *T. erecta* lure compared with unbaited traps over the whole season. Mean *H. armigera* catch per trap per night (both sexes) over the whole season in unbaited traps, floral odour traps, pheromone traps and light traps were 0.004, 0.11, 8.8 and 1.35 respectively. The floral baited traps were non-selective catching large numbers of Hymenoptera and Diptera as well as other moth species. Field trapping experiments in Pakistan indicated that the floral lure was significantly attractive to *Earias* spp. and other Lepidoptera although very few *H. armigera* were caught due to low population density. Olfactory cues are discussed in relation to host-plant finding behaviour of *H. armigera*. They are involved in early stages of host seeking behaviour prior to alighting on the plant and stimulate searching behaviour.

Table of Contents

Declaration i

Abstract ii

Table of Contents iii

Acknowledgements ix

Chapter 1: THESIS OVERVIEW

1.1 Principle Objective 1

1.2 Background 1

1.2.1 *Helicoverpa armigera* 1

1.2.2 Floral Volatiles 2

1.3 Thesis Plan 3

Chapter 2: GENERAL INTRODUCTION

2.1 *Helicoverpa armigera*: Pest Status and Biology 5

2.1.1 Pest Status 5

2.1.2 Mobility and Nocturnal Cycle of Activity 6

2.1.3 Host Plants 8

2.2 Pest Management 13

2.2.1 Use of Insecticides 13

2.2.2 Alternative Control Methods 14

2.2.3 Area Wide Management 17

2.2.4 Trap Crops 18

2.3 Semiochemicals 20

2.3.1 Types of Semiochemicals 21

2.3.2 Semiochemicals and Host-Plant Location 21

2.3.3 Use of Semiochemicals to Improve Attractiveness of Trap Crops 22

2.3.4 Rationale for Identification of Floral Odours to Attract *H. armigera* 22

2.4 Host-Plant Location by Phytophagous Insects 23

2.4.1 Searching Behaviour in Insect Host-Plant Finding 23

2.4.2 Sensory Cues Involved in Host-Plant Finding 27
2.4.3 Floral Attractants and other Kairomones for Moth Pests 37
2.5 Use of Floral Odours in *H. armigera* Control 41
2.5.1 Floral Lures: Potential Problems and Possible Advantages 41
2.5.2 Factors Influencing Performance of Baited Traps 42
2.6 Research Hypothesis 43

Chapter 3: METHODS USED TO REAR INSECTS AND COLLECT PLANT VOLATILES

3.1 Introduction 45
3.2 Insect Rearing 45
3.2.1 Materials and Methods 45
3.2.2 Discussion 49
3.3 Plant Cultivation 49
3.3.1 Materials and Methods 49
3.3.2 Discussion 50
3.4 Collection of Floral Volatiles by Air Entrainment 51
3.4.1 Materials and Methods 51
3.4.2 Discussion 53

Chapter 4: IDENTIFICATION OF ELECTROPHYSIOLOGICALLY-ACTIVE COMPOUNDS IN FLORAL VOLATILES USING LINKED GAS CHROMATOGRAPHY AND ELECTROANTENNOGRAPHY

4.1 Introduction 55
4.2 Methods and Materials 57
4.2.1 Electoanntennography (EAG) Preparations 57
4.2.2 EAG analysis of crude extracts of floral volatiles 59
4.2.3 Linked GC-EAG analyses 60
4.3 Results 63
4.3.1 EAG analyses of extracts of floral volatiles collected on Porapak Q 63
4.3.2. GC-EAG analyses 64
4.4 Discussion 70

Chapter 5: CHARACTERISATION AND QUANTIFICATION OF FLORAL VOLATILES BY LINKED GAS CHROMATOGRAPHY - MASS SPECTROMETRY

5.1 Introduction 74

5.2 Methods and Materials 75

5.2.1 Synthetic chemicals 75

5.2.2 GC-MS Analysis of Natural Samples 76

5.2.3 GC Analysis of Chiral Compounds. 76

5.2.4 Quantification of Compounds in Natural Samples by GC Analysis 76

5.2.5 GC Analyses to Quantify Release Rate of Compounds from Live Flowers 77

5.3 Results 78

5.3.1 *Tagetes erecta* 78

5.3.2 *Lathyrus odouratus* 86

5.3.3 Quantification of EAG-active Compounds in Floral volatiles 91

5.4 Discussion 94

Chapter 6: EAG ANALYSIS OF SYNTHETIC COMPOUNDS

6.1 Introduction 98

6.2 Methods and Materials 99

6.2.1 Comparison of relative EAG activity of synthetic host odour volatiles at a single dose 99

6.2.2 Effect of dose on the magnitude of EAG responses elicited by synthetic host volatiles 100

6.3 Results 101

6.3.1 Comparison of relative EAG activity of synthetic host odour volatiles at a single dose 101

6.3.2 Effect of dose on the magnitude of the EAG response elicited from female *H. armigera* by synthetic floral volatiles 109

6.4 Discussion 113

Chapter 7: WIND TUNNEL BIOASSAY WITH AIR ENTRAINED SAMPLES AND PUTATIVE SYNTHETIC KAIROMONE BLENDS

7.1 Introduction 119

7.2 Methods and Materials 119

7.3 Results 123

7.3.1 Bioassay Series 1: Behavioural response of male *H. armigera* to the female sex pheromone 123

7.3.2 Bioassay Series 2: Behavioural responses of female *H. armigera* to cut *T. erecta* flowers 124

7.3.3 Bioassay Series 3: Behavioural responses of female *H. armigera* to entrained floral volatiles from cut flowers of *T. erecta* 125

7.3.4 Bioassay Series 4: Behavioural responses of female *H. armigera* to blends of synthetic compounds based on the composition of floral volatiles isolated from flowers of *T. erecta* 127

7.3.5 Bioassay Series 5: Behavioural responses of female *H. armigera* to blends of synthetic compounds based on the composition of floral volatiles isolated from flowers of *T. erecta* 129

7.3.6 Bioassay Series 6: Behavioural responses of female *H. armigera* to different doses of a synthetic blend of volatiles based on the composition of floral volatiles isolated from flowers of *T. erecta* 132

7.3.7 Bioassay Series 7: Behavioural responses of female *H. armigera* to a floral volatiles from a live flower of *T. erecta* and synthetic blends of floral volatiles identified from flowers of *T. erecta* 133

7.3.8 Bioassay Series 8: Behavioural responses of female *H. armigera* to synthetic blends of volatiles based on *T. erecta* flowers 136

7.3.9 Bioassay Series 9: Behavioural responses of female *H. armigera* to a 3-component synthetic blend based on floral volatiles identified from flowers of *T. erecta* and phenylacetaldehyde presented alone 138
7.3.10 Bioassay Series 10: Behavioural responses of female *H. armigera* to odours ducted from a live *T. erecta* flower and synthetic blends of volatiles based on *T. erecta* flowers 140
7.3.11 Bioassay Series 11: Behavioural responses elicited from a Pakistani strain of *H. armigera* to a synthetic blend of volatiles based on *T. erecta* flowers 141
7.3.12 Bioassay Series 12: Behavioural responses of female *H. armigera* to natural cut *L. odouratus* and *Buddleia davidii* floral headspace volatiles 143
7.3.13 Bioassay Series 13: Behavioural responses of female *H. armigera* a synthetic blend of volatiles based on *L. odouratus* flowers 145
7.4 Discussion 147

Chapter 8: DEVELOPMENT OF A CONTROLLED RELEASE FORMULATION

8.1 Introduction 151
8.2 Methods and Materials 152
8.2.1 Determination of Release Rates from Filter Papers used in Bioassays 152
8.2.2 Characterisation of Release Rates from Polyethylene Sachets 153
8.3 Results 155
8.3.1 Determination of Release Rates from Filter Papers used in Bioassays 155
8.3.2 Longevity of Release Rates from Polyethylene Sachets 158
8.4 Discussion 167

Chapter 9: FIELD TRIAL

9.1 Introduction 169
9.2 Methods and Materials 170

9.2.1 Israel Field Trial 170
9.3.2 Pakistan Field Trial 175
9.3 Results
9.3.1 Israel Field Trial 176
9.3.2 Pakistan Field Trial 201
9.4 Discussion 207
9.4.1 Trapping Experiments in Israel 207
9.4.2 Trapping Experiments in Pakistan 210
9.4.3 Potential Practical Applications of Floral Odour Baited Traps in Pest Management 211

Chapter 10: GENERAL DISCUSSION

10.1 Introduction 217
10.2 Evidence obtained supporting a role for olfaction in the process of host-plant finding by *H. armigera* 217
10.3 Identification of behaviourally relevant volatiles from headspace samples 221
10.4 Host-plant Finding Behaviour of *H. armigera* 225
10.5 Future Work 231

REFERENCES 233

Acknowledgements

I wish to thank Prof. Hall for advice (especially on racemisation of (-)-piperitone and interpretation of the mass spec of diacetone) and encouragement, and his patience going through various drafts of the thesis, my supervisor Dr Alan Cork for advice on the research, and comments and help with the thesis manuscript, and Mr Dudley Farman for technical advice and support. I thank Dr K Srinivasan, Nagarjuna Agricultural Research and Development Institute, India, for suggesting use of African marigold and provision of seed material, Quest International for samples of synthetic myroxide and Daniels Ltd. for a sample of synthetic piperitone, and ICRISAT, Patancheru, India for supplying the pupae used to start the *H. armigera* culture. I thank Chanban Black for assistance with cultivation of plants in the glasshouse. I am grateful to Dr Ezra Dunkelblum of the Agricultural Research Organisation, The Volcani Center, Bet Dagen, Israel for access to facilities and kind participation in field trapping experiments. I thank Mr Ali Khan Tareen for his hospitality during my stay on Ali Tareen Farm, Lohdran, Multan, Pakistan and David Chamberlain for his enthusiastic support during field trapping experiments in Pakistan. Thanks be to God that this project, which I found interesting and enjoyable, has been completed. I also wish to take this opportunity to thank my parents. The research was funded by a Natural Resources Institute Research Studentship.

Chapter 1

THESIS OVERVIEW

1.1 Principle Objective

The objective of this work was to investigate whether or not floral odours play a role in host-plant location by the cotton bollworm, *Helicoverpa armigera*.

1.2 Background

1.2.1 *Helicoverpa armigera*

Helicoverpa armigera Hübner (Lepidoptera: Noctuidae) is a widely distributed, polyphagous and mobile Lepidopteran insect pest which attacks the fruiting structures of important cash and subsistence crops including cotton, tobacco, maize, tomato, chickpea and pigeonpea. It is one of the most serious agricultural insect pests worldwide:

> *"The economic significance of Helicoverpa damage to crops and the human misery that has resulted are well documented and paralleled only by the devastating locust plagues of Africa"*, McDonald (1990).

The damaging stage is the larva. Historically *H. armigera* control has depended almost exclusively on larvicidal insecticides (King, 1994). Its status as a pest has risen due to the spread of strains resistant to most conventional insecticides. This has led to outbreaks such as in 1987-88 in Andhra Pradesh, India (Venugopal Rao *et al.*, 1996; Jayaswal, 1989). The high demand for cotton and its importance as a cash crop has made the achievement of high cotton yields a main objective of growers. Use of monocultures of pest-susceptible, input-responsive varieties has hastened the spread of insecticide resistance in *H. armigera* since this system of cultivation requires much insecticide application to protect the lush fertilised crop. According to King (1994) the four major species of *Heliothis* and *Helicoverpa* have been responsible for

the use and misuse of more agricultural insecticide than any other insect species. Intensive use of insecticides has created a high selection pressure for insecticide resistant strains meaning that intensified production has often been associated with increased pest management problems. Pyrethroid resistance in *H. armigera* was first detected in Australia in 1983 (Gunning *et al.*, 1984). Since then resistance to this relatively new class of insecticides has become more widespread and has exacerbated control problems in many places.

1.2.2 Floral Volatiles

Female *H. armigera* are known to oviposit preferentially on the flowering stage of their host plants (Parsons, 1940). African marigold, *Tagetes erecta*, has been used as a trap crop for *H. armigera*, drawing ovipositing female moths away from cotton (Patel & Yadav, 1992; Srinivasan *et al.*, 1994). This study set out to examine whether floral volatiles from *T. erecta* were attractive to *H. armigera* and to identify any components responsible for attraction. Volatiles from sweet pea, *Lathyrus odouratus* (Leguminosae), one of the many other host plants of *H. armigera* (Zalucki *et al.*, 1986) were also examined. This is well known for its strong scent and has larger flowers than other Leguminosae.

At a practical level, traps baited with synthetic floral volatiles might be used for monitoring and control of *H. armigera*. A synthetic lure would have the advantages over a trap crop of not taking up land that might otherwise be used for the main crop and of operating throughout the cropping season. The trap crop is only attractive for the short flowering stage (Srinivasan *et al.*, 1994). The study also provides an improved understanding of the mechanisms of host plant location used by *H. armigera*, providing insights into plant characteristics which might be manipulated in plant breeding programmes to obtain more resistant crop cultivars.

1.3 Thesis plan

The various stages of the project are outlined in Fig 1. Volatiles were collected from cut and intact flowers of *T. erecta*, *L. odouratus* and also *B. davidii* (Chapter 3). It was assumed that *H. armigera* moths would detect odours by means of receptors on their antennae and electroantennographic (EAG) recording was used to determine whether the volatile collections were detected by the insect (Chapter 4).

Gas chromatography linked to EAG was used to determine which particular components of the collections were detected by the insect (Chapter 4), and the chemical structures of these active compounds were determined by comparison of GC retention times and mass spectra with those of synthetic standards (Chapter 5). The EAG activity of synthetic compounds was then confirmed (Chapter 6).

In parallel with this the behavioural activity of the volatile collections was evaluated in a laboratory wind-tunnel. The synthetic compounds identified in floral headspace collections, particularly those shown to be EAG active, were then tested in the wind-tunnel and the most attractive blend developed (Chapter 7). In order that the synthetic blends could be evaluated in the field, dispensing systems were devised that protected the compounds and released them at the required rate for a sustained period (Chapter 8). Field trapping experiments were carried out in Israel and Pakistan to determine whether *H. armigera* and / or any other insects could be captured with the blends of synthetic compounds identified in floral volatiles (Chapter 9).

Finally the results are discussed in relation to whether or not floral volatiles play a role in host location by female *H. armigera* and their importance in the overall process of host location.

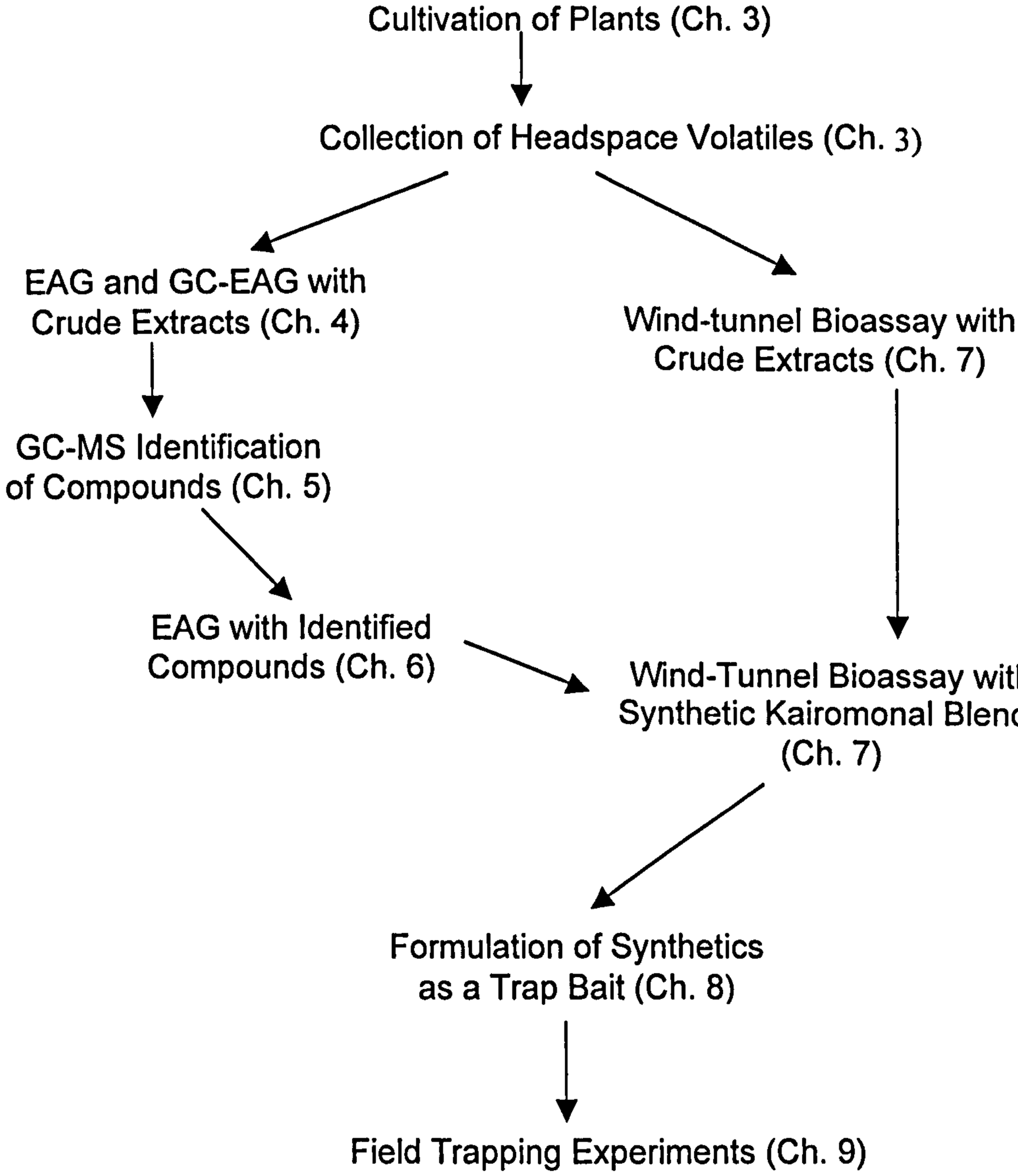

Fig 1. Flow Diagram of Various Stages of the Project

Chapter 2

GENERAL INTRODUCTION

2.1 *Helicoverpa armigera*: Pest Status and Biology

2.1.1 Pest Status

H. armigera has one of the widest distributions of any agricultural pest, occurring throughout Africa, the Middle East, southern Europe, India, central and southeastern Asia, eastern and northern Australia, New Zealand, and many Pacific Islands (Fitt, 1989). The related species *Helicoverpa zea* (Plate 2.1) and *Heliothis virescens* are important agricultural pests in the New World.

A combination of life history traits make it a threatening pest if not managed effectively (Fitt, 1989). These are its high fecundity, high adult mobility, polyphagy, capability for diapause, and a larval stage which is often concealed within the fruit or boll (which makes it a difficult target for insecticide application). *H. armigera* is highly fecund with one female being capable of laying up to 3000 eggs, usually laying 1000-1500 (Fitt, 1989). In the tropics it may breed continuously completing a generation in as little as 28-30 days and pass through 10-11 generations per year. Mobility and facultative diapause help it to cope with changing spatial and temporal distributions of host plants. It is a particularly damaging pest because larval feeding preference is for growing points and reproductive structures which are economically important parts of a crop. One larva destroys several bolls or fruits during its development. Its pest status is further elevated by the ease with which it develops resistance to insecticides. As control has traditionally depended on insecticides the lack of products to which it is not resistant is currently a major problem. In high value crops such as cotton and tomato damage thresholds are low. Serious yield losses of up to 80% can be caused by *Helicoverpa* species (Rosier, 1990).

Plate 2.1 *Helicoverpa zea* on a Cotton Boll. Source: USDA Website (http://www.ars.usda.gov/is/graphics/photos/k4695-6.jpg)

2.1.2 Nocturnal Cycle of Activity and Mobility

Description of Behaviour

The nocturnal cycle of activity for *H. armigera* has been characterised (Roome, 1975; Riley *et al.*, 1992; Topper, 1987): both sexes fly and feed, and females oviposit in the first three hours after sunset. Later in the night (02.00 to 04.00h, Roome 1975) males fly above the crop in a "purposeful" manner while the females are stationary and release pheromone which leads to mating. Topper (1987) emphasised the importance of feeding for adult survival since laboratory reared moths fed sugar solution rather than just water had a significantly longer lifespan. This means that feeding is a high priority activity.

The first flight activity period is prolonged at poor quality adult feeding sites and a redistribution of moths towards more favourable host plants occurs (Topper, 1987). Emergence starts around sunset and virtually ceases by 01.00 h. On the night of emergence moths fly very little and flight activity of one-day old moths is

concentrated within the first hour after sunset which is the nectar foraging part of the nocturnal cycle (Riley *et al.*, 1992). Complementary laboratory studies also showed that the most important female ages for oviposition were 4, 5 and 6 days after emergence when 74% of the total eggs were laid (Topper, 1987). Radar recordings carried out by Riley *et al.* (1992) showed that aerial densities at 4m were almost two orders of magnitude higher than at 26m indicating that most flight was at low altitude.

Relationship with Nectar Production

The periodicity of feeding is similar in *Helicoverpa zea* (Adler, 1987; Beerwinkle *et al.* 1993) occurring just before and up to 2 hours after sunset. Adler suggests that this might be linked to the diel rhythm of nectar production in the host plants. For *Helicoverpa punctigera* Adjei-Maafo & Wilson (1983) found a strong correlation between oviposition and nectar production, during the first 13 weeks of a cotton crop's development. This suggests that oviposition depends on there being adequate adult food as proposed by Quaintance & Brues in 1905 (Topper, 1987). However, it is not clear whether more eggs are produced because of the good nectar supply or whether the moths are drawn in by the attractive nectar and, as they alternate between bouts of feeding and oviposition, lay the eggs that otherwise would have been laid elsewhere. According to Beerwinkle *et al.* (1993) feeding is a high priority activity among very young female moths: the majority of moths, that were collected by sweep net whilst feeding, were less than one day old. Dissection analyses of sampled females showed that 95% were unmated, indicating that the characteristic age of the feeding moths was one day or less.

Mobility

Mark recapture experiments have documented movements by individual moths over distances ranging from 25 to 160 km (Fitt, 1989). Migration behaviour is characterised by suppression of appetitive responses to food-associated external stimuli that might otherwise arrest the insect. Smaller scale movements can be divided into dispersal flight and foraging flight (Ramaswamy, 1988) or into 'non-directed movement' and 'directed movement' (Schyleter, 1992). These movements influence sampling programmes (Wall & Perry, 1987) by affecting the 'range of

sampling' which is the distance from which insects can be shown to reach the source in a given time period and 'range of attraction' which is the maximum distance over which insects can be shown to direct their movement to the source (definitions from Schyleter, 1992). According to Schyleter (1992) maximum range of attraction for Lepidoptera may reach 200-400m with a sex pheromone lure.

2.1.3 Host Plants

In a review of host-plant records Zalucki *et al.* (1986) presented a table detailing 102 recorded potential host plants of *Helicoverpa armigera* or '*Heliothis* spp.'. However they were sceptical about accepting all of them since it was not confirmed that the full life cycle could be completed on all of these species. Nevertheless it is clear that host plants certainly are numerous. An extensive survey of host plants in Australia by Zalucki *et al.* (1994) found 26 additional previously unknown host plants that *H. armigera* could be reared from. According to Jayaswal (1989) it feeds on 157 species of plant in India, of which 96 are crops.

Ecological factors favouring a broad host range are lower searching costs, less chance of completely failing to locate a suitable host-plant and avoiding crowding and density-dependent mortality (Rausher, 1992). The main host families include Asteraceae, Fabaceae, Leguminaceae, Malvaceae, Poaceae and Solanaceae. Well developed detoxification systems are needed to cope with the range of secondary metabolites encountered in these plants (Fitt, 1989) which partly explains the ease with which it has evolved resistance to man-made insecticides. Of the hundreds of compounds found in host-plant extracts by Breeden *et al.* (1996), none were observed to act as oviposition deterrents, indicating the tolerance *H. armigera* has to a range of chemicals. As can be seen in Table 2.1 several important cash and subsistence crops are attacked by *H. armigera*.

Table 2.1 Crop Host Plants of *Helicoverpa armigera*

FAMILY	COMMON NAME	SCIENTIFIC NAME
Asteraceae	Sunflower	*Helianthus annus*
	Lettuce	*Lactuca sativa*
Brassicaceae	Rape	*Brassica napus*
	Black mustard	*Brassica nigra*
	Cabbage	*Brassica oleracea* var. *capitata*
	Broccoli	*Brassica oleracea* var. *italica*
	Turnip	*Brassica rapa*
Caricaceae	Pawpaw	*Carica papaya*
Chenopodiaceae	Beetroot	*Beta vulgaris*
Cucurbitaceae	Melon	*Citrullus lantanus*
Euphorbiaceae	Castor oil plant	*Ricinus communis*
Fabaceae	Peanut	*Arachis hypogea*
	Pigeonpea	*Cajanus cajan*
	Chickpea	*Cicer arietinun*
	Soybean	*Glycine max*
	Sweet pea	*Lathyrus odouratus*
	Lucerne	*Medicago sativa*
	Bean	*Phaseolus vulgaris*
	Pea	*Pisum sativum*
	Cowpea	*Vigna unguiculata*
Lamiaceae	Marjoram	*Origanum vulgare*
Linaceae	Linseed	*Linum usitatissimum*
Malvaceae	Okra	*Abelmoschus esculentus*
	Cotton	*Gossypium hirsutum*
Myrtaceae	Tea tree	*Melaleuca incana*
Poaceae	Wheat	*Triticum aestivum*
	Maize	*Zea mays*
Rutaceae	Lemon	*Citrus limon*
	Orange	*Citrus sinensis*
Solanaceae	Tomato	*Lycopersicum lycopersicum*
	Tobacco	*Nicotiana tabacum*
	Potato	*Solanum tuberosum*
Vitaceae	Grape	*Vitis vinifera*
Zingiberaceae	Ginger	*Zingiber officinale*

(from Zalucki *et al.*, 1986)

Preferred Growth Stage of the Host Plant

Although the host plants of *H. armigera* are numerous, including both monocotyledons and dicotyledons, the flowering stage is most preferred for oviposition. Parsons (1940) states:

> "*For all practical purposes egg-laying may be considered as confined to the period of florescence*".

According to Roome (1975) oviposition coincides almost without exception with the commencement of flowering of the host. Fitt (1989) considers the strong preference by all species for the flowering stage of their hosts as "the most consistent pattern in host selection". The increase in oviposition on the flowering stage of host-plants could be due to an increase in the chemical attractiveness of these crops (Zalucki *et al.*, 1986). If eggs are laid on the flowering stage of the host-plant then the larvae can feed on the fruit (Johnson *et al*, 1975) which is their preferred feeding site. This explains why preference for the flowering stage has been selected for. Neonate larvae of *Helicoverpa* spp. do not move far from their egg shells before they begin feeding and if young larvae try to leave a plant their chances of finding another suitable host are low (Jackson, 1990). Thus, oviposition by the female controls choice of larval host-plant.

Attractiveness is strongly moderated by the growth stage of the host and growth stage preferences tend to override preferences for different host species. Roome (1975) noted that more mated female *H. armigera* were caught in light traps sited in flowering sorghum and tasseling maize than in traps sited in maturing crops. Oviposition even occurred on non-hosts that larvae did not survive on when they were presented in the flowering stage in a choice test along with hosts that were not in flower (Firempong & Zalucki, 1990a). Firempong & Zalucki (1990a) also found that taller plants were preferred for oviposition. A noticeable drop in oviposition preference for sunflower flowers occurred when the flowers were lowered to the same height as the other host plants it was compared with. This indicated that more apparent plants are preferred and it was suggested that moths might use the silhouette in host-plant location.

Preferred Host Plants

There are preferences between host-plant species (Roome, 1975; Hillhouse & Pitre 1976). Preference is moderated by the relative abundances of the different host species (Fitt, 1989). What Freeman *et al.* (1967) have described as an "adaptive host-plant shift" occurs when the primary host-plant decreases in number and suitable secondary hosts increase (Johnson *et al.*, 1975). This high adaptability and potential to utilise many different hosts means *H. armigera* can survive well in the off-season of the predominant host crop. In the Sudan Gezira groundnuts are an important alternative host when sorghum and cotton are not available or are not at the attractive growth stage (Topper, 1987). It is interesting that cotton, *Gossypium hirsutum*, although particularly susceptible to damage by *Heliothis* spp., is not actually a preferred host (Fitt, 1989; Ramnath *et al.*, 1992) and in many areas cotton is heavily attacked only after alternative hosts have senesced. Although there is considerable information on the distribution or pattern of host-plant utilisation, little is known about the actual process of host location and recognition (King, 1994).

In laboratory choice tests Firempong & Zalucki (1990b) found that oviposition preferences between host species occurred. Three consistent groupings were found, sunflower, *Helianthus annus*, tobacco, *Nicotiana tabacum*, and maize, *Zea mays*, being most favoured followed by cotton, *G. hirsutum*, soybean, *Glycine max*, and lucerne, *Medicago sativa*, which were of intermediate preference, and then pigweed, *Trianthema portulacastrum*, cabbage, *Brassica oleraceae*, and linseed, *Linum usitatissimum* which were least favoured. It is notable that cotton was not in the most preferred grouping. It may be chosen in the field because it is more abundant than other host plants of *H. armigera.* Cotton has a longer growing season than other crop host plants which means it is available for a longer time (Wardhaugh *et al.* 1980). Although Firempong & Zalucki (1990b) found slight differences in preferences in populations collected from different localities, the overall ranking of hosts was the same. They suggest that *H. armigera* migration, overlap in the distribution of its host plants and the unpredictability of its hosts in time and space make strong genetic differentiation in preferences between populations unlikely.

Oviposition response was investigated by Ramnath *et al.* (1992) and again the finding was that cotton was not the most preferred host-plant. This coincides with the findings of Goyal & Rathore (1988) regarding growth and fecundity of *H. armigera* on different host plants: gram, *Cicer arietinun*, being most favourable followed, in order, by pea, *Pisum sativum*, linseed, *Linum usitatissimum*, tomato, *Lycopersicum lycopersicum*, and then cotton, *G. hirsutum*. In a field study of a cotton-okra intercrop Singh *et al.* (1993) found an oviposition preference for okra, *Abelmoschus esculentus*. However, despite this more larvae were found on the cotton than on the okra.

Alternative Hosts

As well as the main crop hosts, other plants including weeds and wild plants have also been found to be important as alternative hosts. *H. armigera* is so versatile with host-plant requirements that it always has a good chance of finding some host or other, especially when combined with the high mobility of the adult. This opportunistic life strategy enables it to survive well as a residual population when the main crop hosts are not available. As mentioned earlier it also has capability for diapause in the pupal stage if environmental conditions become unfavourable. The weeds *Chenopodium album* and *Melilotus alba*, which are abundant in the chickpea ecosystem of N. India, were preferred to chickpea, *C. arietinum*, in caged oviposition bioassays (Bajpai & Sehgal, 1993). Reasonable larval survival was obtained when larvae were reared on these two weed hosts in the laboratory though survival was greater on chickpea. Damage to winter flowering ornamentals by *H. armigera* was reported by Singh & Arora (1989), with notable damage reported on snapdragon, carnation, dahlia, aster, sweetwilliam and hollyhock. Having so many different host plants means that active stages can extend over a longer period than the growing season of any single species of host and in tropical conditions there can easily be year round reproduction.

Oviposition Sites on the Plant

According to Zalom *et al.* (1983) the majority of eggs are laid on the ventral leaf surface of tomato *L. lycopersicum*, within one leaf of an inflorescence with the terminal half of branches being preferred to the interior. Singh *et al.* (1995) found

that eggs were distributed mostly in the upper canopy and that the upper leaf surface was the preferred site for oviposition in cotton, *G. hirsutum*, gram, *C. arietinum*, and tomato, *L. lycopersicum*. However, Singh *et al.* (1995) found that the underside of flower buds was preferred in sunflower, *H. annus*. Zalom *et al.* (1983) suggested that because of the frequency with which eggs were found on the leaf immediately below the inflorescence, to save time, crop inspections might be restricted to that leaf. Patel & Yadov (1992) found that, with marigold, *T. erecta*, opened flowers were preferred over buds. Zalucki *et al.* (1986) stated that hairy or rough textured surfaces attracted more oviposition.

2.2 Pest Management

Details of pest management practices used in a particular area depend on the agro-ecosystem, pest incidence and socio-economic conditions (Matthews, 1997). Whether *H. armigera* infestations originate from local or distant sources can affect which control practices are appropriate. Early season suppression and destruction of pupae are tactics which are more suited to locally originating infestations.

2.2.1 Use of Insecticides

The dominant control method that has been used in cotton at least since the 1950s for protection against *H. armigera* has been application of insecticides, mainly directed at the larval stage (King, 1994; Wilson, 1982). This reliance on chemical control has not declined despite research into other control methods and problems of periodic development of insecticide resistance to different classes of chemical (organochlorines, organophosphates and pyrethroids). Nevertheless insecticide use is becoming more rational in places where pest-scouting is implemented to time applications and where there is improved targeting of insecticide applications (Russell *et al.* 1998). Examples of improved targeting are timing sprays to reach earlier instars which are easier to control and minimising losses of pesticide caused by runoff and drift (Matthews, 1979). This means that quantities of insecticides used can be reduced. Monitoring pest occurrence with pheromone traps can help improve spray timing. The need for insecticide resistance management has become more recognised. Insecticides of different categories are

alternated and attempts are made to reduce selection pressure for resistance by reducing the number of sprays (Denholm & Rowland, 1992).

The dominance of insecticide usage has meant that more biologically based approaches to pest management have been neglected and sometimes cannot be used because they are incompatible with it. The complexity of having to control a range of pests and the seriousness of insect damage in cotton has meant that it has been hard even to avoid application of insecticide early in the season in order to conserve natural enemies (Matthews, 1997). Application of insecticides against *H. armigera* can disrupt biocontrol of other pest species such as red spider mites and whitefly. Natural control has been considered insufficient in cotton and the many environmental and socio-economic factors influencing economic thresholds have even made need-based pesticide application difficult to adopt (King, 1994). However, the development of insecticide resistance by *H. armigera* even to relatively new insecticides such as pyrethroids and concern about side-effects of pesticides have been incentives to integrate other control tactics into pest management programmes.

2.2.2 Alternative Control Methods

Cultural control, biological control and the use of host-plant resistance are the main categories of alternative control methods. Cultural control involves manipulation of the growing conditions, biological control involves use of natural enemies, and host-plant resistance involves the selection of pest resistant crop varieties. Cultural control methods include the use of short season cultivars to reduce the time over which the crop is at risk, trap cropping, post-harvest cultivation to destroy pupae, avoiding over-application of fertiliser which makes the crop more attractive to pests, and removal of alternative hosts of *H. armigera* (King, 1994; Sundaramurthy & Chitra, 1992; Russell *et al.*, 1998). Mating disruption using sex pheromone did not control *H. armigera*, probably due to its high mobility which increases the chances of mated females immigrating from outside the treated area (King, 1994).

Natural Enemies

Natural enemies play an important role in reducing pest population densities. Biocontrol can be a valuable component of an IPM programme where several tactics are used for lowering pest population densities. The mortality it causes can be useful in combination with other strategies such as insect resistant crop cultivars or pesticides. It may even slow down the development of resistant pest strains by causing selection to act in a different direction than solely towards insecticide resistance. However, for effective pest management the mortality caused by natural enemies has to be greater than the fecundity of the pest, which is high in the case of *H. armigera.* Sudden upsurges of *Helicoverpa* spp. have to date only been successfully controlled with hard (high toxicity) insecticides (Wilson, 1982). For most situations it would be advantageous if any natural enemies released were insecticide-resistant.

H. armigera, H. zea and *H. punctigera* were considered major pests before the widespread use of pesticides and remain so even in areas where insecticide use is minimal according to Fitt (1989). However, King (1994) stated that *H. armigera* problems in South India have been exacerbated by insecticide applications against other pests. In the absence of pesticides in many places natural biological control of *Helicoverpa* spp. may be insufficient to avoid economic damage if it does not cause irreplaceable mortality. However, in the south-eastern and western US *H. virescens* became a major pest only after introduction of synthetic organic pesticides and this has been attributed to disruption of natural enemy populations by insecticides (Fitt, 1989).

Biological control can be achieved by augmentation or conservation of native natural enemies or by importation of exotic natural enemies. Parasites, predators or pathogens of *H. armigera* may be used. According to Russell *et al.* (1998) the establishment levels of exotic natural enemies are generally low and augmentation with native species is a better option. The parasitoid hymenopteran genus *Trichogramma* is often used because these species are amenable to mass production (King, 1994). *Trichogramma pintoi* and to a lesser extent *Bracon herbetor* have been used successfully in Uzbekistan (Matthews, 1997), where there are over 700 bio-factories producing these parasitoids which are released when pheromone traps

detect *H. armigera* or *Agrotis* moths. Matthews commented that severe winter cold in Uzbekistan caused early season pest population levels to be minimised which improved the prospects for using biocontrol.

According to Sundaramurthy & Chitra (1992) the naturally occurring predators *Menochilus sexmaculatus, Coccinella* sp., *Scymus* sp., spiders and birds are important for regulating bollworm populations in the Indian subcontinent and *Chrysopa carnea* was used in an augmentation programme in Gujarat. Examples of biological control by conservation of already existing natural enemies are use of selective insecticides, avoidance of insecticide use when pest levels are low and application of insecticide to alternate rows to provide a refuge for natural enemies.

A commercialised strain of *Helicoverpa* nuclear polyhedrosis virus (NPV) has been found useful and economic in trials in the USA, Mexico and Australia. However, it had problems of low persistence (1-2 days) and there were quality control problems when it was promoted in India (Russell *et al.*, 1998). The distribution network for NPV was more limited than that for conventional insecticides (Shanower, 1999).

Host-Plant Resistance

Use of host-plant resistance can help to prevent the build up of *H. armigera* infestations. If the seeds are available and affordable this can be a method of pest management which is easy to use and convenient for resource poor farmers in developing countries. It is particularly important in pest management of *H. armigera* on another of its crop hosts, pigeonpea (Shanower & Romeis, 1999). However, the insect-resistant lines are frequently less preferred (by humans) in terms of taste, seed colour, and / or size, and are sometimes susceptible to diseases. Resistant varieties sometimes also have a lower yield potential than susceptible ones (e.g. high gossypol lines of cotton). Another important problem is that characters that cause resistance to *H. armigera* might favour other pests or discourage natural enemies.

The three main categories of host-plant resistance are antixenosis, antibiosis and tolerance. Antixenosis means that the crop is less attractive to the pest. Nectarless cotton which has no extra-floral nectaries are less attractive to *H. armigera* (Adjei-Maafo & Wilson, 1993). Smooth glabrous leaves are less

preferred by ovipositing *H. armigera* but are preferred by jassids (King, 1994). Antibiosis involves production of toxins such as gossypol that are harmful to *H. armigera*. Tolerance is when the crop can withstand damage by compensatory regrowth so that the yield is not reduced. Many cotton varieties can compensate for a considerable amount of presquaring damage (Sterling *et al.* 1989). Early maturing varieties such as Abadhita, SH131 and SV213 are 'resistant' by avoiding damage that might have occurred had there been a longer season (Sundaramurthy & Chitra, 1992). Frego bract and okra leaf characters enhance penetration and coverage by insecticides rather than directly conferring resistance (King, 1994). Transgenic cotton incorporating genetic material encoding toxin production from *Bacillus thuringiensis* has recently become available. So far these only use one gene known as *cry* 1A and expression of the toxin is too low for effective *H. armigera* control although it has been efficacious against pink, spiny and spotted bollworms and *H. zea* and *Heliothis virescens* (Russell *et al.*, 1998).

2.2.3 Area Wide Management

Fitt (1989) emphasised the need for a regional approach to *H. armigera* research and management in coordinated area-wide systems and Zalucki *et al.* (1986) stressed that a 'single host approach' ignores one of the basic features of the biology of *H. armigera*, namely its highly polyphagous nature. Polyphagy, combined with mobility, means that it is not confined to specific localities but tends to cross borders so that agronomic or pest management practices in one area may have repercussions for pest management in another area. The sudden appearance of pyrethroid resistant insects in Andhra Pradesh 250km downwind from highly resistant populations in cotton (McCaffrey *et al.*, 1989) is an example of this. Fitt (1989) mentioned that there can be problems on cotton due to local production of maize and that in many areas uncultivated hosts are important in the initial build-up of the first spring generation before crop hosts are widely available. Johnson *et al.* (1975) also stated, "in many areas *Heliothis* has been reported to exhibit an early season build-up on corn and a late season movement to other crops such as cotton, soybeans and tobacco". Light *et al.* (1993) again mentioned this phenomenon calling corn an "infestation reservoir from which emergent moths emigrate".

Attempts could be made to destroy weed hosts that allow early season population build-up (Fitt, 1989) but it is not clear whether or not this would be counter-productive due to elimination of insecticide-susceptible pest genotypes and natural enemies. Nevertheless it is apparent that the ease with which *H. armigera* moves from one host-plant to another is part of the reason why it is difficult to control and that regional movement has to be expected and prepared for. Moth immigration is a key issue influencing the long-term effectiveness of any control strategy aimed at suppressing more than one generation since local control may have little point if crop infestation is mainly by immigrant moths of distant origin (King, 1994).

2.2.4 Trap Crops

Host-plant preferences have already been used in pest management in the practise of trap cropping. Trap crops are "plant stands that are grown to attract insects or other organisms to protect target crops from pest attack" (Hokkanen, 1991). Pests are diverted away from the main crop that they would have done damage to and become easier to control when concentrated on the trap crop. This depends on having a suitable crop available that is significantly more attractive to ovipositing *H. armigera* than the main crop. The oviposition step is important to the evolutionary fitness of Lepidoptera because the larvae are relatively immobile after hatching (Renwick & Chew, 1994). Trap crops also have the additional function of making the habitat more suitable for natural enemies (Fitt, 1989; Hokkanen, 1991; Rao *et al.* 1994; Abate, 1991; Wu *et al.* 1991). Thus, the observed reduction in pest density on the main crop is partly due to enhanced biological control by natural enemies in addition to disruption of pest host-plant recognition or diversion of oviposition onto the trap crop. Increased shelter and alternative food sources are two possible factors that would favour natural enemies. There have been several research reports of potential trap crops for control of *H. armigera* but according to Fitt (1989) trap cropping has seldom been commercially applied.

Disadvantages of Trap Cropping

Disadvantages of trap cropping are that the stand of the main crop is lowered and effectiveness is much lower when the trap crop is not in the flowering stage. Because cotton is a long season crop and most potential trap crops are only briefly more attractive than the main crop sequential sowings would be necessary (Fitt, 1989). According to Saxena (1982), "the concept of a trap crop is only meaningful when fields are likely to be invaded with a high pest population", in other words farmers are unlikely to sacrifice an area which could be used for their main crop and have the extra labour of growing the trap crop unless the risk of pest damage is high. For highly mobile pests, such as *H. armigera*, an extensive network of trap crops may be required (Hokkanen, 1991). Another problem is that, unless a trap crop can be destroyed promptly, it may become a concentrated source of moths (Fitt, 1989). Latheef & Ortiz (1983) warned that intercropping with attractive species could increase overall oviposition. Care should be taken that the larvae cannot migrate to the main crop after hatching: blocks of treated non-host would probably be more effective than intercropped rows.

Reports of Trap Crops Attractive to H. armigera

Srinivasan *et al.* (1994) tested okra, field bean, pigeonpea, sunflower, maize and marigold as trap crops to protect a main crop of tomato. Marigold, *Tagetes erecta*, was found to give maximum reduction of eggs and larvae in intercropped tomato but the other crops tested did not flower synchronously with the tomato and thus were ineffective. Synchronisation of flowering in tomato and marigold was essential for the marigold to act as an effective trap crop and attract early pest arrivals. Simultaneous planting of 25 day old tomato with 40 day old marigold seedlings resulted in appearance of flower buds in both crops at approximately 26 days after planting (Srinivasan *et al.*, 1994). Patel & Yadav (1992) and Rao *et al.* (1994) also found that marigold could be used as a trap crop by reducing oviposition on tobacco crops. Despite having a lower stand of the main crop, a significantly higher yield was recorded by Patel and Yadav (1992) with a 2 rows marigold: 6 rows tobacco combination than with pure tobacco. In this treatment 86% of *H. armigera* eggs were laid on marigold.

Other trap crops that have been suggested for control of *H. armigera* include *Nicotinia rustica* (Singh *et al.*, 1993; Rao *et al.*, 1994), maize, *Zea mays* (Abate, 1988; Karel, 1993), lupin, *Lupinus termis*, and pigeon pea, *Cajunus cajan* (Abate, 1988), okra, *Abelmoschus esculentus*, (Nagangoud & Thontadarya, 1989; Singh *et al.*, 1993), and coriander (Singh *et al.*, 1993). Singh *et al.* (1993) commented that the high incidence of *H. armigera* eggs on okra suggested the possibility of using it for cultural control in cotton but harvesting the fruit seemed sensible because they found that despite there being more eggs laid on okra, larval populations were higher on the cotton. Shrivastava & Sharma (1991) discovered a similar trend with chickpea and soybean: soybean was oviposited on more than chickpea but larval densities were higher on chickpea. This implied that care should be taken that larvae do not develop through early instars on the trap crop and then move to the main crop.

2.3 Semiochemicals

Semiochemicals are the chemical signal compounds used in interactions between organisms. They are emitted by one individual and cause a behavioural response in another. Volatile semiochemicals are perceived by olfaction and involatile semiochemicals are perceived by contact chemoreception. The terms attractant and repellent are two categories of semiochemicals referring to the behaviour modifying olfactory active compounds which do not require contact with the source. The terms stimulant and deterrent are categories of behaviour modifying chemicals that are active only at close range (Dethier *et al.*, 1960; Foster & Harris, 1997).

These compounds, once identified, could be of practical use in crop protection when used for disrupting harmful insect behaviours. Compounds which are attractive could be used for improving trapping, either in mass trapping, provided that attraction to the bait is sufficiently high, or in population monitoring. However, this depends on having an adequate knowledge of the volatile chemicals pest insects use and whether they can be manipulated to achieve a predictable effect. The first step in this where disruption of foraging behaviour of *H. armigera* is concerned is to ascertain whether olfactory cues are important in host-plant finding.

2.3.1 Types of Semiochemicals

Intraspecific communication chemicals are known as pheromones and comprise alarm pheromones and spacing or epideictic pheromones that can be used as repellents, and sex and aggregation pheromones that can be used as attractants. Mating disruption using sex pheromone has been successfully achieved in Egypt for the control of pink bollworm, *Pectinophora gossypiella* (Critchley, 1991) and sex pheromones are widely used for monitoring.

'Allelochemicals' are interspecific communication chemicals and include 'allomones' (chemical signals that benefit the emitter) which could be used as herbivore repellents in the case of host-plant volatiles, and 'kairomones' (that benefit the receiver) which could be used as attractants either for pests using plant chemicals, or for natural enemies using herbivore emitted chemicals (Nordlund, 1981). There are also 'synomones' that benefit both emitter and receiver such as in the case of the chemicals mediating pollination. This terminology is somewhat limited since the same chemical compound may have several functions, e.g. a pheromone that also acts as a kairomone for another species, hence Dicke & Sabelis (1988) propose the use of the term "infochemical" which may be particularly appropriate in situations where tritrophic interactions are being considered. Certain semiochemicals may be important in the biology of beneficial as well as pest insects and this should be considered when their use for disruption of pest behaviour is being considered. As well as chemicals which act as signals in nature, unnatural chemicals may also have behaviour modifying effects (Beroza, 1970).

2.3.2 Semiochemicals and Host-Plant Location

Particular host-plant chemicals can affect the behaviour of phytophagous insects as attractants and oviposition and / or feeding stimulants (kairomones), and as repellents and oviposition and / or feeding deterrents (allomones). The adaptation of phytophagous insects to secondary plant chemicals is the consequence of a long period of association and coevolution (Hsiao, 1985). There is a vast array of compounds produced by the 200,000 or more species of flowering plants: at least 100,000 according to Metcalf & Metcalf (1992). The challenge is to discover which

of these are important in the life cycles of pest species and then to devise methods for utilising them for pest management. It is hardly surprising that there are intricate chemical interactions between plants and insects when it is considered that they have been coevolving for almost 400 million years (Metcalf & Metcalf, 1992).

2.3.3 Use of Semiochemicals to Improve Attractiveness of Trap Crops

It is possible that a semiochemical attractant could be applied to a trap crop to increase and / or prolong its attractiveness (Agelopoulos *et al.*, 1999). Boll weevil trap cropping was greatly enhanced after the discovery and synthesis of the boll weevil pheromone grandlure at the beginning of the 1970s (Hokkanen, 1991). Unnithan & Saxena (1990) suggested spraying attractive kairomones derived from host plants onto non-host plants on which the larvae would not survive thus disrupting successful oviposition. They found that applying sorghum seedling extract onto maize could dramatically increase oviposition by the sorghum shootfly, *Atherigona soccata*, on the non-host maize. An alternative way of interfering with host finding behaviour would be if attractive plant volatiles (kairomones) from the trap crop could be identified and incorporated into an artificial trap device for luring ovipositing females away from the crop. This would depend on the olfactory cues or kairomones from the trap crop being the main factor responsible for its attractiveness.

2.3.4 Rationale for Identification of Floral Odours to Attract *H. armigera*

The current study focuses on volatile host-plant floral compounds in relation to whether they function as kairomones for *H. armigera* and whether they can be used as attractants for trapping purposes. Compared with the sex pheromone (1 : 33 blend of (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal, Nesbitt *et al.*, 1979), which only attracts males, a floral attractant would have the benefit of attracting both sexes. In a review of the application of pheromones for crop pest management in the Indian subcontinent Cork & Hall (1998) state, "despite extensive scientific investigation the optimised pheromone of *H. armigera* remains of uncertain value in population surveillance and control". One of the reasons for this is that the size of the damaging larval population is more directly related to the size of the adult female population

than to the size of the adult male population. Targeting the adult female moth for control would be advantageous since this would directly interfere with oviposition which is an essential part of the *H. armigera* life cycle.

Night observations have shown that nectar foraging precedes oviposition (Beerwinkle *et al.*, 1993) so it is possible that floral lures could attract females before they oviposit. It is also relevant that adult moths are 10-100 times more susceptible to insecticide than larvae (Wood, 1991) if the attractant is to be used as part of a 'lure and kill' behavioural manipulation method such as the one for cabbage looper moth, *Trichoplusia ni*, described by Landolt *et al.*(1991). However, the more fundamental question of the role of olfaction in host-plant finding by phytophagous insects needs to be addressed first because if olfactory cues only play a minor role they would be less effective for any applications in pest management. Landolt & Molina (1996) suggest that attraction to generalised compounds found in the odour of many plants may be more important in finding plant patches or host habitat rather than location of individual preferred hosts for oviposition.

2.4 Host-Plant Location by Phytophagous Insects

2.4.1 Searching Behaviour in Insect Host-Plant Finding

Selection Pressure for Efficient Foraging Behaviour

Efficient searching mechanisms and accurate assessment mechanisms are crucial for an individual's chances of survival and reproduction and hence evolutionary fitness (Bell, 1990). Oviposition site choice is critical in Lepidoptera where larvae are relatively immobile (Renwick & Chew, 1994). The mechanisms used must be sufficiently flexible to allow for variation in environmental circumstances such as host-plant availability. Natural selection is expected to favour searching mechanisms that minimise searching costs and maximise benefits but also reduce various types of risk incurred while searching.

Damman & Feeny (1988) point out that there is strong selection pressure in favour of females that can discover accurately and rapidly plant tissues that will best support the development of their offspring. If females can reject unsuitable plants

early in evaluation using olfactory or visual cues, they can concentrate their time and risk on plants more likely to provide an oviposition site. Optimal foraging theory predicts that within certain constraints an animal will forage in such a way as to maximise its fitness. However, insect foraging is rarely directly related to the plant nutritional value or caloric content and because natural selection is an ongoing dynamic process the "optimal" state is unlikely to be achieved (Hsiao, 1985). Because plants are also evolving counter-adaptations, it is unlikely that insect adaptations to host-plants are ever perfect at any given time.

Foraging Behaviour and Polyphagy

The basic behavioural patterns involved in foraging are probably similar in most insects but the types of stimuli required for host finding and recognition vary from case to case. The vast majority of phytophagous insect species are monophagous (feeding on one host-plant) or oligophagous (feeding on a few host plants) (Hsiao, 1985; Bernays & Chapman, 1994). They may have specialised refinements for host-plant finding and their life history strategies are intimately related to their diet specialisation. *H. armigera* is unusual as it is polyphagous (it feeds on many host plants, see Table 2.1). A diet comprising many plant species also needs specialised adaptations to avoid species which are too toxic for larval metabolism. However, any attractive host-plant odours would not be expected to involve volatiles specific to any given host-plant species. A specialist responding to host-specific cues might be confused or even repelled by nearby non-host plant species, whereas a polyphagous herbivore, orienting to generalised plant characteristics, might perceive diverse plant mixtures merely as dense stands of food (Bell, 1990). Polyphagous insects must have mechanisms for selecting the most suitable host-plant species available and they may select specific plant tissues (Lance, 1983).

Dispersal Phase

There is an innate tendency to disperse prior to foraging in mobile life cycle stages (Hsiao, 1985) although this may be reduced if local conditions are favourable. Dispersal enables species to colonise new sites. New foraging sites could be some distance away from the emergence site in the case of *H. armigera*. Mark-recapture

experiments indicate that there is a rapid movement of *H. armigera* moths from the emergence site (King, 1990). During the dispersal phase insects may not respond to environmental cues that would normally elicit food-finding and in some insects feeding and locomotion are antagonistic behaviours.

Directional and Non-Directional Movements

Food searching or foraging includes random (non-directional) and non-random (directional) movements. The former occur before any directional host-plant cues are perceived. A non-directional cue of one sensory modality may change a sensory threshold or input template of another modality (e.g. olfactory cues increasing visual pattern recognition), a process called "cross-channel potentiation" (Bell, 1990). Environmental cues may be used to aid orientation such as in upwind anemotaxis.

Mechanism of Flight Towards Host-Plant Odours

Odour-conditioned anemotaxis was declared by Visser (1986) as the mechanism involved in early stages of host-plant finding and was assumed to operate as soon as the odour concentration is above the threshold for detection. Instead of trying to follow a non-existent concentration gradient the insect simply flies upwind. It appears that odour molecules are effective chemotactically only within a short distance and current views suggest that chemotaxis is not necessary to guide an insect toward a distant upwind odour source because odour-conditioned anemotaxis can be used to achieve the same effect (Hsaio, 1985). Haynes & Baker (1989) found upwind anemotactic flight in female navel orangeworm moths in a wind-tunnel in response to host-plant odours was very similar to upwind anemotactic flight in males in response to the sex pheromone albeit that it was somewhat slower. Casting at right angles to the wind direction occurred in response to loss of the odour in an attempt to relocate it.

Learning Behaviour

Hsiao (1985) stated that most behavioural patterns of insects, including feeding, are stereotyped with genetically controlled species-specific motor patterns (fixed action patterns) being released according to set sensory stimuli from one or

several sense organs. However, the precise nature of the cues associated with host-plant recognition has been shown to depend more on prior foraging experience than was previously assumed. A cue may be genetically specified but experience allows for alterations in quality assessments (Bell, 1990). With deprivation motivation increases and an insect might be attracted to stimuli that otherwise would not be responded to. Plant recognition is thought to occur when a particular stimulus or configuration of stimuli originating in the external world matches a model in the neural world (Dethier, 1982).

Learning is a process resulting in a change in behaviour as a result of experience. Habituation and associative learning are important in defining or modifying the feeding patterns of insects (Hsiao, 1985). Habituation is a decline in response after continuous stimulation and is quite common. Associative learning includes induction where cues previously experienced on a host-plant become more favourable when encountered later and aversion learning cues associated with toxic or distasteful plants are avoided. Associative learning may permit more efficient location and exploitation of host plants, particularly when host plants are unpredictable and vary with habitat, geography and season (Landolt & Molina, 1996).

Learning causes an increase in the use of a genetically-preferred host when it is the most abundant host-plant but when it is rarer than another host-plant learning can cause individuals to use less of that host than individuals that do not learn (Jaenike & Papaj, 1992). This could be important for polyphagous insects such as *H. armigera*. Learning mechanisms can allow even polyphagous insects to achieve some degree of specialisation (Hsaio 1985). Specialisation on more favourable resources is referred to as conditioning by Bell (1990). Flower constancy, in which the most abundant suitable flower is preferred and insects restrict their visits to a single host species, is one example of this type of experiential effect. Learning effects may mean that the attractiveness of particular floral volatiles is less robust under field conditions where the response to them depends on prior experience by foraging moths.

Associative learning of host odour has been documented in Noctuid moths. Landolt & Molina (1996) found that mated female cabbage looper moths, *Trichoplusia ni*, that were caged with cotton, celery, or soybean foliage were attracted significantly more often than inexperienced moths to the odour of the same species the following night. Only brief contact by a moth or a single oviposition on plant foliage was sufficient to increase subsequent attraction to plant foliage. The role of learning in oviposition and nectar foraging behaviour of *H. armigera* itself was studied by Cunningham *et al.* (1998a, 1998b). They found that for both these behaviours previous experience with a particular host species increased the probability of that species being selected again later. Experience on a host significantly increased the proportion of moths selecting that host, compared with moths experienced on the alternative host or artificial sites and for nectar foraging preference was exhibited by both males and females. Cunningham *et al.* (1998b) suggest that learning in nectar foraging and oviposition is linked with flowering host-plant species initially favoured for nectar foraging also becoming preferred host plants for oviposition. Geographical differences in host-plant preference by *H. armigera* have been noted (Reed & Pawar, 1982) and these may be related to learning effects, such as specialisation on the most abundant host, rather than genetic differences because the gene pool is quite well mixed due to the high mobility of *H. armigera* (Zalucki, 1986) which prevents the formation of different races.

2.4.2 Sensory Cues Involved in Host-Plant Finding

> *"However comforting our schemes, insects know nothing of them."*
> Miller & Strickler (1984)

Cues Used in Host Location

Most authors agree that the sense of smell or olfaction plays some role in host-plant finding by phytophagous insects. However it is less well known how important this role is and the relative importance of olfactory cues is likely to vary from one insect-plant interaction to another. Honda (1995) lists plant apparency, foliar shape, colour, surface texture, presence of conspecific eggs and learning as other factors involved in host-plant selection for oviposition. The chemical ecology

of host-plant detection can involve contact chemoreception as well as olfaction. Visser (1988) contrasts two possibilities regarded as extremes: insects could make their host-plant choice prior to landing on the plant (host-plant "finding") or alternatively they could make their choice on contact after alighting on plants randomly (host-plant "recognition"). Renwick (1989) makes similar remarks contrasting volatile stimuli used in orientation and landing, and contact stimuli which are used in assessment of the plant surface. Physical characteristics of the plant surface, such as surface texture and arrangement of leaves, can be important as well as chemical characteristics (Foster & Harris, 1997). It is important to consider how much information about the plant can be gleaned by the insect by particular cues. The information from volatile plant chemicals can be quite limited because they only make up a small fraction of the chemicals belonging to a particular plant and have simple low molecular weight structures (Lance, 1983) and contact cues may be required to distinguish between host and non-host plants. Plant characteristics used for searching should be correlated with the quality of plants (Damman & Feeny, 1988).

Hypotheses of Insect Host-Plant Finding

In describing the theories of host-plant finding proposed in the literature a historical approach is useful for seeing how the understanding of the subject has developed over the years (Panda & Khush, 1995). Brues (1920) suggested inferentially that the vague notion held at the time of a 'botanical instinct' by which insects find their host plants was based on responsiveness to the complex of chemical and physical stimuli from plants. This early theory fits quite well with present understanding. However, more restrictive theories (better considered as hypotheses) were subsequently proposed. Many of these models assumed that host selection is predominantly based on chemical cues stimulating olfactory and contact-chemo-receptors. The "Token Stimuli" theory of Fraenkel (1959) suggested that certain secondary plant metabolites ("odd substances") played a key role in host-plant recognition, functioning positively as attractants and feeding or oviposition stimulants. However this simple lock and key model of insect decision-making, in which a few 'token stimuli' trigger stereotyped host-investment behaviours, is not upheld by accumulating facts in many instances (Miller & Strickler, 1984). Beck

(1965) proposed the Dual Discrimination theory that host-plant choice was based on use of secondary plant metabolites and plant nutrient constituents. Although slightly less restrictive than the 'Token stimulus' theory it still only involved chemical stimuli. A theory opposing the idea that the stimuli from the plant are attractive was put forward by Jermy (1966), partly due to the lack of discovery of token stimuli causing overt behavioural responses (Dethier, 1982). This negative stimuli theory was contrary to preceding theories as it suggested that host selection is largely based on negative reactions to repellent stimuli. However, according to Hsiao (1985) known examples of natural repellents from plants are rare.

The early theories, with the possible exception of the more general ideas of Brues (1920), suffered from being too simplistic. In the last two decades it has been realised that the behavioural processes mediating insect-plant interactions are more complex, multifactorial and labile than previously supposed. Although examples of insect-plant interactions might be found that agree broadly with the above hypotheses of Fraenkel (1959), Beck (1965) or Jermy (1966) this does not necessarily mean that the predictions of these hypotheses also apply to the interactions of all other phytophagous insect species with their host plants. It has now been realised that the mechanisms of host-plant finding are likely to vary from insect to insect and even within the same insect species according to the physiological state, previous experience and environmental circumstances of the insect. Wind can influence foraging through the aerodynamics of upwind flight or downwind movement of floral odours. Temperature and humidity can also have important effects on flight behaviour.

A more sophisticated mechanism for host-plant recognition was described by Dethier (1982), in which a mixture of stimuli leads to complex responses, modified by the physiological state of the insect, and this has been found to agree best with our current knowledge of insect-plant interactions (Miller & Strickler, 1984). According to Dethier (1982) recognition and preference of host plants involves:

> "*the integration of a complex of neural and metabolic events*" which include "*the sensing and encoding characteristics of the sense organs, decoding mechanisms in the central nervous system, assessment of across-fibre*

patterns and deterrent / stimulant ratios, pre- and post-ingestion factors such as level of satiety, nutritional balance, and experiential factors such as induction and aversion learning".

Dethier (1982) introduced the term "across fibre patterning" to signify the complex multineural integration of stimuli. The sensory input received by an insect and its subsequent processing in the nervous system are two critical aspects influencing the type of behavioural response elicited. Although there have been studies correlating sensory input and behavioural responses (including the current research), little is known about the neural processing events that govern the response. Dethier also made the important point that the concepts of token stimuli and complex mixtures are not mutually exclusive:

"*that they are complementary can be appreciated by analogising with a tone consisting of a fundamental and harmonics or, probably more accurately, as a chord*" (Dethier, 1982).

The concept of 'both' instead of 'either / or' probably holds true in a wider context too: there have been debates about whether post-alighting cues are more important than pre-alighting cues, about whether chemical cues are more important than visual or tactile cues, and about whether olfaction is more important than contact chemoreception; however, a sequence of cues may be necessary in which all of these factors are involved instead of just one or the other. It is possible that something which at first sight may seem an unimportant or trivial factor may through some subtle effect bring the total stimulus above the threshold required for acceptance of a plant. It should also be kept in mind that host-finding behaviour, in most cases, is probably more than a simple cause and effect relationship between one type of cue and one type of outcome such as feeding or oviposition. Many experiments on phytophagous insect host-plant selection have been carried out under controlled conditions in the laboratory. Under these conditions a clear significant response to a particular host-plant cue may be demonstrated for a particular insect. This means that that cue probably has a role in host-plant finding. It does not, however, mean that no other cues are involved in nature where insects are locating their host plants in the field and are exposed to a great variety of different stimuli.

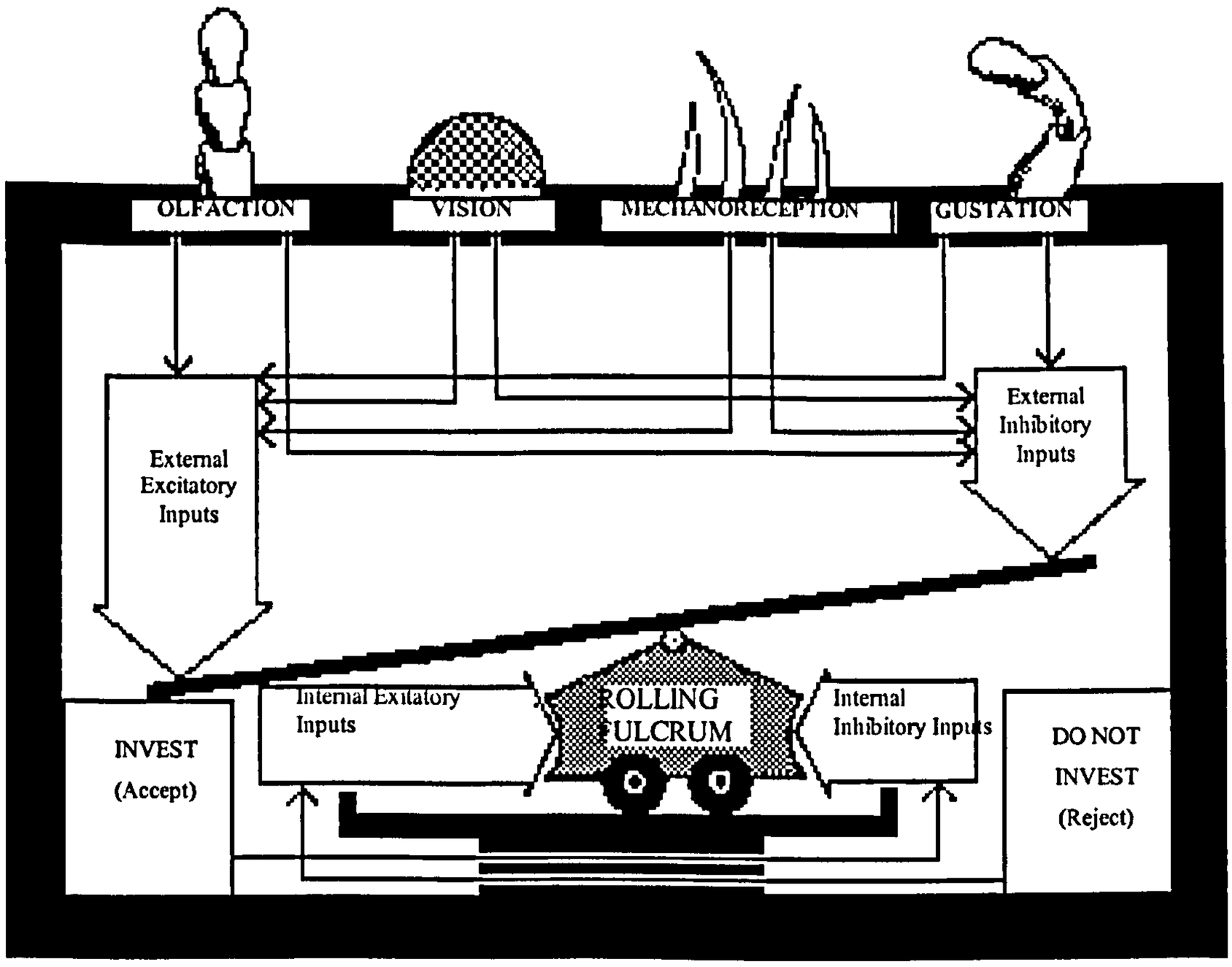

Fig 2.2 Mechanical analogue of Dethier's 1982 model for the influence of external and internal factors on insect investment behaviours (e.g. feeding, oviposition) [from Miller & Strickler (1984)]

Miller & Strickler (1984) were in favour of the complex interpretation of insect host finding advocated by Dethier. Although Dethier only referred to "sense organs" and presented his review from the perspective of neural processing of the sensory input using chemical stimuli as examples, Miller & Strickler (1984) made it explicit that vision and mechanoreception are involved in host-plant recognition as well as olfaction and gustation with their "mechanical analogue" diagram of Dethier's model (Fig. 2.2). Miller & Strickler (1984) brought an evolutionary perspective into the discussion of insect host-plant finding:

> "*Those insects whose neuronal wiring leads to better correlations between host-plant cues and host-plant suitability should be favoured evolutionarily*".

They considered the cues for host-plant finding from the perspective of what is most effective for locating suitable plant resources and stated:

"*the more characteristics that interact with the sensory system to shape the resultant reading, the higher the probability that it will be unique and constitute useful information*".

Miller & Strickler (1984) saw the use of as many host-plant cues as possible as a way of increasing the probability of making a correct choice of host-plant. According to them opinions that visual stimuli merely supplement the more important chemical cues might require some re-evaluation. They made the point that information provided by photons is more directional than that provided by volatile molecules which are distributed according to air movements. As an example they gave oviposition by caged onion flies, *Delia antiqua*, which is maximal when there is a synergistic interplay of onion-produced alkyl sulphides, a smooth yellow cylinder positioned vertically, and moist sand (Harris & Miller, 1982).

Examples of Insects Using Olfactory Cues in Host-Plant Location

Visser (1986) presented two hypotheses concerning the possible role of volatile olfactory cues in host-plant location,

1. "Plant odours are highly specific and composed of compounds not found in unrelated plant species"

2. "Plant odour specificity is achieved by the particular ***ratio*** *between constituent components which are generally distributed among plant species"*

This is likely to vary according to the particular insect-plant interrelationship being considered as the following examples illustrate:

A study illustrating the complexity of the role of volatiles in host-plant recognition by phytophagous insects is that of Visser & Ave (1978). The odour of potato plants was found to be attractive to the Colorado potato beetle, *Leptinotarsa decemlineata*, eliciting a positive anemotactic response. However, component chemicals were not attractive when tested alone. Also, when individual components were added to the potato odour attraction was disrupted. The conclusion was that not only did a blend of volatile chemicals have to be present but that they also had to be in the correct ratio. Visser and Ave (1978) made the point that a greater degree of selectivity would be needed in the approach to potential host plants with

oligophagous insects such as Colorado potato beetle and hence there might be more constraints on the range of blends that would be attractive when compared with a polyphagous insect. Specialists would seldom find suitable food if they contacted foliage at random (Lance, 1983). Nevertheless it appears in this case that the volatiles used to orient towards the host-plant are not specific to potato and are common "green leaf volatiles", the main components being (*E*)-2-hexen-1-ol, hexan-1-ol, (*Z*)-3-hexen-1-ol, (*E*)-2-hexenal and linalool. The specificity arises from the ratio and blend composition of these compounds. Isothiocyanates which are emitted by brassica species have been shown to attract cabbage root fly, Delia radicum, and have been used to bait traps in the field (Finch & Skinner, 1982). However all isothiocyanates tested on their own had to be emitted at much higher than natural release rates and plant extracts caught similar numbers of insects at lower release rates. Finch & Skinner (1982) interpret this as being due to the response to a mixture of chemicals in the plant extracts. Certain isothiocyanates were found to be repellent.

An oligophagous insect in which the chemical ecology of host finding has been investigated is the carrot fly, *Psila rosae*. In contrast to the Colorado potato beetle, more specific olfactory cues from the host-plant were used in host recognition. Gas Chromatography-Electoanntennographic (GC-EAG) studies indicated a strong EAG response to the compound *trans*-asarone and this compound, when used alone, significantly increased trap catches compared with unbaited controls in field trapping experiments (Guerin *et al.*, 1983). Thus, it appears that when compounds more specific to the host-plant are used there is less of a requirement for being presented in a blend with a particular ratio of compounds. However, when a blend of hexanal and *trans*-asarone was used there was a significant increase in trap catch compared with either compound used singly. Addition of (*E*)-methylisoeugenol suppressed trap catches suggesting that it was competing for the same receptor site *trans*-asarone. *trans*-Asarone appears to be acting as a "token stimulus" *sensu* Fraenkel (1959). The EAG perception threshold for *trans*-asarone (1.4×10^6 molecules/ml) is the lowest recorded for an insect to a plant volatile. Sensitivity was correlated with a lower emission rate of this compound than the green leaf volatiles. Selectivity of traps baited with *trans*-asarone was good with few other insects apart from carrot fly being caught. In this example

the plant volatile seemed to play a selective and specific role in attraction which is similar to the attraction elicited by sex pheromones.

Use of Visual Cues in Host-Plant Finding

Insects see in a different way from humans (Kevan & Baker, 1983): The insect visual spectrum is shifted 100nm to the shorter wavelength end compared with humans and extends from ultraviolet at about 300nm to yellow-orange at about 650nm. The visual acuity of insects, even those that are highly developed optically (e.g. bees), is almost an order of magnitude less than ours. However, what we may recognise by outline pattern, an insect may recognise just as readily or more so by colouration and colour pattern. Some flowers reflect UV and other wavebands strongly, others weakly, and others not at all. Insects are better able to resolve rapid motion. As a flower-visiting insect approaches a plant, the outline form may play an important role. Flowers with highly broken outlines have longer outline lengths and are more stimulating. Similarly, the motion of floral parts enhances floral visibility. A flower, whether seen in colour or not, must contrast with its background. Flowers growing in shaded areas and against dull backdrops tend to be pale and those blooming in open situations and visible against the sky or a bright backdrop tend to be darker (Kevan & Baker, 1983).

Prokopy & Owens (1983), in their review of visual detection of plants by phytophagous insects, concluded that flying insects are not likely to perceive pattern details of a distant plant on the horizon several meters or more away and that a colour response is improbable if the insect is travelling directly toward or away from the sun in diffused light conditions. The gross silhouette of the plant against the horizon line appears to play a greater role than hue or form at a distance (Prokopy & Owens, 1983). However, plant spectral qualities, particularly hue and intensity, are thought to be the principal stimuli eliciting insect alightment on living plants. The combined scent of a large stand of flowers can potentially act over considerable distances (Kevan & Baker, 1983). Thus visual cues could be utilised subsequent to olfactory attraction. In fact, Bell (1990) suggests that a switch to the more precise directional information obtained from visual stimuli occurs whenever possible. Prokopy & Owens (1983) were of the opinion that most insects use a combination of

olfactory and visual stimuli to locate their host plants. Olfactory cues have the advantage that they can still act when the flower or plant part is obscured from direct view whereas photons only travel in straight lines.

Aluja & Prokopy (1993) investigated responses of apple maggot flies, *Rhagoletis pomonella*, to host fruit visual stimuli (apples or models of apples) and chemical stimuli (a synthetic apple volatile blend) in semi-dwarf field-caged apple trees. The flies discover a point source of odour, such as an odour-bearing tree in a patch of trees, by flying upwind in response to intermittent response to odour. On arrival at a host tree they discover individual apparent and abundant host fruits on the basis of vision. Only if fruits are less apparent or scarce, odour appears to interact with vision during the fruit-finding process. As the visual stimulus became progressively weaker (red to green to clear), odour became more important during the fruit finding process.

However, as it has already been emphasised in the present discussion, what occurs in one insect-plant interaction could be different in another. For *H. armigera* visual cues probably play a lesser role due to the low illumination during foraging which is nocturnal. Nevertheless they could still have a role even with low illumination as the visual apparatus is adapted to those conditions. Visual cues are likely to be important in the final approach to the plant where they could provide useful directional information despite the low light intensity. Callahan (1957) suggested that low intensity, short wavelengths of light reflected from the field may present wavelength or pattern stimuli to moths and thus attract them to oviposit or feed. The contrast between the short wavelengths of skylight and the longer reflected wavelengths from the plants may present a stimulus to moths. Using light of different colours transmitted through cloth oviposition surfaces, Callahan (1957) found that *Helicoverpa zea* preferred areas on the oviposition substrate with shorter wavelength light. Moth pollinated flowers tend to be lightly coloured (Kevan & Baker, 1983).

Interaction between Olfactory and Visual Cues

In a recent review of host-plant selection by phytophagous insects Bernays & Chapman (1994) were also proponents of a multifactoral approach concerning the

sensory cues mediating the ensuing locomotory behaviours. They consider olfactory and visual cues as reinforcing each other in host-plant finding. They state that plant odours can be taxon-specific and that the insect's olfactory system often has the capacity to distinguish these odours from others whereas plant shape and colour are usually less characteristic because they are variable even within a particular plant species. However, when made in the context of a specific odour visually mediated responses can play a key role in host recognition.

The Role of Contact Cues in the Host-Plant Selection Process

It has already been found that post-alighting cues play an important role in host-plant selection by the polyphagous Noctuid *Heliothis virescens* (Ramaswamy *et al.* 1987). When their eyes were painted over and antennae removed the moths were still able to discriminate between host and non-host plants but this was at close range in cylindrical plastic containers of 20 x 22cm dimension. Olfactory and visual cues, if important, would be expected to operate more in the earlier stages of host-plant finding. Compared with vision and olfaction distant from the source, mechanoreception and gustation occur in direct contact with the plant where signal levels are high and noise levels should be low. Due to their co-occurrence with chemoreceptors the precise role of tactile receptors in feeding behaviour is difficult to determine (Hsiao, 1985). Because they are relatively non-volatile and effectively compartmentalised, many phytochemicals generating inhibitory inputs may influence insect behaviour more during or after the examining phase when direct contact has been established (Miller & Strickler, 1984).

Sequence of Cues Used in Host Location

It seems likely that there is a hierarchical sequence in which plants are found: first an appropriate habitat is found, then a patch in which resource items are concentrated is found, and then an individual flower for nectar foraging or an oviposition site is located (Bell, 1990; Miller & Strickler, 1984). An insect may detect cues from the host-plant at some distance from it but may not be able to complete an accurate assessment until it has alighted and uses contact cues. Multiple cues can be associated with host-plants.

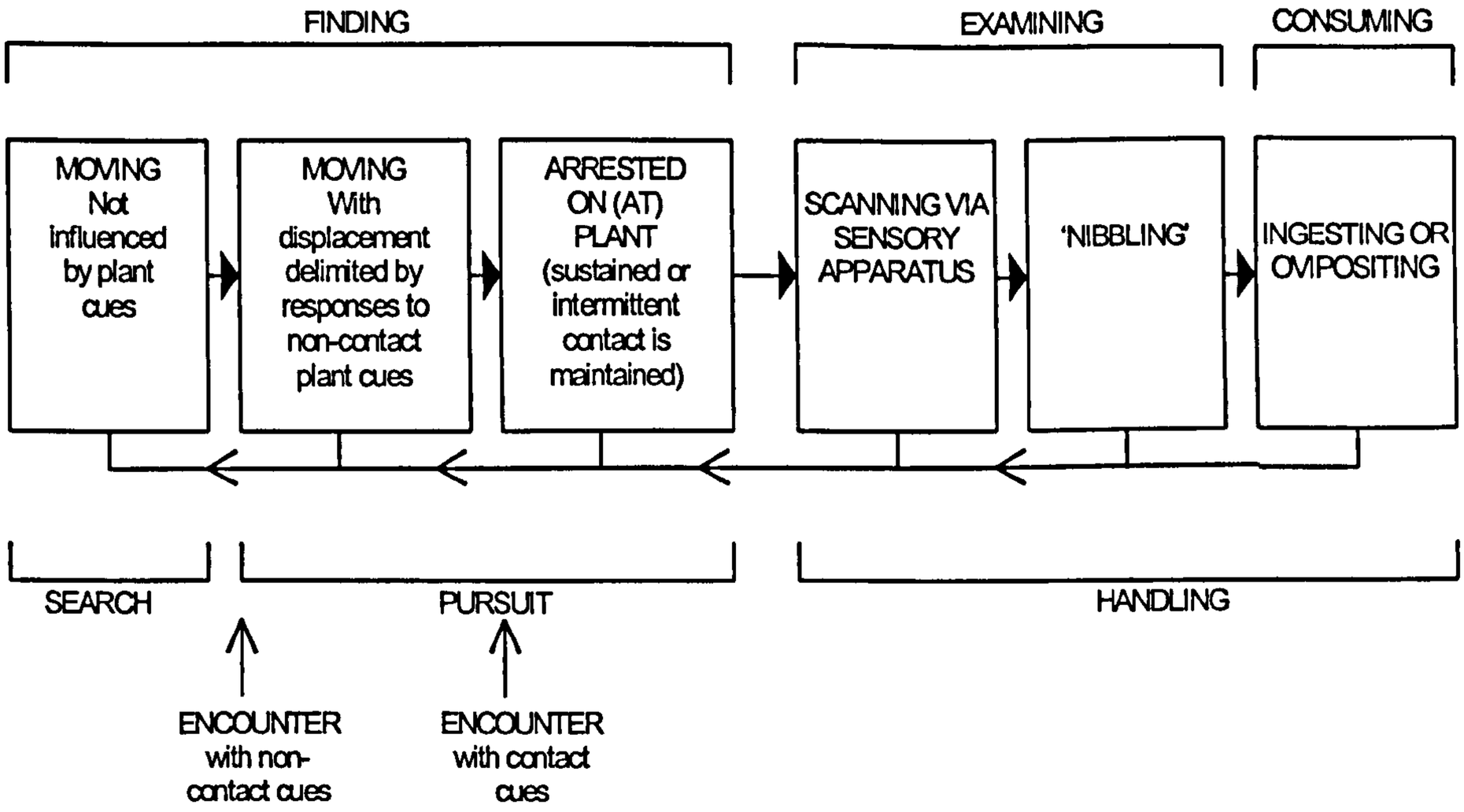

Fig 2.3 Sequence of Behaviours Involved in Host-Plant Location. "Finding", "Examining" and "Consuming" are terms approximating behaviours. "Search", "Encounter", "Pursuit" and "Handling" are optimal foraging terms. (from Miller & Strickler, 1984).

Miller and Strickler (1984) emphasised that a sequence of cues is involved in host finding leading up to host utilisation, providing a diagram to aid visualisation of the process and understanding of the terminology involved (Fig.2.3). Host use was described as a chain of behavioural responses in which an insect can progress through the steps, remain for an extended time at a step, or regress to any prior step in the sequence if conditions are unfavourable (Miller & Strickler, 1984). Visser (1986) was also in favour of a sequential approach in phytophagous insect host finding. He suggested a sequence of behavioural responses to an array of stimuli (emphasising olfactory stimuli) with the number of perceived stimuli increasing as the insect proceeds in host selection.

2.4.3 Floral Attractants and other Kairomones for Moth Pests

Many adult insects, including *H. armigera*, feed on floral nectar and attractant karimones could play a role in their host finding processes. Most Lepidoptera feed extensively on floral nectar as adults. As mentioned earlier the presence of flowers on the host-plant is particularly important for oviposition by

female *H. armigera*. The flowers that nocturnal moths visit are frequently pale, whitish to yellow in colour, and strongly scented (Kevan & Baker, 1983) especially if the flowers only open at night. Kevan and Baker suggested that these moths were attracted from a distance by the scent and at close range by the more reflective and contrasting light coloured flower on a background of dark foliage which is the converse of what is generally believed to occur with diurnal Lepidoptera. If the chemical nature of these floral scents could be better understood then it might be possible to manipulate them to improve pest management, provided that they are sufficiently attractive.

Helicoverpa species

One of the earliest identifications of a floral compound that attracts moths was that of phenylacetaldehyde from the bladder flower, *Araujia sericofera*, by Cantelo & Jacobson (1979). Phenylacetaldehyde was characterised by GC-MS and NMR in a steam distillate of bladder flowers. They found that traps baited with this compound caught a variety of different moth species, including *H. zea*, the American congener of *H. armigera*. This finding inspired Pawar *et al.* (1983) to test phenylacetaldehyde in the field in India as a trap bait for *H. armigera*. Over an 8 week period 484 *H. armigera* were caught period in an unspecified number of traps. Slightly more than 60% of the catch consisted of female moths. However, performance of nearby sex pheromone traps in terms of numbers of moths captured was said to be considerably better.

Light *et al.* (1993) discovered that one of the prominent leaf volatiles of maize, (*Z*)-3-hexenyl acetate, could significantly increase the capture of *H. zea* males in sex pheromone baited traps as these traps were preferred over traps baited with sex pheromone only. This could be of commercial importance because the volatile (*Z*)-3-hexenyl acetate costs a fraction of the cost of the pheromone component which would justify its use as a synergist. They also noted that the odour of corn leaves has been shown to change as the corn plants mature from silking to senescent growth stage, as evidenced by the decline of certain volatile constituents eg. (*Z*)-3-hexenyl acetate which corresponds with change in attractiveness.

Attraction of *H. armigera* to chickpea and pigeonpea volatiles was investigated by a research group at the Max-Planck Institute in Germany. Rembold *et al.* (1989) found the chickpea (*Cicer arietinum*) seed volatiles pentan-1-ol, Δ-3-carene, myrcene and α-pinene attractive to *H. armigera* larvae in an olfactometer bioassay which prompted Rembold *et al.* (1991) to test the same kairomone mixture for attractiveness to the adult stage. The kairmone blend was more attractive to egg-laying female moths than the control in a wind-tunnel bioassay although no source contacts were obtained. In a field trial a total of 88 moths were caught during 3 weeks of trapping using 5 baited traps which is on average less than one moth per trap-night. The moths trapped in plant odour baited traps were predominantly female though and control traps were reported to have caught no moths at all. However, no independent assessment of the *H. armigera* moth population with pheromone or light traps was reported and so the sensitivity of the traps was not clear.

Hartlieb & Rembold (1996) studied pigeonpea, *Cajunus cajan*, volatiles. A synthetic mixture of sesquiterpenes, β-caryophyllene, α-humulene, α-guajene, α-muurole, γ-muurole and α-bulnesene, in the same proportions as in the pigeonpea steam distillate, elicited increased upwind flight by *H. armigera* females. Although the natural steam distillate did elicit a greater number of source contacts than the control treatment, contacts with the odour source were not significantly increased relative to the control with the synthetic blend. Surprisingly, in an EAG test very little EAG activity was reported when the sesquiterpenes were tested individually even with a 1:10 dilution of the pure compounds. The whole steam distillate had higher EAG activity but then all the compounds were presented simultaneously. Better EAG responses were found for the chickpea compounds (pentan-1-ol, Δ-3-carene, myrcene and α-pinene) than for the pigeonpea compounds. No field trials were conducted for the pigeonpea based synthetic kairomone.

Blaney & Simmonds (1990) found that *H. armigera* oviposition on several crop legumes and their wild predecessors was correlated with the presence of relatively high amounts of certain compounds in the favoured host plants. These were hexanal, β-pinene, (*Z*)-3-hexen-1-ol acetate, limonene, α-cedrene, methyl-cyclopentane and 2,3,4-trimethylhexane. When applied to an inert substrate in the laboratory these compounds stimulated oviposition. However, they commented that

the presence of these compounds does not automatically mean that females are stimulated to oviposit as some of them are present in non-preferred plants.

Other Moth Species

Cabbage looper moth, *Trichoplusia ni*, attraction to *Abelia grandiflora* flowers was studied by Haynes *et al.* (1991). On the basis of retention times on GC columns of different polarities and mass spectrometry they identified the four dominant GC peaks in the floral headspace sample. These compounds, phenylacetaldehyde, benzaldehyde, 2-phenyethanol and benzyl alcohol, in a blend in the natural ratio, gave upwind flight to the odour source in a wind-tunnel that was as good as an actual cluster of *A. grandiflora* flowers. Either phenylacetaldehyde or 2-phenyethanol on their own performed almost as well as the complete synthetic blend in the wind-tunnel. Heath *et al.* (1992) also studied the cabbage looper moth but this time using night-blooming jessamine, *Cestrum nocturnum*, flowers as the source of attractant. The three principal chemicals in these flowers were benzaldehyde, benzyl acetate and phenylacetaldehyde, identified by GC-MS and NMR, constituting 95% of the total volatiles released. A mixture of these 3 compounds in the natural ratio caused upwind flight and contacts with the odour source in a wind-tunnel. Testing the compounds individually indicated that attraction was primarily due to phenylacetaldehyde although there was some response to benzyl acetate on its own. Attraction of Lepidoptera by phenylacetaldehyde alone was also reported by Creighton *et al.* (1973) and Smith *et al.* (1943) and it has been used in a lure and toxicant system for *T. ni* described by Landolt *et al.* (1991).

The European grapevine moth, *Lobesia botrana*, has also been studied in relation to floral attractants. Field observations showed that it was attracted to tansy, *Tanacetum vulgare*, flowers and EAG tests indicated that the terpenoid fraction of the steam distillate of flowers was active (Gabel *et al.*, 1992). The early flowering stage was more attractive than the late flowering one (Le Metayer *et al.*, 1991). Using linked GC-EAG analyses Gabel *et al.* (1992) located nine electrophysiologially active compounds in a tansy steam distillate (of over 200 peaks) which were identified by GC-MS as ρ-cymene, δ-limonene, α-thujone, β-thujone, thujyl alcohol, terpinene-4-ol, (*Z*)-verbenol and piperitone. A synthetic

blend of these compounds gave consistent attraction in a field cage and was almost as attractive as the tansy steam distillate itself.

Salama *et al.* (1984) in an olfactometer bioassay found that the petroleum ether extract of cotton, *Gossypium barbadense*, flowers contained compounds that were attractive for *Spodoptera littoralis*. They used TLC and GLC to identify the components of this extract and out of these compounds caryophyllene oxide, citronellol, borneol and α-pinene proved to be attractive to both sexes of the moth. Tingle & Mitchell (1992) found significant attraction in a wind tunnel to an extract of cotton flowers by *Heliothis virescens*. Zhu *et al.* (1993) investigated electrophysiological responses of another Noctuid moth, *Agrotis ipsilon*, and carried out field observations. They found that the flowers that were more attractive in the field also elicited higher electroantennogram (EAG) responses.

2.5 Use of Floral Odours in *H. armigera* Control

2.5.1 Floral Lures: Potential Problems and Possible Advantages

There are a number of considerations that have to be made regarding the potential for commercial use of a floral kairomone based lure for traps. Attraction is unlikely to depend on one compound alone (Jackson, 1990) thus a blend of compounds in a specific ratio should be identified. The formulation of attractant, release method and trap design are also critical aspects that could determine feasibility for use in pest management. The considerable mobility of *H. armigera* means that local control of mated females might not be sufficient in the face of a large influx of more mated females from outside the trapping area (Fitt, 1989). This could mean that a coordinated, area-wide approach is necessary if mass trapping is to be effective. It also means that the lure should be a strong attractant. Another consideration is the selectivity of the lure since it has to be ensured that lures do not attract honey bees or other beneficials (Wood, 1991; Griffiths, 1990).

The main advantage of using a floral odour attractant is that it attracts female moths unlike the *H. armigera* sex pheromone which only attracts males. A mated female moth typically lays about 1000 eggs and on average 500 will hatch into crop-damaging larvae (Fitt, 1989). Thus, it is important to control their emigration into

crop areas. By reducing the number of egg laying females it might be possible to control *H. armigera* before its population builds up (Rembold *et al.*, 1991). Another advantageous aspect is that adult insects such as moths are typically around 10 to 100 times easier to kill with insecticides than are caterpillars (Wood, 1991).

A floral lure could be used for monitoring of ovipositing females or, if it was strong enough, as a bait in mass trapping programmes. These two uses parallel the applications that have been made of pest sex pheromone attractants. Floral odours could be used together with sex pheromone in traps so that both males and females were captured. Sex pheromone traps containing one of the prominent leaf volatiles of certain *H. zea* hosts, (*Z*)-3-hexenyl acetate, not only significantly increased the capture of *H. zea* males but were preferred over traps baited only with sex pheromone (Light *et al.*, 1993).

2.5.2 Factors Influencing Performance of Baited Traps

Trap design and positioning influence whether moths readily detect the attractant stimulus and whether or not they are effectively captured once they have located the source. There is quite an extensive literature on attractant baited trap design for traps for moth pests due to the use of pheromone baited traps. In summary it appears that sticky traps are not a good option for capturing strong flying moths and that funnel type traps seem to be best for these insects, especially where large numbers are expected to be caught and there is the danger that a sticky trap would saturate (e.g. Beasley & Adams, 1994). It also appears that varying dimensions of a trap can sometimes significantly influence trap catch. The ideal trap would maximise capture rate and minimise rate of escape from the trap but this need not be the same trap when different insects and different situations are considered. Cost and availability are also important considerations. The ideal trap might be too expensive and a greater overall capture of insects might sometimes be achieved with a less costly and more widely available trap.

The scale at which monitoring is carried out should be considered: do traps give a local or regional indication of the moth population? Timing and size of pheromone trap catches during the night may reflect the timing and extent of the

natural mating activity of the males (Dent & Pawar, 1988). The parallel with this for a floral lure would be the amount of naturally produced floral volatiles present in the area around the trap. The longevity of release of volatile attractant is a concern for both monitoring and mass trapping. Sinha & Mehrotra (1993) found that pheromone septa were significantly less effective after 3 weeks and thus needed replacing. The turbulence generated by foliage, tree trunks, or other features of the habitat near the trap modify the dispersion of the pheromone plume creating a more diffuse plume, and possibly lowering the proportion of insects attracted Cardé & Elkinton (1984).

2.6 Research Hypothesis

H. armigera is a major pest of a range of important crops. Control of this pest still relies heavily on conventional insecticides, but their use has led to widespread development of resistance, resurgence of secondary pests and harmful environmental side effects. There is thus an urgent need to develop alternative control technologies.

Even for a polyphagous insect such as *H. armigera*, finding a suitable host plant at an appropriate stage of development for oviposition is a critical stage of the life cycle, since the young larvae must emerge close to a source of food and protection in order to survive. Mechanisms of host-finding in insects are poorly understood and probably involve a range of stimuli including those acting at a distance such as olfactory and visual cues and those acting on contact such as gustatory and mechanical cues. There is good evidence that female *H. armigera* oviposit preferentially on the flowering stage of host plants, in or close to an inflorescence (Parsons, 1940; Roome, 1975; Fitt, 1989). Given that *H. armigera* is nocturnal it would seem likely that visual stimuli might be relatively unimportant and act at close range, unless the silhouette of the plant was important, with olfactory stimuli more likely to have a role in host-plant location from a distance.

Given the apparent uncertainty in the literature about the role of plant odours in host selection by polyphagous moths (for example see Ramaswamy, 1988), the present study was undertaken to determine whether or not floral odours play a role in host-plant location by *H. armigera* and to investigate the nature of the response. In

particular, it was intended to determine whether odours simply stimulate increased searching or whether they cause a more directed response that could be used to lure gravid female moths to some form of trapping device and hence provide potential control systems for the pest.

Flowers of marigold, *T. erecta*, and sweet pea, *L. odoratus*, were used as odour sources as these are known to be highly attractive to *H. armigera* (Srinivasan *et al.*, 1994; Patel & Yadav, 1992; Rao *et al.,* 1994). Responses to floral volatiles were measured in a laboratory wind-tunnel bioassay. Given that insects generally perceive olfactory stimuli by means of receptors on their antennae, gas chromatography linked to electroantennographic recording (GC-EAG) was used to detect and characterise components of the volatile blends that might be responsible for their biological activity. Blends of the corresponding synthetic compounds were evaluated in the laboratory bioassay and optimised as far as possible for attraction of female *H. armigera* moths, and suitable dispensing systems developed. These were then evaluated as lures for *H. armigera* in traps in the field.

The hypothesis being tested by the research was that floral odours play no role in the attraction of the polyphagous moth species *Helicoverpa armigera* to the flowers of its host plants. The alternative hypothesis was that they do play a role.

Chapter 3

METHODS USED TO REAR INSECTS AND COLLECT PLANT VOLATILES

3.1 Introduction

In order to carry out electrophysiological and wind-tunnel bioassays described in later chapters an adequate supply of *H. armigera* was required. Similarly, plants were needed for collection of floral headspace odours. Insect rearing and plant cultivation methods are described in this chapter together with details of methods used for collection of headspace volatiles.

3.2 Insect Rearing

3.2.1 Materials and Methods

A laboratory culture of *Helicoverpa armigera* was maintained on a semi-synthetic chickpea-based diet in the insectary at NRI. This was founded on 100 pupae procured from ICRISAT (International Centre for Research in the Semi-Arid Tropics, India) on 31.10.97. On 19.11.99 another 50 pupae were obtained from the same source to restart the culture after it had been closed down while overseas fieldwork was conducted. The culture was maintained at 25°C, with a relative humidity of 50% and a light-dark regime of 14 : 10 h light : dark.

Plate 3.1 Adult Cages and Larval Rearing Pots

Pupae

These were surface sterilised using 1.8% (v/v) sodium hypochlorite solution and sexed using the presence or absence of a small dark spot on the tip of the abdomen to indicate a male or female respectively (Armes *et al.*, 1992). They were then split into groups of about 22 insects with an equal sex ratio and placed inside a polypropylene pot (10 cm diameter) and kept moist with dampened vermiculite. The pots were placed in Perspex cylindrical containers (36 cm high x 20 cm diameter) with ventilated metal lids which served as the adult cages on emergence (See Plate 3.1).

Adults

The Perspex cylinder cages had a circular piece of filter paper at the base on which the pot containing pupae was placed along with two other smaller pots (4 cm diameter). One small pot had water for increasing humidity and the other had adult diet. To make the adult diet the ingredients listed in Table 3.1 were poured into a flask which was shaken well until the sucrose dissolved:

Table 3.1 Ingredients of the adult *H. armigera* diet

Ingredient	Amount	Supplier
Sucrose	50g	BDH Laboratory Supplies, Poole, UK
Vandersant vitamin mix	10mL	United States Biochemical Corporation, Cleveland, Ohio
Methyl-4-hydroxybenzoate	1g	BDH Laboratory Supplies, Poole, UK
Distilled water	500mL	(distilled from tap)

Cotton wool was placed in the pots to prevent adults drowning in the liquid. A lid with holes to provide ventilation formed a cover at the top of the cylindrical cages. This allowed adults to emerge from the pupal cases, find a perch on which to expand and dry their wings (e.g. the sides of a pot), obtain food and mate. Strips of nappy liner (Boots, Nottingham, UK) were hung inside the cage to provide a substrate for oviposition. The first eggs were laid three days after the first adults had emerged. It was important to ensure that there was mixing of pupae of different ages because males emerge later and have a longer pre-reproductive period than females (Colvin *et al.*, 1994). Putting younger females with older males improved the likelihood of obtaining successful matings. The female moth can be distinguished from the male by her more rounded abdomen, lack of tufts on the abdomen tip and by large valves at the end of the abdomen through which the ovipositor can be extruded (Armes *et al.*, 1992).

Larvae

Once adult females had started oviposition liner strips hung inside the adult moth cages were replaced every two days. The old liners with eggs on them were cut in half and each half was placed in a transparent Perspex pot (12 cm diameter) with a

clip-on lid with some larval diet crumbled into small pieces. In this container the eggs hatched and the young larvae were reared. Once the larvae had grown to about 0.7 cm length (2^{nd} or 3^{rd} instar) they were removed from the shared pot and transferred to individual pots (4 cm diameter) using forceps. Growth to this size took approximately 7 days at 25°C. A cube of diet approximately 1.5 x 1.5 x 1.5 cm which was sufficient to sustain them until pupation was put in each pot. A piece of filter paper was placed under the lid of the pot to prevent it becoming too humid, thereby discouraging mould growth. Holes were made in the lid using a sharp pin or cut using a scalpel to ensure sufficient ventilation. Pots with dead or underweight larvae were discarded. Once pupation occurred the cycle started again.

Larval Diet Recipe

The ingredients listed in Table 3.2 were used.

Table 3.2 Ingredients of the *H. armigera* larval diet

Ingredient	Amount	Supplier
Distilled water	2400 mL	(distilled from tap)
Agar powder	50g	ACROS Organics, Geel, Belgium
Chickpea flour	600g	Doves Farm Foods Ltd., Hungerford, UK
Dried active yeast	270g	DCL Yeast Ltd., Sutton, UK
Sorbic acid	15g	Fisher Chemicals, Loughborough, UK
Methyl-4-hydroxybenzoate	20g	BDH Laboratory Supplies, Poole, UK
Wheatgerm oil	24 ml	Sigma Chemical Co., St Louis, MO, USA
Vandersant vitamin mix	20ml	United States Biochemical Corporation, Cleveland, Ohio
Ascorbic acid	10g	BDH Laboratory Supplies, Poole, UK
Sulphamethazine	1.5g	Sigma Chemical Co., St Louis, MO, USA

The water and agar were measured out and transferred to a large bowl where they were mixed together. The water-agar mix was then cooked in a microwave oven (1.4kW, Philips, Sweden, Space Cube 50 M734) at full power for 30 min. The chickpea flour, yeast, sorbic acid, methyl-4-hydroxybenzoate and wheatgerm oil were then measured out and mixed together in another container. Once the water-agar mix had cooked the other ingredients were added and mixed in well with an electric mixer (Kenwood Chef). The bowl was covered with cling-film (Somerfield, UK) and four small holes made in it. Then the contents of the bowl were cooked at half power in the

microwave oven for 60 min. When cooled to <60°C the remaining ingredients (vitamin mix, ascorbic acid, sulphamethazine) were added and mixed well. The diet was spread out onto trays, covered with cling-film and put in a freezer (-22°C).

3.2.2 Discussion

The rearing method was similar to that described by Armes *et al.* (1992). Important features were that the larvae had to be kept in individual pots after they had reached a certain size since they are cannibalistic and that care had to be taken to avoid the spread of viral infections or diet mould in the culture. A modification was made that moths were kept in groups in adult cages. This method was less complicated than rearing from individual pairs of adults but nevertheless was effective enough for obtaining an abundant supply of eggs. The larval diet recipe used was found to provide a diet that was less prone to mould than the diet made with an earlier recipe (Armes *et al.*, 1992) which was cooked for a shorter time and used less yeast and fungicides, methyl-4-hydroxybenzoate and sorbic acid. The wheatgerm oil provided a source of fatty acids that are indispensable for promoting proper larval growth, adult emergence and maintenance of reproductive potential in successive generations of *H. armigera* (Gurusubramanian *et al.*, 1991).

3.3 Plant Cultivation

3.3.1 Materials and Methods

An Indian strain of African marigold, *Tagetes erecta* (Compositae), was grown in glasshouses using a 14:10 hr light : dark cycle. At each sowing 45 marigold seeds were sown with 5 seeds per pot in John Innes No. 2 compost. In-pushings were made in the compost and seeds were sown individually at 1-2 cm depth. Once seedlings had germinated and grown to a large enough size they were transplanted to 20 cm diameter pots, one plant per pot, and were supported using wooden stakes. Pots were placed on trays which were partially filled (2 - 3 cm depth) with water. This allowed water to seep into the bottom of the pots and avoided over-watering which prevents

water to seep into the bottom of the pots and avoided over-watering which prevents the roots from being sufficiently aerated. Sweet Pea, *Lathyrus odouratus* (Papilionaceae) was also grown under similar conditions.

Plate 3.2 *Tagetes erecta* growing in the Glasshouse

Flowers of *Buddleia davidii* (Buddlejaceae) were obtained from established plants growing on the campus. The history of these and details of any chemical treatment were unknown.

3.3.2 Discussion

The main experimental work was on *T. erecta* which was grown under controlled conditions in the glasshouse and was unsprayed. Growing plants on site enabled a good supply of plant material for air entrainments. At least 14 hr of daylight were required for flowering and this was provided by artificial illumination when natural daylight was insufficient.

3.4 Collection of Floral Volatiles by Air Entrainment

3.3.4 Materials and Methods

Entrainment of Cut Flowers

T. erecta was the main species from which floral volatile collections were made. Air entrainments of *L. odouratus* and *B. davidii* flowers were also carried out. Samples of floral headspace volatiles were initially collected by air entrainment using freshly cut flowers. Air entrainment was done immediately after flowers were cut from the plants in the glasshouse so that they were as fresh as possible and was conducted at a temperature of 24°C.

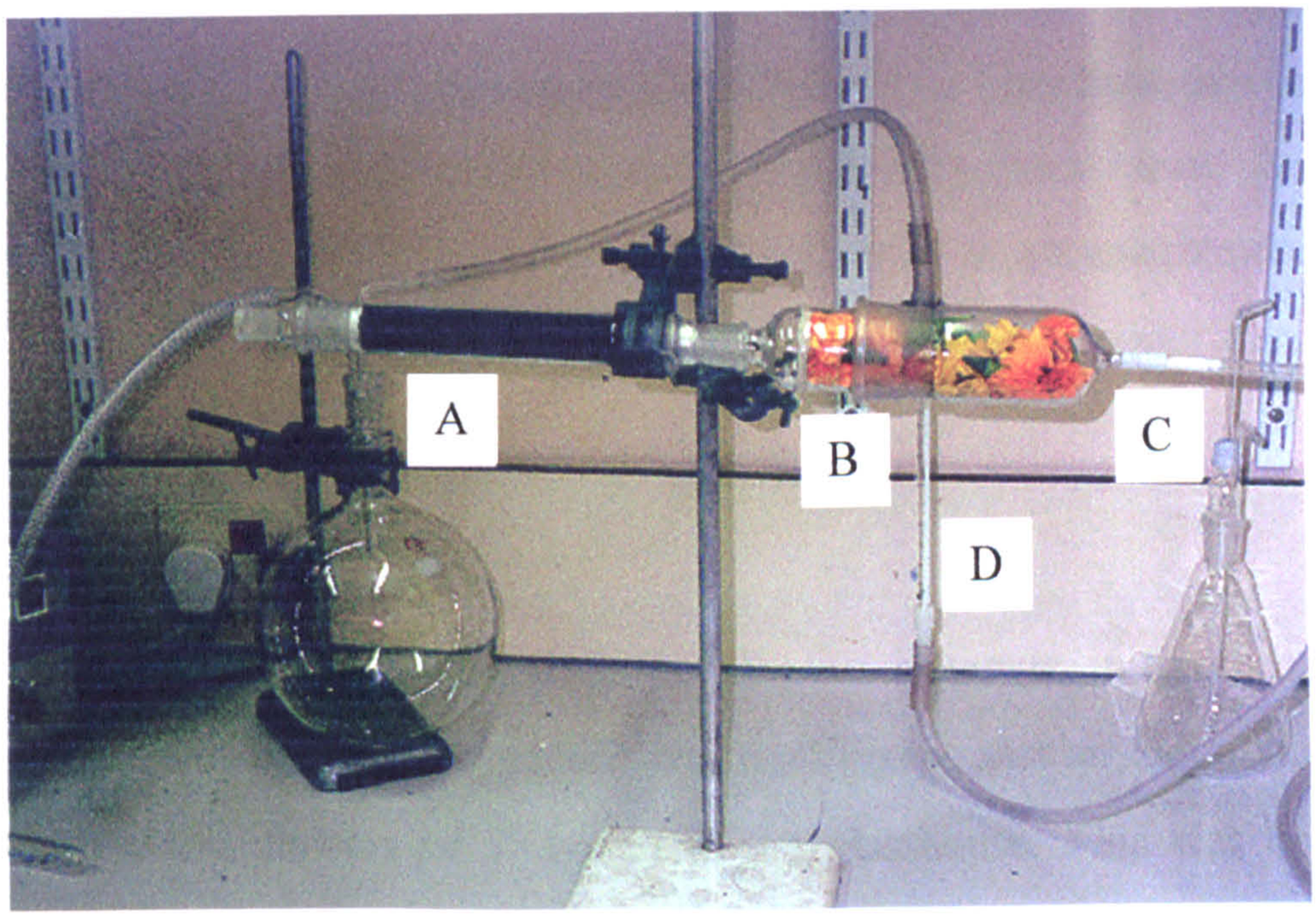

Plate 3.3 Air Entrainment Apparatus. The flask used for entraining between 1 and 20 flowers. A, Charcoal filter on inlet; B, entrainment chamber for cut flowers; C, Porapak Q filter; D, flowmeter on outlet to pump.

Four to 55 flowers were placed in clean glass Quickfit flasks, typically 500 ml capacity, although flask size was varied with the number of flowers available (Plate 3.3). Charcoal (20g, 8-15 mesh size, BDH, UK) filtered air was drawn through the flask at 2 L/min by a suction pump (CAPEX L2C, Fisher Scientific, Loughborough, UK). Airflow was smoothed by passing through a conical flask before entering the suction pump and flow rate was measured using a flowmeter. A time duration of typically 3 to 8 hr was chosen for entrainments because samples from shorter

collection times were found to be less electrophysiologically-active. Longer entrainment times simply enabled a larger quantity of volatiles to be collected. On exiting the container the volatiles were adsorbed on Porapak Q (200mg, 60-80 mesh size, Phase Separations, UK), packed inside a Pasteur pipette and held in place by silanised glass wool.

Procedures were implemented to avoid contamination of headspace samples. Care was taken that glassware used was clean (washed in Teepol solution, rinsed with distilled water, acetone and air dried). The Porapak Q was Soxhelet extracted with chloroform for 8 hr before it was put in the Pasteur pipette and rinsed with dichloromethane (2 x 5 ml) to remove any residual contaminants from previous use. A control air entrainment with an empty entrainment container was conducted. GC analysis of the dicholoromethane extract of the Porapak Q filter from this entrainment was carried out to ensure that the equipment and filter were clean. Another test consisted of two Porapak Q filters connected in series in an entrainment of floral volatiles to verify that there was no breakthrough of volatiles from the first filter during an 8 hr collection period.

Entrainment of Live Flowers

Initial work was undertaken with cut flowers. However, in the second year of the project the air entrainment apparatus was modified so that air entrainments could be made from live flowers of *T. erecta* in the glasshouse. This was because cut flowers could conceivably have a emitted a different blend of volatiles. However defensive compounds are only released after specific herbivore damage and not after mechanical damage (Vet & Dicke, 1992). Samples of floral headspace volatiles were collected by air entrainment using single live flowers, still attached to growing plants. These were enclosed between two Quickfit flange lids, clamped together to form a 3-necked flask (80 ml) and held by another clamp over the flower. Quickfit glass containers were rinsed with acetone and dried before use. Where the flower entered the flask, the gap between the stem and the flask was packed with charcoal (8-15 mesh size, BDH, UK) and sealed with aluminium foil. Air was drawn through the apparatus by a suction pump (CAPEX L2C, Fisher Scientific, Loughborough, UK). Incoming air was filtered through a charcoal-filled glass column, to remove volatile

contaminants. Volatiles from flowers were entrained for 10-14 h at 2 l/min. After exiting the flask the volatiles passed through a Porapak Q filter (200mg, 50-80 mesh size, Phase Separations, UK) that had previously been cleaned by rinsing with dichloromethane (3 x 5 ml). Entrained volatiles were eluted from the Porapak Q filters with dichloromethane (2ml) and stored at -20°C. Using both live and cut flowers meant that any qualitative and quantitative differences in the odour blend emitted from cut and live flowers could be investigated by GC analysis. Once live flowers were entrained release rates could be calculated so that doses used in bioassays could be expressed in terms of natural flower-hour equivalents.

Elution of Samples from Porapak Q filter

The entrained volatiles were eluted from the Porapak Q filters with dichloromethane (1 ml). The Pasteur pipette containing the Porapak Q was clamped vertically and the solvent was pipetted in at the top. The sample was collected under gravity in a sample tube (3.5ml, Fisher Scientific, Loughborough, UK) held at the base of the Porapak filter. A rubber teat fitted to the top of the tube was used to force remaining drops of solution out of the Porapak Q filter. The sample tube was immediately fitted with the screw top lid (PTFE lined) and the sample stored at - 20°C.

3.4.2 Discussion

A variety of methods is reported in the literature for the collection of plant volatiles. The main techniques are solvent extraction, steam distillation and headspace collection methods. Extraction and distillation remove substances from the plant tissue whereas headspace methods collect from the air surrounding the plants. This means that headspace samples contain fewer non-volatile contaminants (Dobson, 1991). Knudsen *et al.* (1993) considered extraction and distillation methods to be unreliable due to the artefacts (compounds which would not normally be released into the air surrounding the live plant) that they can produce.

Headspace sampling is non-destructive and thus can be carried out on live flowers. Headspace adsorption involves accumulative trapping of volatiles onto an adsorbent such as Porapak Q over time. Porapak Q was chosen in this research

because a larger sample can be collected from it than with other adsorbents such as Tenax. Air entrainment has become a widely used method and was the method chosen in this study. Compared with direct headspace sampling, headspace adsorption has the advantage of allowing concentration of the sample during the period of collection which means larger quantities of collected compounds are available for analysis. Adsorbed volatiles can be thermally desorbed as well as being eluted with organic solvents. Another headspace technique that is less widely used is cold-trapping which uses condensation of the headspace volatiles at low temperatures for collecting them. A problem with cold-trapping is that samples can become contaminated with water (Knudsen *et al.*, 1993).

Solid Phase Microextraction (SPME) is a relatively new headspace sampling method which involves adsorption of volatiles onto a polymer coated fused silica fibre (Martos *et al.,* 1998). It is different from air entrainment using Porapak Q in that a dynamic equilibrium is set up between the SPME fibre and the headspace whereas trapping of volatiles is accumulative on a Porapak Q filter. The ratio of compounds sampled by SPME is not the same as that obtained when headspace volatiles are collected, without adsorption, in a syringe and Porapak Q gives a more representative ratio (Agelopoulos & Pickett, 1998). Other disadvantages are that the sample collected can only be used once and the capacity is inadequate for collection of the large amounts of material required for behavioural bioassays. A larger amount of material can be collected from Porapak Q and repeated aliquots can be taken from the same sample. At least 20 μg of entrained sample was required for each replicate in the bioassay in the current research.

Chapter 4

IDENTIFICATION OF ELECTROPHYSIOLOGICALLY-ACTIVE COMPOUNDS IN FLORAL VOLATILES USING LINKED GAS CHROMATOGRAPHY AND ELECTROANTENNOGRAPHY

4.1 Introduction

Floral odours comprise complex mixtures of volatile compounds (Knudsen *et al.*, 1993). Such blends are highly complicated to reproduce synthetically if a complete blend is attempted and thus it is preferable to formulate simplified blends of compounds. In order to produce a synthetic mimic of a floral odour that can elicit the same behavioural responses from *H. armigera* as the natural odour a technique is needed that can identify 'behaviourally-relevant' compounds in floral odours. Insects perceive volatile compounds by means of sensilla on their antennae. The simplest means of recording electrophysiological responses from antennae is to use the electroantennogram (EAG) technique. The EAG response was first observed by Schneider (1957) who reported that the response was a summation of electrical depolarisations from sensilla across an antenna in response to olfactory stimuli. Since it records responses from all olfactory receptors it can be used as a detector for electrophysiologically-active compounds in natural mixtures of behaviour-modifying chemicals.

Gas chromatography provides a sensitive and versatile means of separating volatile compounds in a mixture in the gaseous state. By coupling the two techniques Moorhouse *et al.* (1969) developed a powerful technique for identifying components of insect semiochemicals. This technique, and related techniques (Arn *et al.*, 1975, Cork *et al.*, 1990), has been successfully applied to the identification of behaviour-

modifying compounds from a wide range of insect semiochemcials, typically components of sex pheromones (e.g. Roelofs & Comeau, 1971), but also aggregation pheromones (Hodges *et al.*, 1984) and more recently kairomones of haematophagous pest species (Cork & Park, 1996).

The technique relies on the assumption that compounds that are found to elicit significant electrophysiological responses from olfactory sensilla on the antennae of insects are most likely to be behaviourally-relevant. Thus, it could in principle be used to identify behaviourally-relevant compounds in the headspace samples of volatiles from flowers of African marigold, *Tagetes erecta* and sweet pea, *Lathyrus odouratus*, hosts of *H. armigera*. Similarly, the EAG technique could be used to both confirm the presence of electrophysiologically-active compounds in volatiles collected by air entrainment and to confirm the electrophysiological activity of synthetic compounds once they had been characterised.

Most of the work reported here was undertaken with female *H. armigera* reared in the laboratory culture rather than male moths since female moths are dependent on host plants for sources of nutrition and for ovipositing progeny whereas male moths are only dependent on host plants for nutrition. Thus for example, Raguso *et al.* (1996) observed that female *Hyles lineata* (Lepidoptera: Sphingidae) were more electrophysiologically sensitive to volatile compounds from *Clarkia breweri* (Onagraceae) than male conspecifics. Also for pest management applications attractants developed for female moths would be more useful for both monitoring adult populations and as baits for lure-and-kill control technologies.

EAG responses are proportional to stimulus concentration over a short dynamic range and so the EAG technique could not be used to quantity the relative amount of electrophysiologically-active compounds present in the floral volatiles collected. Similarly, the more volatile components of a floral odour would be lost if entrainments were conducted over prolonged time periods. Thus, entrainments (see Chapter 3) were made over different time periods and with different quantities of flowers in the expectation of collecting sufficient material to enable the identification of all the behaviourally-active compounds present in the floral volatiles.

4.2 Methods and Materials

4.2.1 Electoanntennography (EAG) Preparations

Fixing Insects

Electroantennographic (EAG) recordings were carried out as described by Cork *et al.* (1990) using adult female *H. armigera* of up to one week old. The EAG preparation was set up on a vibration free table, separate from the GC. Test insects were anaesthetised using CO_2. Whole insects were held down in a groove on a plasticine block on their dorsal surface and covered with a strip of polystyrene which was pinned down above them. They were positioned so that the head pressed against the plasticine to prevent the moth pushing forward when attempting to escape. The antennae were left exposed on the surface of the plasticine. Small pieces of copper wire (0.2mm diameter, 7mm length) were cut and bent over to form U-shaped staples which were inserted into the plasticine over the antennae to hold them in a 'V' shape in front of the insect to accommodate insertion of the micro-electrodes.

Microelectrodes

A microelectrode puller (Harvard Apparatus, Kent, UK) was used to make the glass microelectrodes (c. 3cm length, tip diameter 20-50 μm) from capillary tubing (borosilicate glass capillaries, 2.0mm outer diameter, 1.16mm inner diameter, Clark Electromedical Instruments, Reading, UK). Fine microelectrode tips were formed by the microelectrode puller by balancing the level of heat applied to the mid-point of the capillary tube and the degree of pull on each end. Once cooled the micro-electrodes were filled with a saline solution isotonic with the insect haemolymph. The saline consisted of NaCl (7.5g/l), $CaCl_2$ (0.21g/l), KCl (0.35g/l) and $NaHCO_3$ (0.2g/l) as described by Roelofs (1984). This saline solution in the microelectrode would provide electrical contact between the antennal haemolymph and the silver/silver chloride junction of the recording apparatus once inserted into an antenna.

EAG Preparation

The microelectrodes were attached to electrode holders clamped in micromanipulators (Leitz, Wetzlab, Germany). The electrode holders contained Ag/AgCl junctions that were connected to a high impedance AC/DC amplifier (Syntech, The Netherlands). The recording micro-electrode was inserted through the interstitial membranes between annuli at the distal end of one antenna and the reference micro-electrode was inserted at the proximal end of the other antenna, viewing the entire procedure through a binocular microscope (6 - 50x magnification, Leica WILD M8, Heerbrugg, Switzerland). The position of the electrodes was chosen to minimise interference from extraneous neuromuscular activity (Cork *et al.* 1990). As reported by Cork *et al.* (1990), the interstitial membranes between antennal segments were easier to pierce with microelectrodes than the annuli themselves in order to obtain good electrical contact between the haemolymph of the antenna and microelectrode.

Amplification and Processing of the EAG Signal

DC signals recorded between the two microelectrodes were amplified through a high impedance AC/DC amplifier (Model UN06, Syntech, Hilversum, The Netherlands), stored and processed on a PC using Turbochrom 4.0 software (Perkin Elmer Ltd., Beaconsfield, UK).

Presentation of Stimuli

Volatile stimuli were delivered to the EAG preparation using two different methods: 'Direct EAG' involved releasing volatiles into a pulsed stream of nitrogen from a filter paper contained inside a Pasteur pipette. The other system involved the use of effluent from a GC column as the source of volatile chemicals in 'linked GC-EAG'. Before experiments with air entrained floral volatile samples were carried out known stimuli were tested to ensure that the EAG preparations were in good condition and that the EAG equipment had been set up correctly. The stimuli used for this purpose were the female sex pheromone tested on male insects (20ng, (*Z*)-11-hexadecenal and (*Z*)-9-hexadecenal in a 33 : 1 ratio, Nesbitt *et al.*, 1979) and phenylacetaldehyde (1 μg) tested on female insects.

4.2.2 EAG analysis of crude extracts of floral volatiles

Stimulus Delivery

Electrophysiological activity of air entrained Porapak Q samples was tested using the EAG technique. This involved placing aliquots (1 - 20 µl) of test solutions onto filter paper strips (Whatman No. 4, 5 x 15 mm) placed inside Pasteur pipettes. The pipettes were connected to a nitrogen supply and a pulse (3 s, 100 ml/min) delivered through the pipette to evaporate off the solvent. The pipette was then repositioned 1 cm above the centre of the EAG preparation and a pulse (3 s, 100 ml/min) of nitrogen passed through the pipette and over the EAG preparation, while recording the electrophysiological response. A 1 min interval was given between samples and replicates to allow the preparation to recover from exposure to each stimulus. A preliminary check was carried out with phenylacetaldehyde (1 µg) to ensure that the 1 min interval was sufficient time for female *H. armigera* to recover from the preceding stimulus (Burguiere *et al.*, 2000).

Procedure used to Measure EAG Responses

Initially the EAG response elicited by a solvent control (1 – 20 µl dichloromethane), prepared as described above, was recorded by exposing the EAG preparation to 5 three sec pulses of nitrogen passed through the Pasteur pipette at 1 min intervals (57 sec delay between pulses). Then air entrained Porapak Q samples were used as stimuli. The mean EAG response of replicates 2 - 5 of the solvent control was compared with the mean EAG responses of replicates 2 - 5 of air-entrained floral samples. The first replicate was not used when measuring responses because it tended to be larger and less consistent than the following responses (Burguiere *et al.*, 2000). The amplitudes of the EAG responses were calibrated by applying a –1 mV spike from the high impedance AC/DC amplifier. The mean EAG response to solvent control was subtracted from the mean sample response to obtain a mean corrected EAG response for the test samples. This corrected response represented the summation of the olfactory response from the sample, having effectively subtracted contributions from mechano- and thermoreceptors to the pulse

of nitrogen and any response to the solvent or volatiles from the filter paper. Entrainment samples that elicited EAG responses that were at least 30% greater than that of the solvent alone were used for linked GC-EAG analyses.

Sample Concentration

Where extracts of headspace samples were collected from small numbers of flowers (Tag-98-01, Tag-98-02 & Tag-98-03 but not Tag-98-04; Table 4.1) it was necessary to concentrate aliquots down before use in EAG and linked GC-EAG analyses. This was done by applying a gentle stream of nitrogen over an aliquot (typically 20 - 40 µl) in a tapered Pyrex glass vial (1.1 ml STVG, Chromacol, Herts, UK) and reducing the solvent volume to 1 - 3 µl. Where required, internal standards were added before evaporation to ensure losses could be quantified by GC analysis.

4.2.3 Linked GC-EAG analyses

Stimulus Delivery and Details of the GC-EAG Link

Linked GC-EAG analyses were conducted as described by Cork *et al.* (1990) using a modified Carlo Erba 5300 Mega series gas chromatograph equipped with two fused silica capillary columns (polar, CPWax52CB, 30 m x 0.32 mm ID, and non-polar, CPSil5CB, 25 m x 0.32 mm ID, Chrompack Ltd., The Netherlands; Fig. 4.1). The capillary column was needed for obtaining good resolution of peaks for GC analysis of compounds eliciting any EAG responses. Samples were introduced into the GC in splitless mode (valve closed for 30 sec) using a Grob split / splitless injector at 200 °C with the FID at 250°C. Two temperature programs were used for analyses, 50°C for 2 min, then temperature programmed at 20°C/min to 80°C and then at 5°C/min to 240°C and 50°C for 2 min and then temperature programmed at 7°C/min to 240°C. The GC carrier gas was helium (40 kPa head-pressure; 2 ml/min flow-rate). GC column effluent was split 50 : 50 between the flame ionization detector (FID) and the EAG preparation. GC column effluent to the EAG preparation was collected for 17 seconds in a silanised glass reservoir inside the GC oven and then passed over the EAG preparation for 3 seconds in a stream of nitrogen (250 ml/min) after which

collection in the reservoir resumed and the cycle repeated. Pulses of GC effluent were thus delivered every 20 seconds to the EAG preparation.

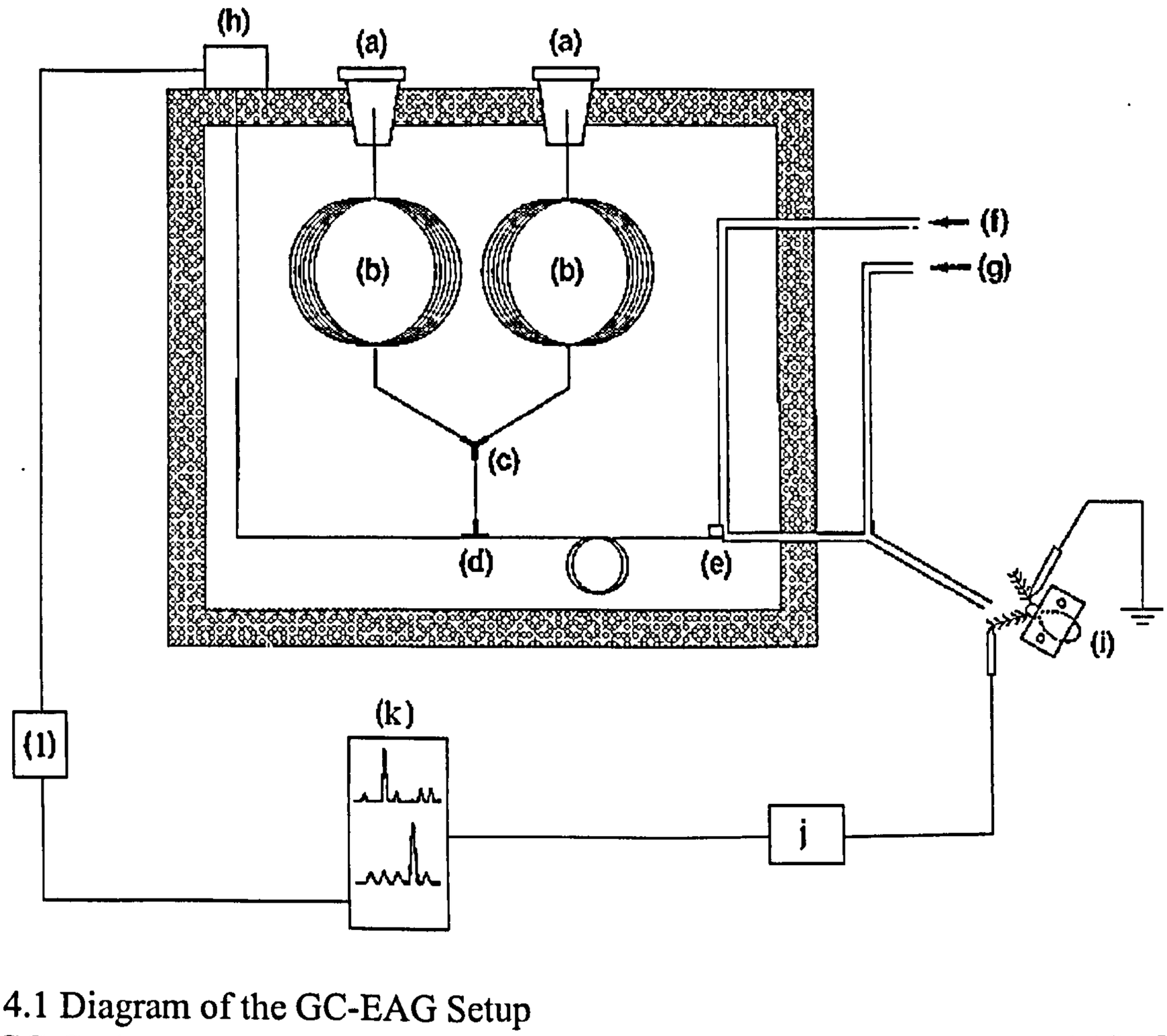

Fig 4.1 Diagram of the GC-EAG Setup
(a) GC Grob type split / splitless injectors; (b) capillary columns; (c) and (d) Y tube connectors; (e) GC column outlet to EAG preparation; (f) inlet for pulsed nitrogen flow (250 ml/min); (g) inlet for constant bypass nitrogen flow (0 ml/min); (h) GC column outlet to flame ionisation detector (FID); (i) EAG preparation; (j) high impedance DC amplifier; (k) computer terminal for data recording (l) FID amplifier
From Cork *et al.* (1990)

Retention Indices of Compounds

Linked GC-EAG analyses of entrained floral volatiles were replicated four times using different insects and carried out on four different *T. erecta* Porapak Q extracts and two different *L. odouratus* Porapak Q extracts. The time taken for a compound to elute from a GC column was dependent on the balance between the tendency of the compound to interact with the stationary phase and the tendency for it to vapourise (Throck Watson, 1985). GC retention times of components associated

with EAG activity were converted into retention indices by comparison with the retention times of saturated, straight-chain hydrocarbons (thus, *n*-tetradecane = 1400). The retention index for a given compound is essentially constant for a give stationary phase. Thus, by calculating the retention indices of compounds eliciting EAG responses in linked GC-EAG analyses they can be located in subsequent linked GC-MS analyses with the same samples using similar GC columns. The retention index of a compound eluting between dodecane and tetradecane (R.I., 1200 and 1400) would be calculated as follows:

Retention Index = 1200 + (200x b/a)

Where b = (retention time of the compound) - (retention time of dodecane)
and a = (retention time of tetradecane) - (retention time of dodecane)

The abundance of compounds in a sample was calculated by comparison of peak area with that of a known quantity of an external standard, e.g. 20 ng, tetradecyl acetate. No correction was made for any difference in FID response to the compounds and the FID response was assumed to be linear for the range of concentrations used throughout the study.

Location of Olfactory Responses from Linked GC-EAG Analyses

Output from the EAG was recorded as a series of EAG responses. In order to determine whether a GC fraction contained an EAG-active compound the amplitude of EAG responses were calculated and those that were >10% larger than the mean of two baseline responses recorded before and after the response were considered to contain compounds that had elicited an EAG response. Confirmation of the presence of an EAG-active compound was obtained by repeating the linked GC-EAG analysis at least four times with other samples and EAG preparations. Where two or more EAG-active compounds eluted closely from the GC (e.g. benzaldehyde and linalool elute close together on the polar column) the mean baseline response had to be calculated without including neighbouring EAG responses.

4.3 Results

4.3.1 EAG analyses of extracts of floral volatiles collected on Porapak Q

T. erecta

EAG tests of crude Porapak Q extracts of floral volatiles collected from *T. erecta* were used to ascertain whether the samples contained sufficient EAG-active material to warrant analysis by linked GC-EAG. Details of samples used are given in Table 4.1

Table 4.1 Porapak Q floral headspace samples collected from *Tagetes erecta*.

Sample Code[a]	No. of flowers[b]	Entrainment time (h)	Flower – hours	Mean corrected EAG response[c] (-mV)	No. of insects used in EAG test
Tag-98-01	6	24	0.55	5
Tag-98-02	6	12	0.37	5
Tag-98-03	3	21	0.51	4
Tag-98-04	3	165	0.45	4

[a] Porapak Q filters were eluted with dichloromethane (1 ml).

[b]the number of flowers used to collect each sample depended on availability and samples were concentrated before EAG tests to give comparable concentrations.

[c]Aliquots (50 µL) of samples Tag-98-01, Tag-98-02 and Tag-98-03 were concentrated down using a gentle flow of nitrogen to 2µL (25-fold concentration). A 1 µL aliquot of sample Tag-98-04 (Table 4.1) was used without concentration.

The EAG data shown in Table 4.1 confirmed that entrainments of floral compounds from *T. erecta* could elicit electrophysiological responses from female *H. armigera*. Approximately one flower-hours of volatiles were required to elicit an EAG response (e.g. for sample Tag-98-01, with 50µL of a 1000µL sample, 50/1000 x 24 = 1.2 flower hours). However this was the amount on the filter paper and the

amount which the antenna was exposed to in each pulse could have been considerably less.

L. odouratus

Details of headspace samples collected from *L. odouratus* flowers are given in Table 4.2. No concentration of samples was required prior to EAG tests.

Table 4.2 Porapak Q floral headspace samples collected from *Lathyrus odouratus*.

Sample Code[a]	No. of flowers	Entrainment time (h)	Flower-hours
L-98-01	30	24	720
L-98-02	30	9	270

[a] Porapak Q filters were eluted with dichloromethane (1 ml).

Aliquots (1µl) of extracts prepared from Porapak Q collections of floral volatiles, L-98-01 and L-98-02, elicited EAG responses from female *H. armigera* that were 331% and 265% greater than the solvent control response respectively. This confirmed that the floral odours from *L.. odouratus* contained electrophysiologically-active compounds. There was evidence that responses were dose dependent since L-98-01 contained 0.72 flower-hr of material while L-98-02 contained 0.27 flower-hr of material.

4.3.2. GC-EAG analyses

Forty-five compounds were detected by the FID on GC analysis of the Porapak Q air entrained collections of floral volatiles from *T. erecta* on a polar GC column (Figure 4.2). In linked GC-EAG analyses with female *H. armigera* five EAG responses above the background level were observed using the polar column and also when analyses were carried out on a non-polar column. Due to highly sensitive mechano- and thermoreceptors on the antennae of *H. armigera*, the baseline response to nitrogen pulsed over the EAG preparation was large: the average background response to the nitrogen pulse for all GC-EAG analyses was -0.78 mV. Typically, EAG-active compounds in the samples elicited responses, as they eluted from the GC,

175% of the background responses. Because of the poor signal to noise ratio (Figs 4.3 & 4.4) it was necessary to conduct at least four linked GC-EAG analyses with each sample in order to establish which compounds consistently elicited EAG responses.

Fig. 4.2 shows the gas chromatogram of a *T. erecta* sample later used in wind-tunnel bioassays on which EAG-active compounds are numbered 1 – 5. Typical linked GC-EAG analyses are shown in Figures 4.3 - 4.5.

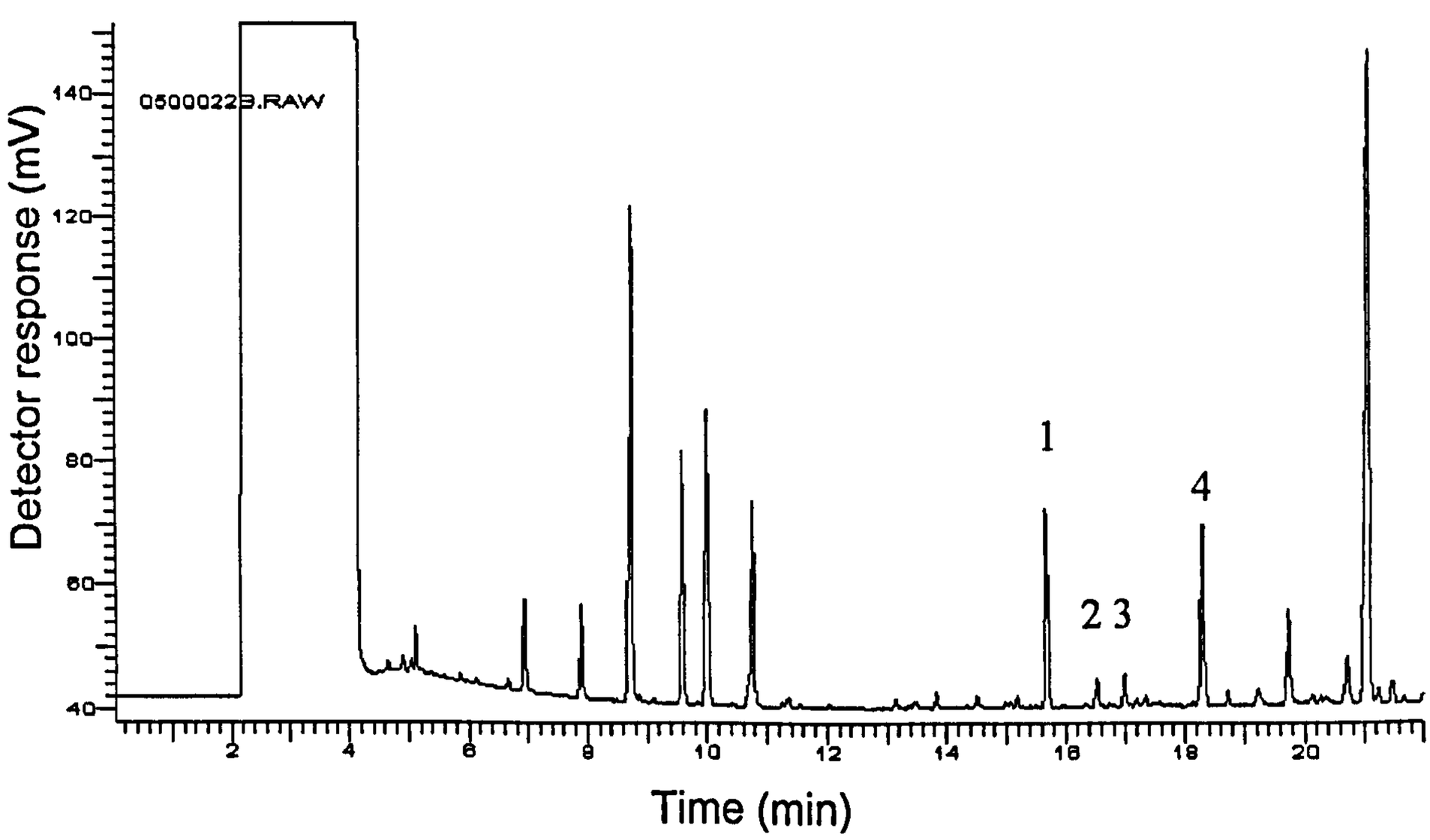

Fig 4.2 Gas chromatogram of *T. erecta* sample Tag-WT1 on a polar column (CPWax52CB, 30 m x 0.32 mm ID) showing compounds associated with EAG activity (T1 to 5). Sample Tag-WT1 was prepared by adding together Tag-98-01, Tag-98-03 and Tag-98-04 and later used in wind-tunnel bioassays.

The retention indices (RI) of the five compounds that eluted at times when EAG responses were observed were calculated on polar and non-polar GC columns (Table 4.3). The compounds constituted between 0.5 and 3% of the total amount of volatile material detected by the GC apart from T5 which constituted 14.5% and consistently elicited the smallest EAG responses.

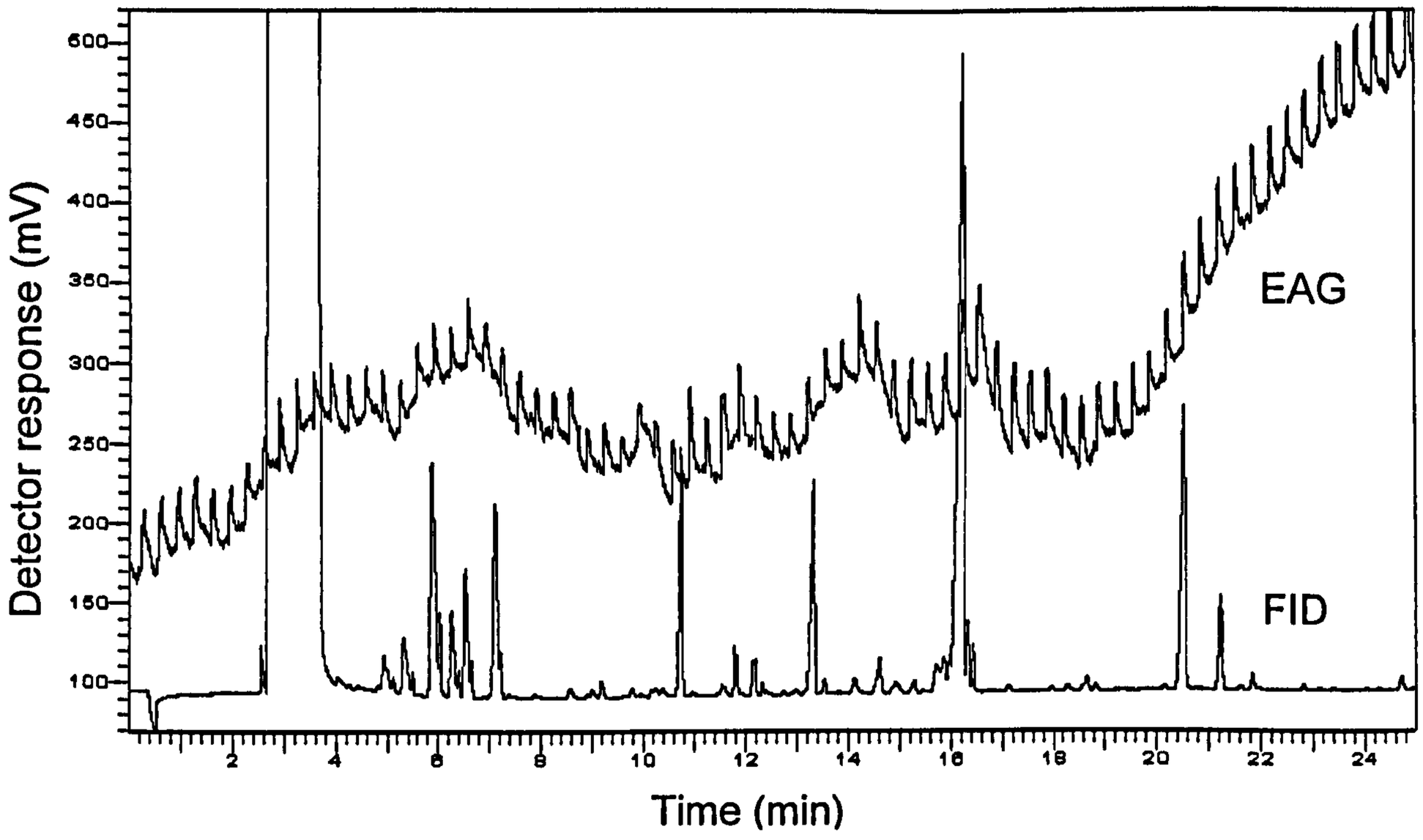

Fig 4.3 Linked GC-EAG analysis of floral volatiles from *T. erecta* (Tag-98-01) eluting from a polar column (CPWax52CB, 30 m x 0.32 mm ID) and exposed to a female *H. armigera* EAG preparation. EAG responses of -1mV were equivalent to +70mV (FID) on the 'Y' axis.

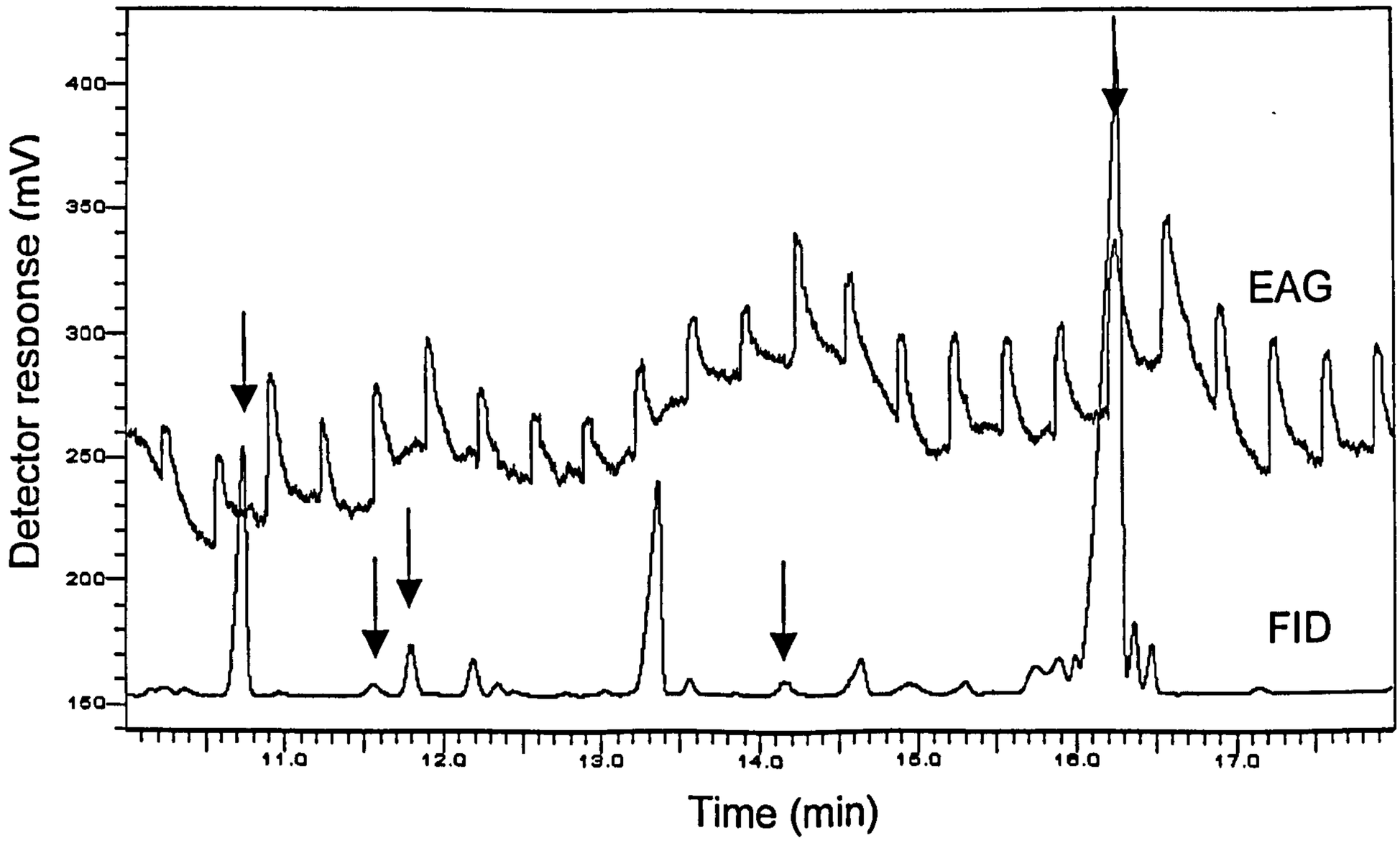

Fig 4.4 Linked GC-EAG analysis (polar column: CPWax52CB, 30 m x 0.32 mm ID) of floral volatiles from *T. erecta* (Tag-98-01) expanded from Fig 4.3. to show EAG-active components (arrows) in detail. EAG responses of -1mV were equivalent to +70mV (FID response) on the 'Y' axis.

GC-EAG analyses carried out with *L. odouratus* air entrained samples indicated that they contained four GC peaks associated with EAG activity (Fig 4.5). The retention indices of these EAG-active compounds were calculated on polar and non-polar columns and are reported in Table 4.4.

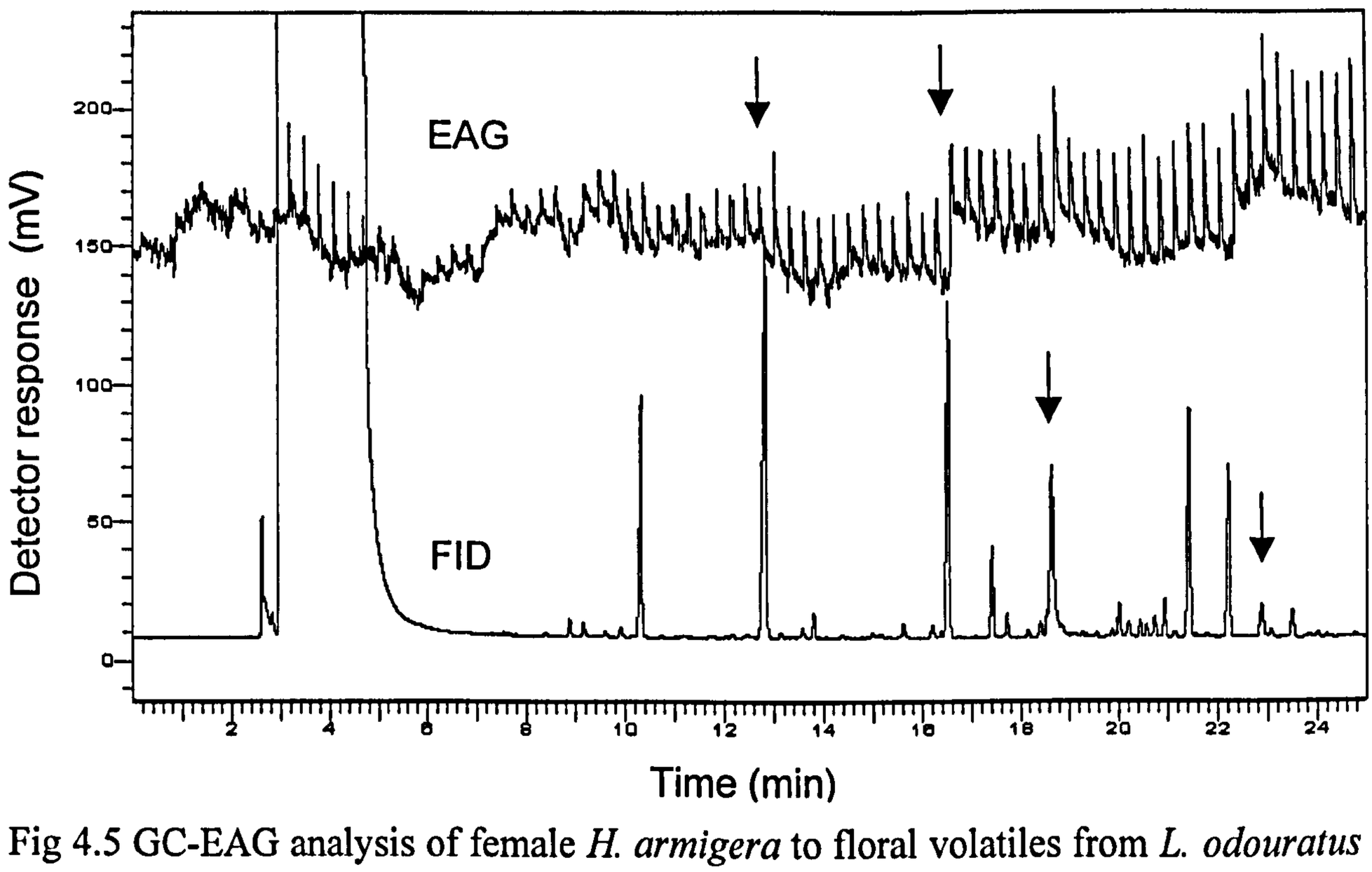

Fig 4.5 GC-EAG analysis of female *H. armigera* to floral volatiles from *L. odouratus* (L-98-03). Analysis was conducted on a polar column (CPWax52CB, 30 m x 0.32 mm ID). Arrows show EAG-active components. EAG responses of -1mV were equivalent to +70mV on the 'Y' axis.

Table 4.3 GC retention indices of EAG-active compounds identified in entrainments of floral volatiles collected from *Tagetes erecta*.

Compound	Relative abundance [a]		Retention Indices		EAG responses[c]	
	Mean percentage	S.E	polar column	non-polar column[b]	Mean (-mV)	S.E.
T1	3.52	1.42	1485	1123	-0.26	0.10
T2	2.36	1.18	1522	925	-0.39	0.04
T3	0.61	0.23	1539	1085	-0.29	0.05
T4	3.99	2.43	1642	1007	-0.54	0.10
T5	14.5	5.67	1725	1226	-0.17	0.10

[a] Percentage of the total area under all GC peaks excluding the solvent, mean of four samples.
[b] Retention times of EAG-active compounds on the non-polar column were correlated with those on the polar column using their mass spectra
[c] n = 4

Table 4.4 GC retention indices of EAG-active compounds identified in entrainments of floral volatiles collected from *Lathyrus odouratus*

Compound	Relative abundance [a]		Retention Indices		EAG responses[c]	
	Mean percentage	S.E	polar column	non-polar column[b]	Mean (-mV)	S.E.
L1	12.2	12.2	1366	821	-0.32	0.11
L2	13.4	1.52	1545	1014	-0.56	0.07
L3	15.1	1.32	1652	1086	-0.55	0.04
L4	1.05	0.42	1887	1022	-0.15	0.02

[a] Percentage of the total area under all GC peaks excluding the solvent. Mean of four samples.
[b] Retention indices of EAG-active compounds on the non-polar column were correlated with those on the polar column using their mass spectra
[c] n=3

4.4 Discussion

EAG analysis confirmed that electrophysiologically-active compounds had been entrained amongst volatiles released by *T. erecta* and *L. odouratus* although increasing the number of flowers per collection did not necessarily result in a proportional increase in electrophysiological activity of the samples collected. Nevertheless, the electrophysiological activity of samples collected did decline with the age of the flowers, but this data provided information on the relative change in composition of electrophysiologically-active compounds collected over time.

EAG Responses of H. armigera to Volatiles from T. erecta and L. odouratus in Linked GC-EAG Analyses

Five EAG-active compounds were identified in the headspace samples of *T. erecta* and four EAG-active compounds in the headspace samples of *L. odouratus* by linked GC-EAG analyses and their retention indices were calculated. However, the EAG responses elicited by the compounds in the GC-EAG analyses were not always apparent compared to neighbouring responses (average -0.78mV) due to the large background responses from mechano- and thermoreceptors and it was necessary to replicate analyses at least four times to confirm results. EAD uses non-pulsed delivery of GC column effluent and consequently avoids chemo-responses being co-occurring with mechano-responses. Despite this it was considered better to use pulsed delivery of GC effluent because the regular series of EAG responses could be readily discerned over baseline drift compared to continuous exposure of the EAG to GC column effluent (Marion-Poll & Thiéry, 1996). Also between pulses from the GC the EAG preparation was allowed to return to the resting potential. Pulsed delivery of GC effluent limited the resolution of the EAG detector to the time interval over which volatiles were collected (17 seconds in the present study) compared to continuous exposure to GC effluent where the resolution was limited by the GC peak width (typically 5 to 12 sec).

Since GC analyses were temperature-programmed, typically 7°C/min, the background EAG response increased progressively with time, although this rarely

increased by more than ±0.1mV from one pulse to the next and so EAG responses elicited by compounds eluting from the GC could be discerned.

Assumptions of GC-EAG Analyses

An implicit assumption in the use of the EAG technique was that a volatile stimulus that elicited a strong electrophysiological response was more likely to elicit a behavioural response from an insect than a compound that elicited a weak electrophysiological response at the same dose (Marion-Poll & Thiéry, 1996). This might not always be true and so a certain amount of caution was needed in interpreting the EAG data - small responses to compounds for which there are relatively few chemo-receptors might still be behaviourally important. High sensitivity would not be expected for compounds that have high ambient concentrations because this would lead to sensory overload. Suckling *et al.* (1996), investigating oviposition responses of lightbrown apple moth, *Epiphyas postvittana*, found that the magnitude of EAG responses showed little relation to the strength of the behavioural response they could elicit. Similarly, Honda *et al.* (1998) found that EAG activity was poorly correlated with proboscis extension activity for cabbage butterfly, *Pieris rapae.* Nevertheless, Zhu *et al.* (1993) did obtain relatively large EAG responses from volatiles collected from blooming plants that were observed to be attractive to adult black cutworm, *Agrotis ipsilon* (Noctuidae), in the field.

Another assumption of the EAG technique is that the principal receptors used in insect olfaction are those located on the antennae (Moorhouse *et al.*, 1969). Sensory perception, recorded by an EAG response, is only the start of the mechanism of host-plant recognition (Dethier, 1982). Other important factors, not measured by EAG, are the processing of sensory input in the central nervous system, across fibre patterns, effects of deterrent / stimulant ratios, level of satiety, and the influence of prior experience which all affect the behavioural outcome. EAG responsiveness may decline after several generations of rearing of insects in the laboratory (Lecomte *et al.*, 1998). These authors noted a significant decline in the threshold concentration required for an EAG response by the leek moth, *Acrolepiopsis assectella*, to dipropyl thiosulphinate, dipropyl disulphide and (*Z*)-3-hexen-1-ol.

Use of GC-EAG Analysis for Identifying Biologically-active Volatiles

GC-EAG analyses indicate which plant volatiles in a crude mixture can be detected by the olfactory apparatus of an insect. The technique was used as a method of screening for compounds to test in the wind-tunnel bioassay with female *H. armigera*. EAG-active compounds were prioritised for identification by GC-MS over non EAG-active compounds. If GC-EAG analyses had not been used it would have been necessary to separate the crude air entrained samples into many fractions and laboriously carry out bioassay tests on each of them. Pivnick *et al.* (1994) used fractionation and EAG to investigate olfactory cues used by diamondback moth, *Plutella xylostella*, for host-plant finding but were unable to identify which host-plant compounds were responsible for attraction. If bioassays are carried out on different fractions of a host kairomone then any synergism between compounds may be lost if they are separated in different fractions. Thus, GC-EAG analyses are potentially useful for identifying individual compounds that together can give a behavioural response but which would not necessarily be behaviourally-active on their own (Moorhouse *et al.*, 1969).

GC-EAG analysis has proved to be of great utility in identifying insect sex pheromones (Cork *et al.* 1990). It has also been successfully applied to the identification of 1-octen-3-ol from cattle odour as an attractant for tsetse (Hall *et al.*, 1984). However, according to Dobson (1994) utilisation of EAG analyses for the identification of volatiles associated with food sources is in general more difficult than for the identification of sex pheromones. Insects appear to perceive a broad spectrum of food-related chemicals and specificity to host plant chemicals may be more apparent at the behavioural than at the odour receptor level if key integration processes occur in the brain (Dobson, 1994). Nevertheless, EAG activity indicated that female *H. armigera* possessed olfactory receptors capable of detecting compounds in host plant volatiles.

Other Reports of GC-EAG Analyses of Plant Volatiles

Gabel *et al.* (1992) used linked GC-EAG analyses for the identification of floral attractants for the European grapevine moth, *Lobesia botrana* and found it a useful technique for locating which of the 200 plus compounds observed in their

tansy, *Tanacetum vulgare*, extract had electrophysiological activity. Nine EAG-active compounds were identified in this way and they were shown to be almost as attractive as the steam distillate of *T. vulgare* flowers in a release-recapture experiment. Thiéry *et al.* (1990) carried out an investigation into the recognition of a sunflower floral extract by worker honeybees using GC-EAG analysis and out of the 100 or more compounds observed, 24 compounds elicited EAG responses. They stressed that behavioural tests were necessary to clarify the behavioural significance of EAG-active chemicals, especially as two of the EAG-active compounds identified, 3-methyl-1-butanol and 1-hexanol, were already known to be present in the alarm pheromone of honeybees (Collins & Blum, 1983).

One of the earliest and most successful identifications of host plant attractants using GC-EAG analyses was that of Guerin *et al.* (1983) in which *trans*-asarone and *trans*-methylisoeugenol were found to have strong EAG activity and were good trap baits in field trapping experiments for carrot fly, *Psila rosae*. A very low perception threshold for *trans*-asarone was recorded, 5×10^{-16}g/ml of air, which they claimed was lower than recorded for any other insect to a plant volatile. *trans*-Asarone is a significant component of volatiles from carrot, *Daucus carota sativa*, and as *P. rosae* is a monophagous species this host odour cue would be expected to more species-specific than most host-plant odours, which was confirmed by Guerin *et al.* (1983) in field trials using traps baited with *trans*-asarone.

Chapter 5

CHARACTERISATION AND QUANTIFICATION OF FLORAL VOLATILES BY LINKED GAS CHROMATOGRAPHY - MASS SPECTROMETRY

5.1 Introduction

Electrophysiological techniques were used to confirm the presence of olfactory stimulants in floral volatiles. When linked to GC, five EAG-active compounds in the *T. erecta* floral volatiles and four in *L. odouratus* floral volatiles were detected. The RI calculated on polar and non-polar GC columns provided some information on the structures of the EAG-active compounds and although these could be compared with tables of retention time data in the literature to obtain a better idea of the likely compounds present it was not possible to characterise the compounds from GC data alone. Mass Spectrometry (MS) was selected as the technique of choice to identify the EAG-active compounds because of its high sensitivity - to pg levels of material (Rouessac & Rouessac, 1998) and compatibility with GC, so avoiding the need for extensive purification of compounds from the extracts.

Electron impact (EI) MS provide characteristic breakdown patterns of compounds that can be used like fingerprints to identify compounds. Ionisation is caused by collision of sample molecules with electrons emitted from a filament (Rouessac & Rouessac, 1998). The pattern of ion fragments obtained is dependent on the molecular structure of a compound. A minute quantity of sample is ionised and the charged ions are analysed by an electric field. The mass analyser separates ions according to their mass/charge ratio (Rouessac & Rouessac, 1998). Although it is possible to reconstruct molecular structures by interpretation of the fragments produced, tentative identifications in this study were based upon comparing mass spectra obtained from natural samples with library spectra. Tentative identifications were confirmed by comparing GC retention indices of the natural EAG-active compounds on both polar and non-polar GC columns with synthetic standards.

Having identified the EAG-active compounds, the quantities released by live and cut flowers were quantified by GC analysis of headspace samples of floral volatiles. With information on the identity and absolute release rate of EAG-active compounds produced by the flowers synthetic blends of the putative kairomone components could then be formulated for bioassay which would closely mimic natural extracts.

5.2 Methods and Materials

5.2.1 Synthetic chemicals

Synthetic compounds used were at least 95% chemically pure by GC analysis. Benzaldehyde, benzyl alcohol, diacetone, (-)-limonene, (+)-limonene, (±)-linalool, (-)-linalool, and phenylacetaldehyde were purchased from Sigma-Aldrich Chemical Company Ltd. (Gillingham, UK). (*E*)-Myroxide was provided by Quest International, Ashford, U.K. as a 50:50 mixture of (*E*)- and (*Z*)-isomers. The isomers were separated on an open liquid chromatographic column (Braithwaite & Smith, 1985) using flash silica gel (Still et al., 1987) as the adsorbent (25g silica gel 60, 0.040-0.063mm particle size, Merck, Darmstadt, Germany) eluted with 40-60 petroleum spirit and diethyl ether, the (*E*)- isomer eluting first. Aliquots (100 ml) of petroleum spirit with 1%, 2%, 3% and 5% diethyl ether (stepwise increase in polarity) were added sequentially after 0.5g of myroxide had been carefully pipetted onto the top of the column. Fractions (1 – 2 ml) collected from the column were checked using thin layer chromatography (TLC) for the presence of compounds and then analyzed by GC for (*E*)-myroxide. Myroxide isomers eluted from the column in 2% diethyl ether. Ocimene purchased from Fluka (Gillingham, UK) contained 40% limonene and 60% (*Z*)-Ocimene. (*Z*)-Ocimene was separated from this mixture using silica column chromatography as described for isomers of myroxide.

(-)-Piperitone was donated by H.E. Daniel Ltd. (Cleveland, UK). An aliquot of (-)-piperitone (0.155g) was racemised in methanoic potassium hydroxide (0.16g KOH, 10 ml methanol) at room temperature overnight. Hexane (10 ml) and a few drops of water were added to the reaction product and the organic phase decanted off, extracted with water (3 x 5 ml) and dried over $MgSO_4$. Analysis on a chiral β-

cyclodextrin GC column (50m x 0.25mm ID, SGE, UK), and ^{1}H-NMR (JEOL EX270, 270MHz) in the presence of a chiral shift reagent, (*R*)-(-)-2,2,2,-trifluoro-1-(9-anthryl)-ethanol confirmed (-)-piperitone had racemised to an approximately 1 : 1 mixture of enantiomers.

5.2.2 GC-MS Analysis of Natural Samples

Aliquots of EAG-active Porapak Q extracts were analysed by GC-MS using a Carlo Erba 5160 Mega Series GC linked directly to a mass spectrometer (ITD 700, Finnigan MAT, Hemel Hempstead, U.K). Ion Trap Detector System (ITDS) software was used on the GC-MS (Version No. 4:10, Finnigan MAT). Compounds in the MS were ionised in electron impact (EI) mode (1 scan/sec, 70eV). EAG-active compounds were located by their retention indices and mass spectra recorded. Tentative identifications of EAG-active compounds were made by comparing their mass spectra with library spectra (Adams, 1995) and confirmed by comparing EI-MS and GC retention indices with synthetic standards under the same operating conditions (CP Wax52, 30m x 0.25mm ID Polar GC column; held at 50°C for 2min, then programmed at 7°C per min to 250°C, 2 ml/min flow-rate). Chemical ionistaion mass spectrometry (CI mode) was used to confirm the molecular weight of one compound. GC-MS analyses were carried out with *T. erecta* air entrained Porapak Q extracts, Tag-98-01, Tag-98-02, Tag-98-03 and Tag-98-04 and *L. odouratus*, air entrained Porapak Q extracts L-98-01 and L-98-03.

5.2.3 GC Analysis of Chiral Compounds.

The enantiomeric compositions of natural and synthetic compounds were determined by GC analysis on a chiral β-Cyclodextrin column (50m x 0.25mm ID, SGE, UK) in a Varian 3700 GC (helium, 0.5 kg/cm^2) fitted with split/splitless injector (SGE, UK) (220°C) and FID (220°C). Split injections (50 : 1) were made onto the chiral GC column at 90°C, held for 2 min and then temperature programmed at 2°C/min to 190 °C.

5.2.4 Quantification of Compounds in Natural Samples by GC Analysis

Quantification of compounds in natural headspace samples was achieved either by addition of a known quantity (10 μg) of an internal standard (tetradecyl

acetate, 14:Ac) to a sample or an aliquot of sample, or by co-injection of a known volume of the sample with a known amount of external standard.

A flame ionisation detector (FID) was used throughout the GC analyses. No correction was made for differences in responsiveness of the FID to compounds (response factor) and a linear dose response from the FID was assumed for the compounds quantified. Most of the compounds had similar structures (oxygenated C10 compounds) and differences in response were assumed to be relatively small and would have been obscured by the relatively high variability in ratios of compounds between entratinments. An FID detector has high sensitivity to virtually all organic compounds and good linearity of response over a wide sample concentration range (Braithwaite & Smith, 1985).

The following formulae were used to quantify the amount of compounds in the various samples analysed:

1. External Standard:
Concentration of compound X (ng/µl) = [(a/b)*c]/d

a= Area under GC peak corresponding to compound X
b= Area under GC peak corresponding to the internal standard (14:Ac)
c= Amount of external standard (ng)
d= Volume of sample injected (µl)

2. Internal Standard:
Concentration of compound X (ng/µl) = (a/b)*e
Amount of compound X (ng) in sample = (a/b)*f

a= Area under GC peak corresponding to compound X
b= Area under GC peak corresponding to the internal standard (14:Ac)
e= Concentration of internal standard in sample (ng/µl)
f= Amount of internal standard added to whole sample (ng)

5.2.5 GC Analyses to Quantify Release Rate of Compounds from Live Flowers

Typically 2 ml extracts were collected from Porapak Q filters that had been used to entrain volatiles from live flowers over a 10 h period meaning that 200µl of sample contained the amount of material released in one hour by one flower (1 flower-hour). By GC analysis with a 14:Ac external standard (10 ng) the concentration of the EAG-active compounds in the samples could be calculated and the release rate of compounds from the flowers estimated (µg/flower/h).

5.3 Results

5.3.1 *Tagetes erecta*

GC-MS Analyses

A typical total ion chromatogram of a *T. erecta* floral headspace sample (Tag-98-04) is shown in Figure 5.1 The compounds tentatively identified (Table 5.1) by EI-MS were predominately low molecular weight monoterpenes apart from benzaldehyde and phenylacetaldehyde eluting at 914 and 1127 RI.

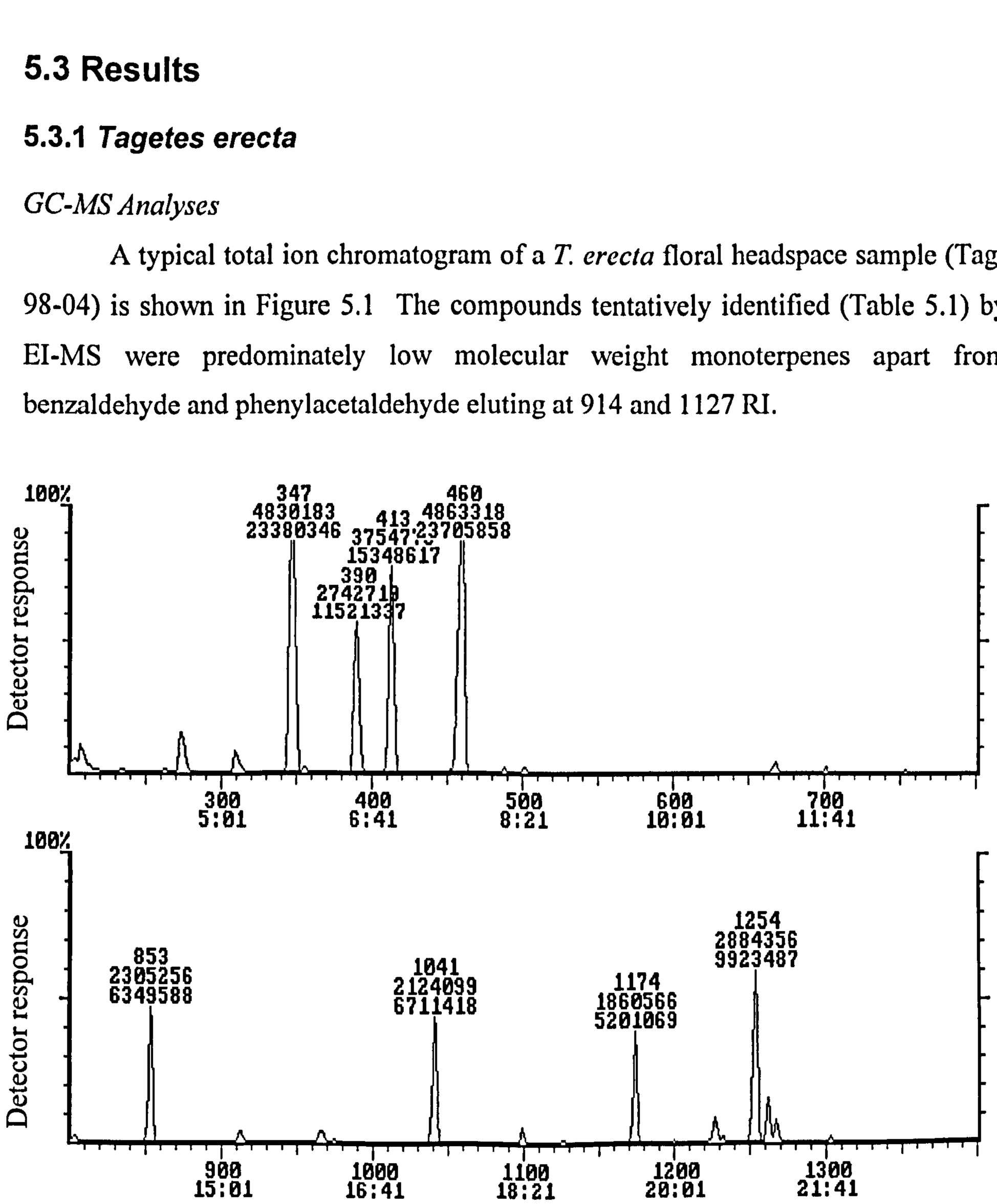

Fig 5.1 Total Ion Chromatogram of *T. erecta* floral headspace sample (Tag-98-04), separated on a polar GC column and ionised in EI mode. Numbers above peaks give scan number, peak height and peak area respectively from top to bottom. The 'x'-axis scale gives scan number (sec, upper number) and retention time (min, lower number).

Table 5.1 Compounds identified in *T. erecta* floral headspace sample (Tag-98-04).

GC-MS Scan No.	Identification [a]	GC-MS fit [b]	% Area [c]	Retention Index on Polar Column		
				Natural	Synthetic	Literature Value (Davies, 1990)
258	Undecane	923	0.68		1100	
275	Sabinene	937	3.17	1118		1130
312	**Myrcene**	938	1.45	1159	1159	1157
350	Dodecane / **Limonene**		20.50	1200	1200	1206
392	**(*Z*)-Ocimene**	938	9.66	1225	1232	1228
416	(*E*)-Ocimene	860	12.53	1239		1250
464	Terpinolene isomer	935	19.44	1267		1279
690	Tetradecane		0.77	1400	1400	
855	***(E)-Myroxide (T1)***		5.04	1487	1483	
914	***Benzaldehyde (T2)***	925	0.61	1519	1522	
968	***Linalool (T3)***	874	0.84	1547	1541	1555
1044	**β caryophyllene**	977	5.09	1587	1600	1617
1068	Hexadecane		0.88	1600	1600	
1100	(*Z*)-Sabinene hydrate acetate	879	0.52	1620		
1127	***Phenylacetaldehyde (T4)***	978	0.26	1637	1642	
1174	Farnesene isomer	948	4.00	1666		1697
1228	Germacrene D	966	0.79	1700		1712
1254	***Piperitone (T5)***	894	7.80	1716	1723	1739
1262	Octadecene	857	1.74	1721		
1389	Octadecane		1.00	1800	1800	
	Unidentified minor peaks		3.23			

[a] Compounds in bold were confirmed by comparison with authentic compounds, italicised compounds were EAG-active in linked GC-EAG analyses.
[b] Comparison with library mass spectrum (where 1000 would be an identical fragmentation pattern).
[c] Percentage of total area under GC peaks.

EI-MS of the five EAG-active compounds observed in linked GC-EAG analyses (Chapter 4) of volatiles collected from *T. erecta* are shown below (Figs 5.2 – 5.9). Four of the five compounds (T2 - T5) had good matches with library spectra (Adams, 1989) (Figs. 5.5 – 5.8). However, the first EAG-active compound to elute on a polar column (T1) did not have a fit greater than 850 with any of the library EI-MS. To identify this compound a more extensive listing of mass spectra was consulted

(Adams, 1995) in which there was an EI-MS with a similar pattern of ions ((*E*)-myroxide). The EI-MS of synthetic (*E*)-myroxide is shown (Fig. 5.2) together with EI-MS of the natural compound eluting from polar (Fig. 5.3) and non-polar GC columns (Fig. 5.4). Chemical ionisation mass spectrometry (CI-MS) confirmed that the molecular weight of the synthetic and natural samples were the same (152 for both). The EI-MS of limonene is also shown (Fig 5.9) since it was subsequently found to elicit electrophysiological and behavioural responses from female *H. armigera*

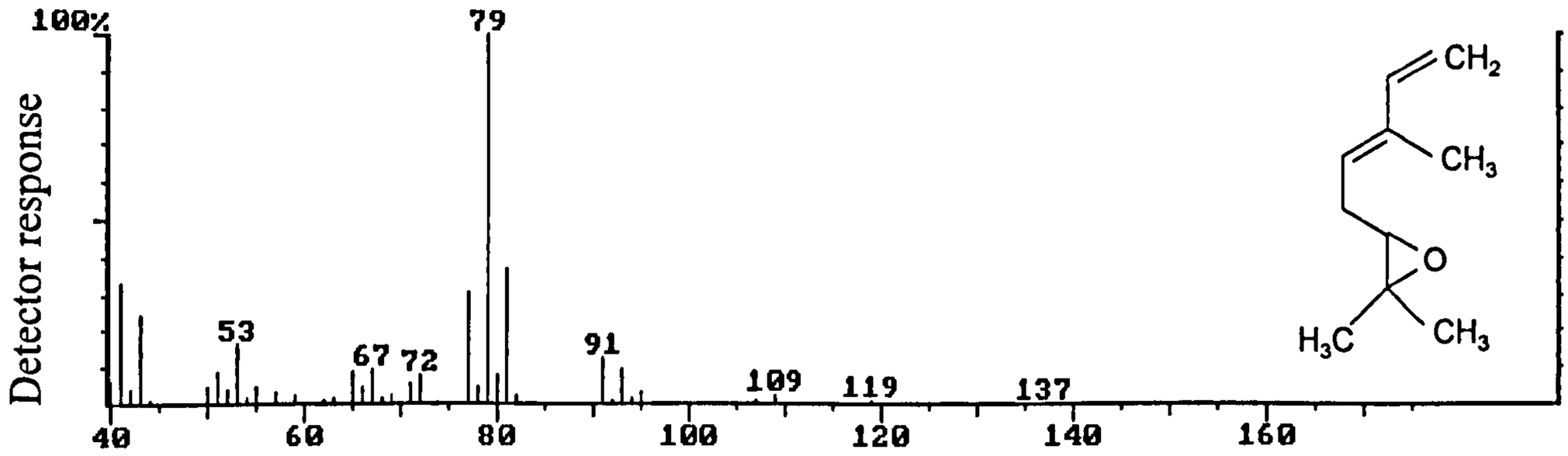

Fig 5.2 Electron impact mass spectrum of synthetic (*E*)-Myroxide: 2,2–dimethyl–3 (3–methylpenta–2,4–dienyl) oxirane; R.I. 1483 (polar), 1126 (non-polar). 'x'-axis shows mass-charge ratio (m/z)

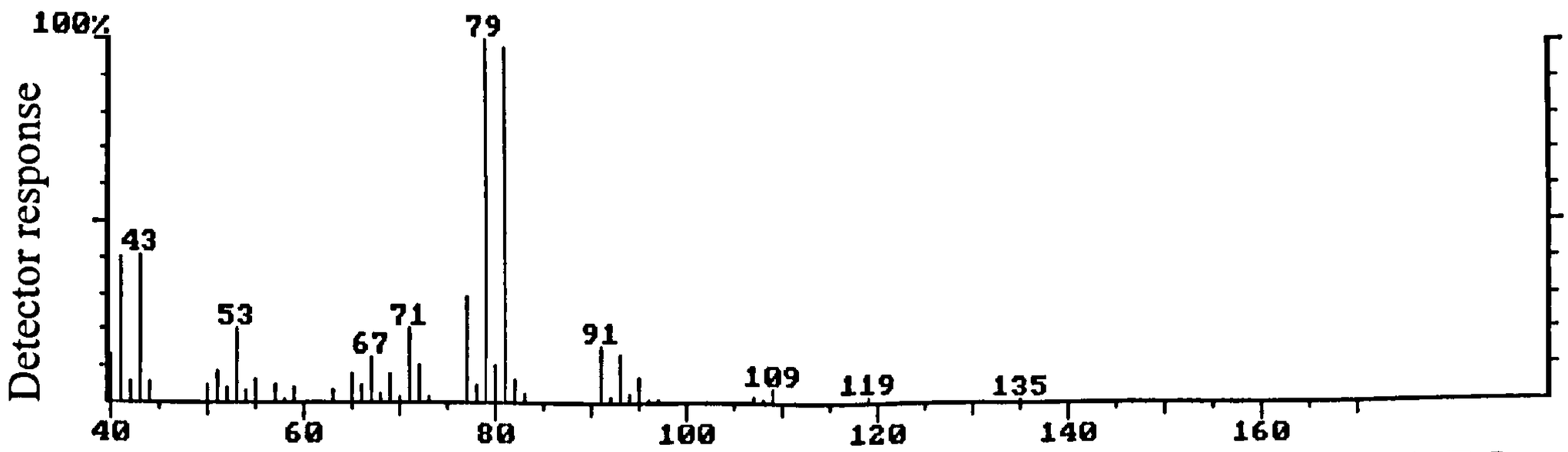

Fig 5.3 Electron impact mass spectrum of *T. erecta* EAG-active compound T1 (R.I. 1485, polar). 'x'-axis shows mass-charge ratio (m/z)

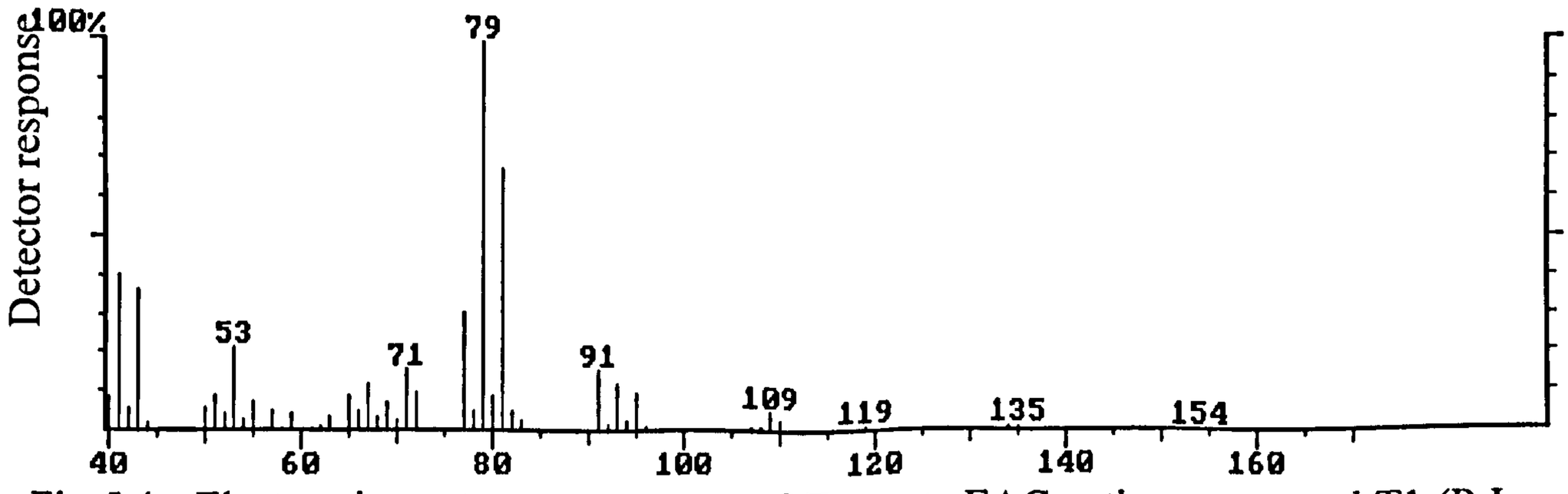

Fig 5.4 Electron impact mass spectrum of *T. erecta* EAG-active compound T1 (R.I. 1123 non-polar). 'x'-axis shows mass-charge ratio (m/z)

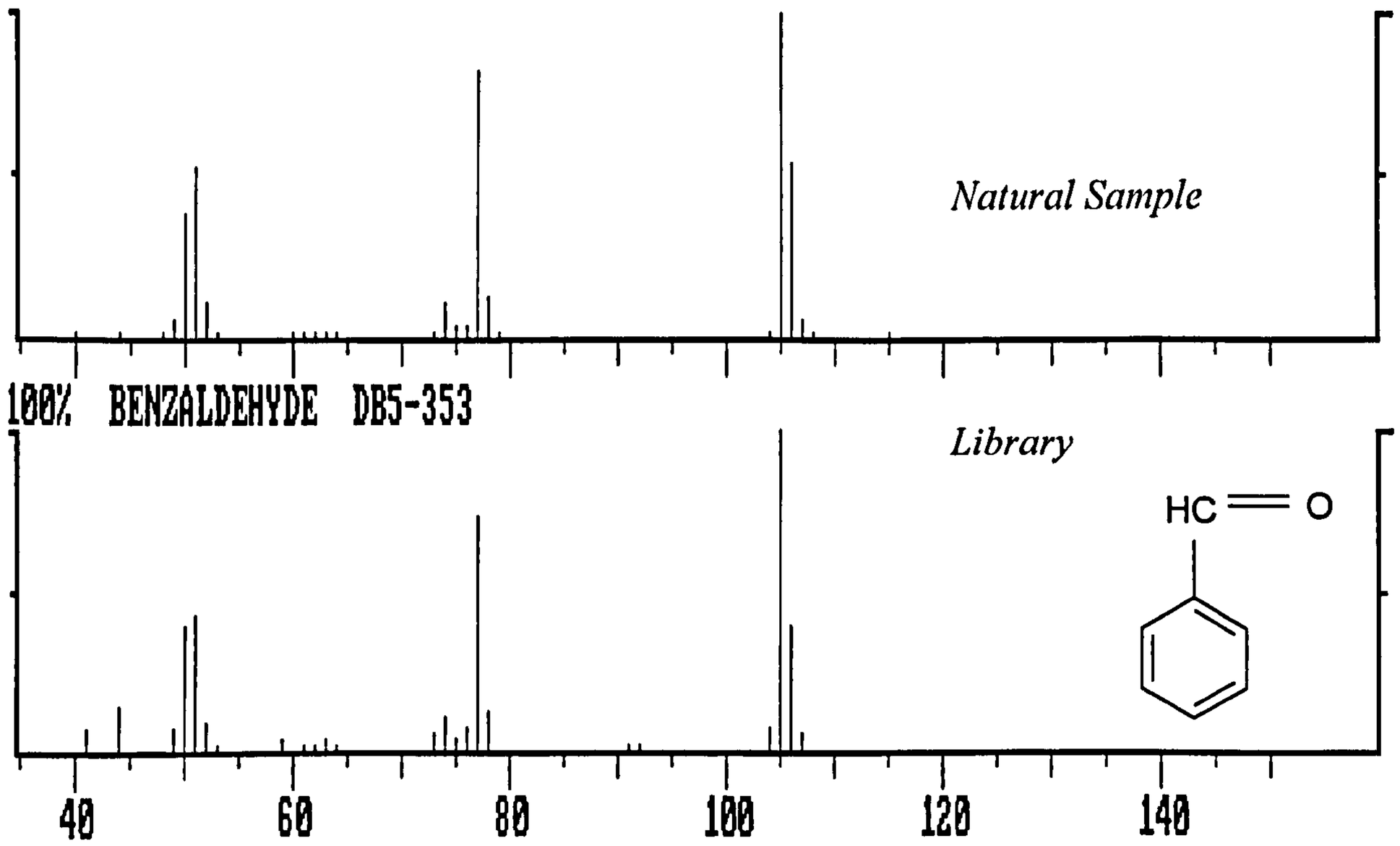

Fig 5.5 Electron impact mass spectrum of *T. erecta* EAG-active compound T2 (top) and best library match, benzaldehyde (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

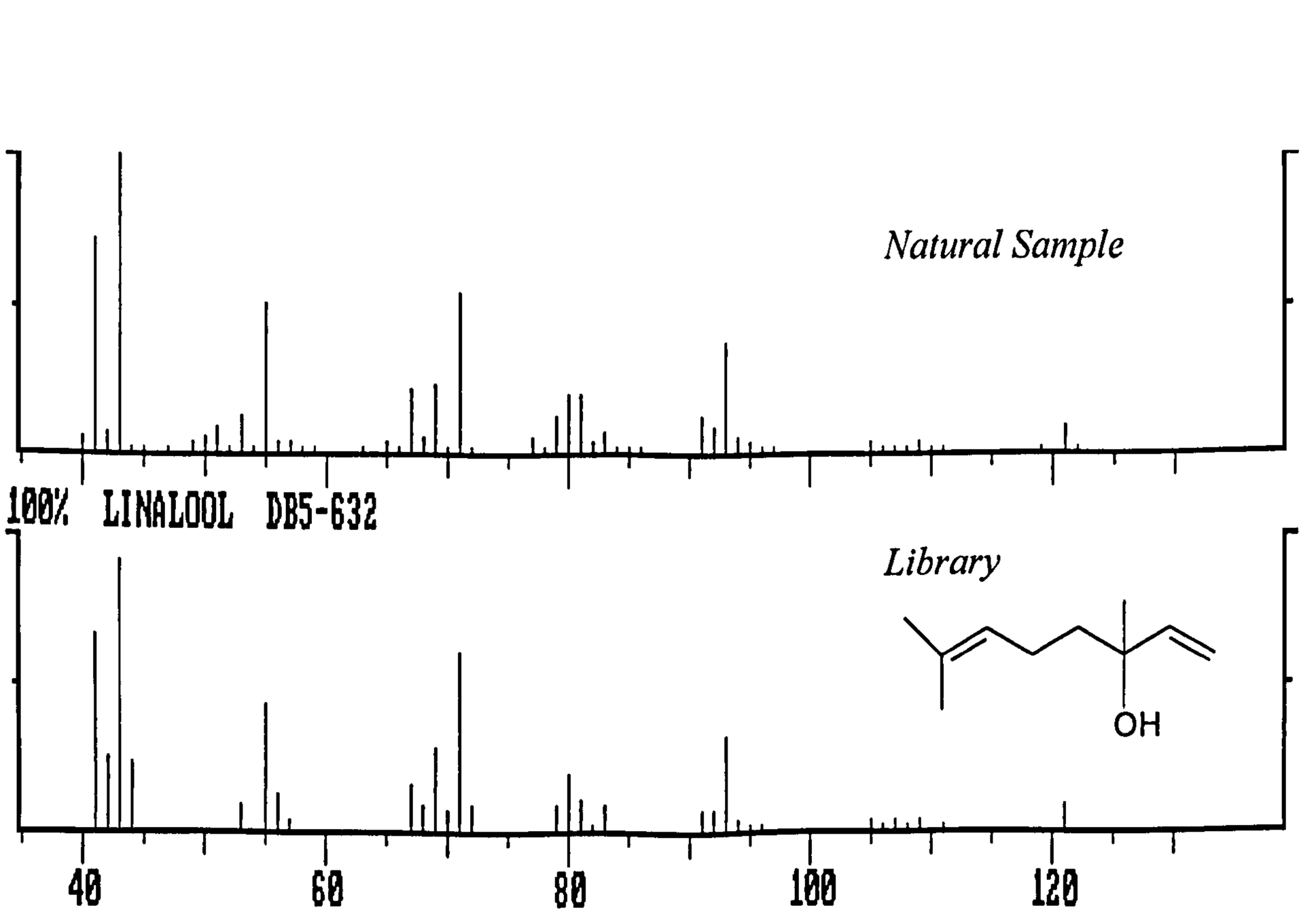

Fig 5.6 Electron impact mass spectrum of *T. erecta* EAG-active compound T3 (top) and best library match, linalool: 3,7–dimethyl-1,6–octadien–3-ol (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

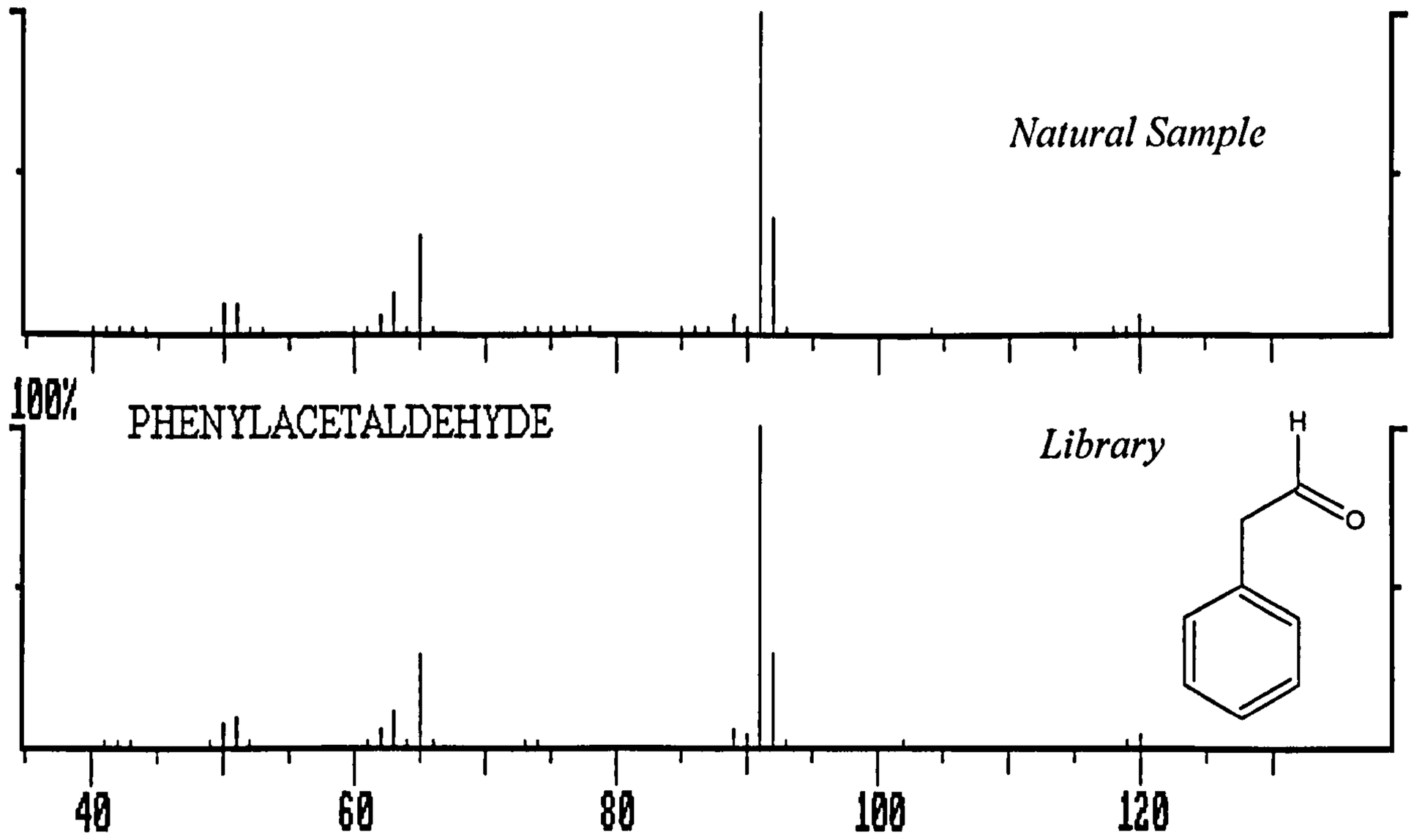

Fig 5.7 Electron impact mass spectrum of *T. erecta* EAG-active compound T4 (top) and best library match, phenylacetaldehyde (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

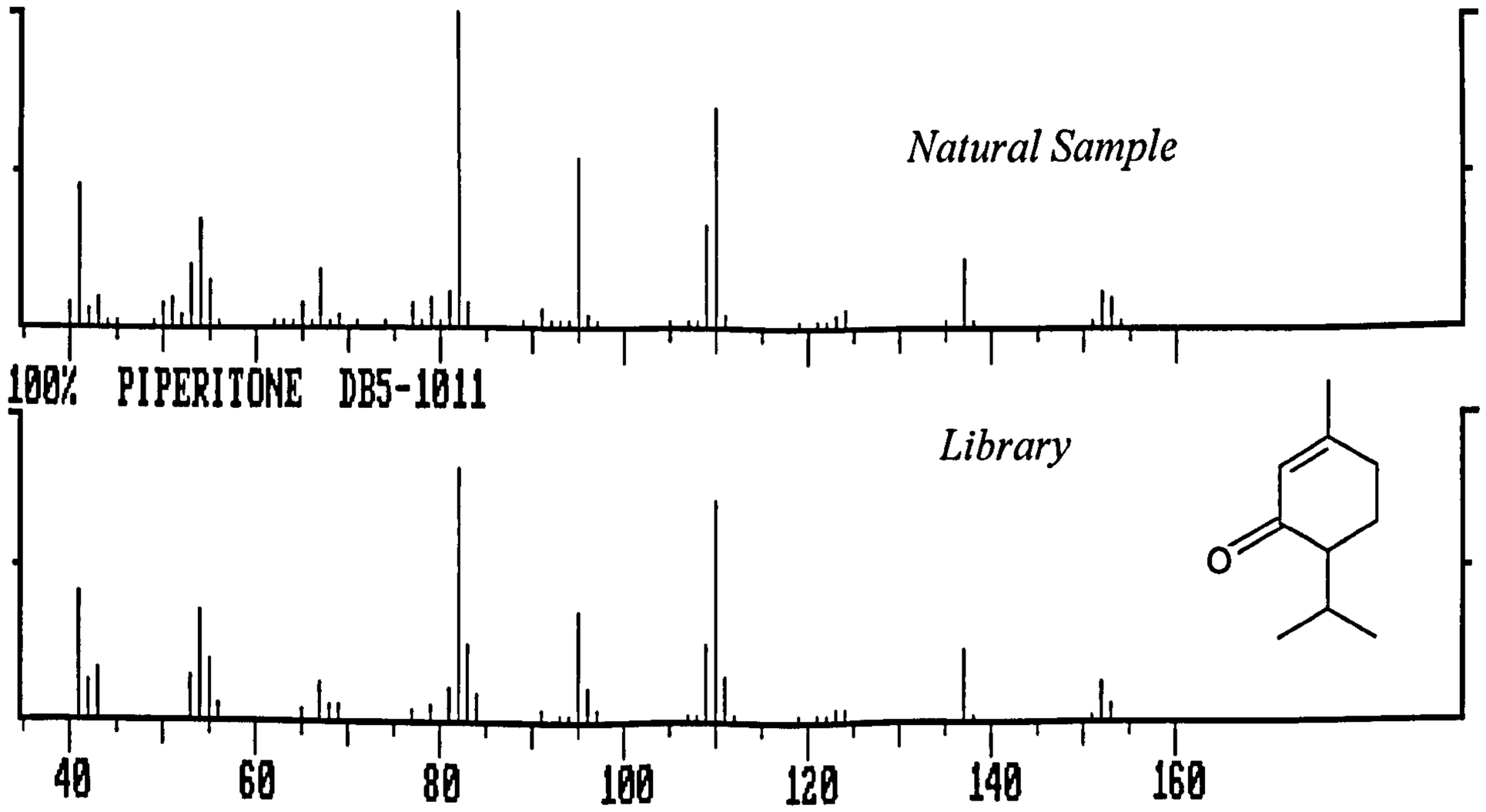

Fig 5.8 Electron impact mass spectrum of *T. erecta* EAG-active compound T5 (top) and best library match, piperitone: 3–methyl–6–(1–methylethyl)–2–cyclohexene–1–one (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

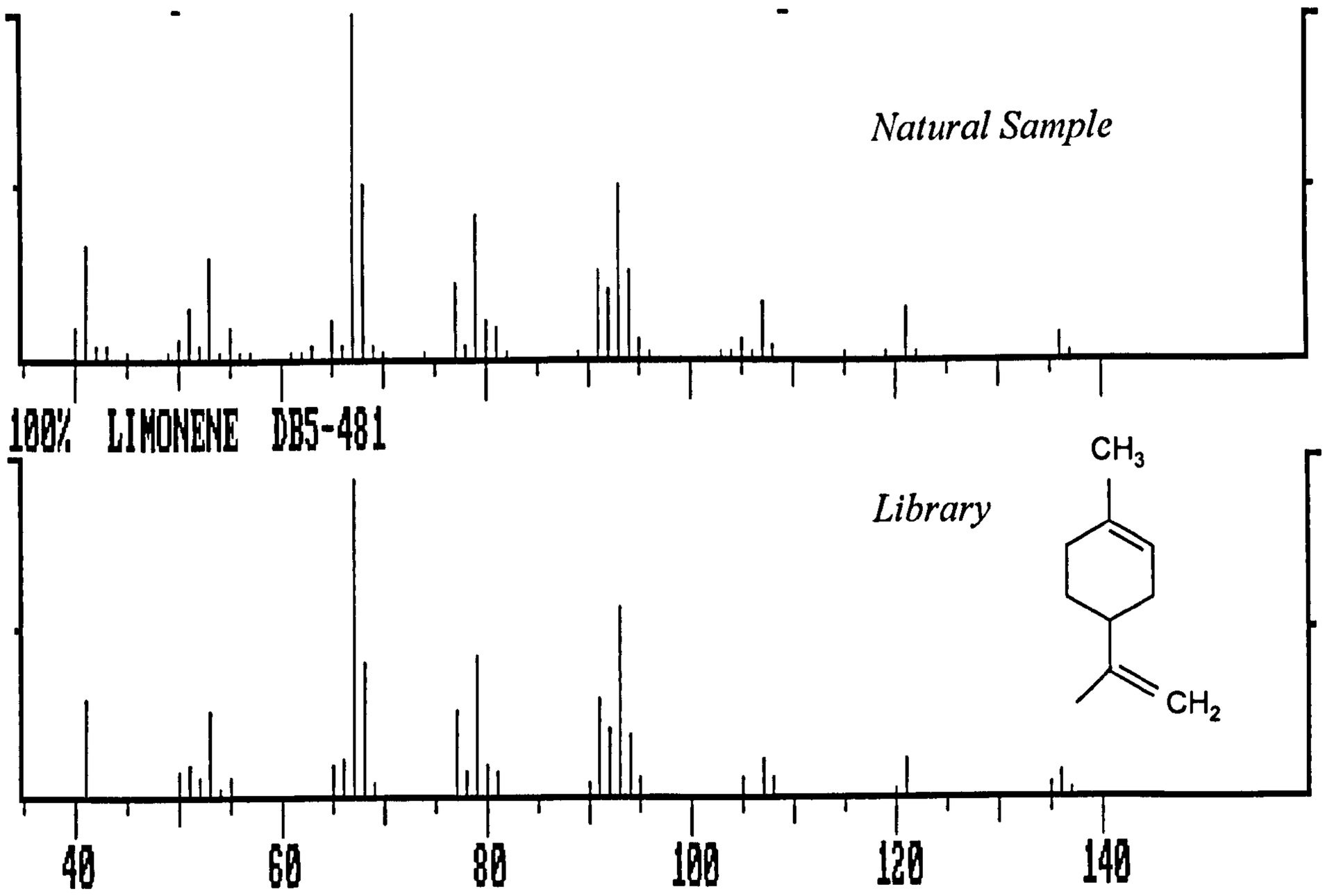

Fig 5.9 EI-MS of *T. erecta* compound 6, (top) and best library match, limonene: 1–methyl–4–(1–methylethenyl)-cyclohexene (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

The best library fits for EI-MS of compounds T1 – T5 were myroxide, benzaldehyde, linalool, phenylacetaldehyde and piperitone respectively. The tentative structural assignments were confirmed by comparison of their RI with authentic synthetic compounds on polar and non-polar columns (Table 5.3) as (*E*)-myroxide, benzaldehyde, linalool, phenylacetaldehyde and piperitone and occurred in a 7.3 : 1.0 : 1.4 : 0.5 : 28.7 ratio in a headspace sample of cut *T. erecta* flowers (sample Tag-WT1) used initially for wind-tunnel bioassays. Four other compounds present in significant quantities in the air entrainments but not eluting at GC retention times associated with electrophysiolgical responses observed in linked GC-EAG analyses were similarly identified as myrcene, limonene, (*Z*)-ocimene and β-caryophyllene (Table 5.2). They had an abundance relative to benzaldehyde of 2.7 : 17.0 : 8.8 : 7.7 respectively in headspace sample Tag-WT1.

Table 5.2 Compounds identified in an extract (Tag-WT1) prepared from Porapak Q entrainment of volatiles from *T. erecta* flowers.

Compound	Ratio relative to benzaldehyde	Retention Index on Polar GC Column		Retention Index on Non-Polar GC Column	
		Natural	Synthetic	Natural	Synthetic
Myrcene	2.7	1160	1159	979	982
(±)–Limonene	17.0	1200	1200	1017	1019
(*Z*)–Ocimene	8.8	1234	1232	1027	1028
(*E*)–Myroxide (T1)	7.3	1485	1483	1123	1126
Benzaldehyde (T2)	1.0	1522	1522	925	926
(±)–Linalool (T3)	1.4	1539	1541	1085	1088
β-Caryophyllene	7.7	1600	1600	1406	1407
Phenylacetaldehyde (T4)	0.4	1642	1642	1007	1011
(±)–Piperitone (T5)	28.7	1725	1723	1226	1225

GC Analyses of Chiral Compounds

Linalool, limonene and piperitone were identified by their EI-MS and RI on polar and non-polar columns. However, the achiral columns could not separate the enantiomers of these compounds and in order to determine the enantiomeric composition of these compounds a chiral β-cyclodextrin GC column was used. It was known from König *et al.* (1990) that (+)-limonene eluted earlier than (-)-limonene and that (-)-piperitone eluted before (+)-piperitone on a β-cyclodextrin column. This was confirmed using synthetic (+)- and (-)-limonene and comparison of the retention times of (-) linalool with those of racemic linalool. Thus, the floral volatiles collected from *T. erecta* were found to contain (+)- and (-)-linalool in a 1 : 1 ratio and S-(-)-limonene (Table 5.3). Likewise piperitone collected from the floral volatiles released from flowers of *T. erecta* was shown to be the (-)-enantiomer by comparison of the retention times of an authentic sample of (-)-piperitone and (+)-piperitone present in the racemised mixture (Table 5.3).

Table 5.3 GC Retention Times of Enantiomers of Chiral EAG-Active Compounds on β-Cyclodextrin Column.

Compound	GC Retention Time (min)		
	(+)-enantiomer	(-)-enantiomer	Natural Sample
Linalool	27.96	27.83	27.87, 27.99
Limonene	18.25	18.39	18.39
Piperitone	40.37	40.22	40.26

5.3.2 *Lathyrus odouratus*

GC-MS Analyses

EI-MS were obtained for compounds associated with EAG activity in linked GC-EAG analyses of air-entrained *L. odouratus* samples (L1 – L4). The total ion chromatogram is shown in Fig. 5.10. Tentative structural assignments were made on the basis of a comparison of EI-MS with library spectra (Adams, 1989; 1995) (Figs 5.11 – 5.15). The tentative structural assignments made for L1 to L4 were confirmed by comparing their RI with authentic compounds on polar and non-polar columns as diacetone (2-hydroxy-2-methyl-4-pentanone), linalool, phenylacetaldehyde and benzyl alcohol in a 13.8 : 7.9 : 6.3 : 1.0 ratio (Tables 5.5 and 5.6).

Interestingly, two of the EAG-active compounds, linalool and phenylacetaldehyde, identified from the floral volatiles of *L. odouratus* had previously been identified in the floral volatiles of *T. erecta* (Table 5.3).

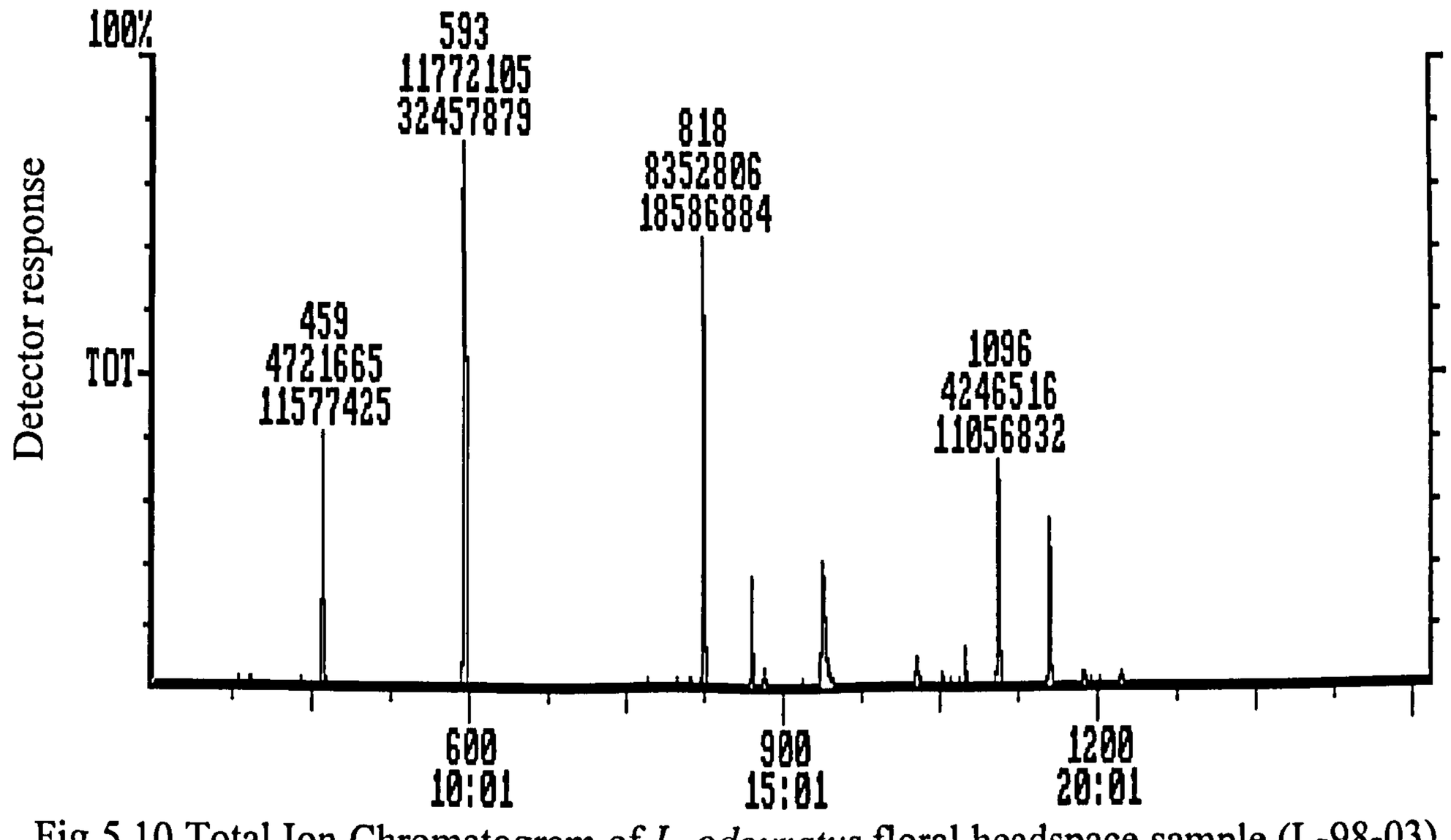

Fig 5.10 Total Ion Chromatogram of *L. odouratus* floral headspace sample (L-98-03), separated on a polar GC column and ionised in EI mode. Numbers above peaks give scan number, peak height and peak area respectively from top to bottom. The 'x'-axis scale gives scan number (sec, upper number) and retention time (min, lower number).

Table 5.5 Compounds identified in a *L. odouratus* floral headspace sample (L-98-03).

GC-MS Scan No.	Identification [a]	GC-MS fit [b]	% Area [c]	Retention Index on a Polar GC Column		
				Natural	Synthetic	Literature Value (Davies, 1990)
459	(*E*)-Ocimene	950	9.9	1255		1250
593	***Diacetone (L1)***	922	24.6	1366	1369	
818	***Linalool (L2)***	884	14.3	1545	1547	
864	Bergamotene	982	3.2	1590		1590
930	***Phenylacetaldehyde (L3)***	972	11.2	1652	1654	
1097	Nerol	965	9.5	1798		1808
1144	Geraniol	965	7.0	1889		1842
1177	***Benzyl Alcohol (L4)***	865	1.8	1888	1888	

[a] Compounds in bold were confirmed by comparison with authentic compounds, italicised compounds were EAG-active in linked GC-EAG analyses.
[b] Comparison with library mass spectrum (where 1000 would be an identical fragmentation pattern).
[c] Percentage of total area under GC peaks.

As with the floral volatiles of *T. erecta*, floral volatiles of *L. odouratus* were dominated by low molecular weight terpenoid compounds. However, a larger proportion of the sample was made up of aromatic compounds and oxygenated monoterpenes.

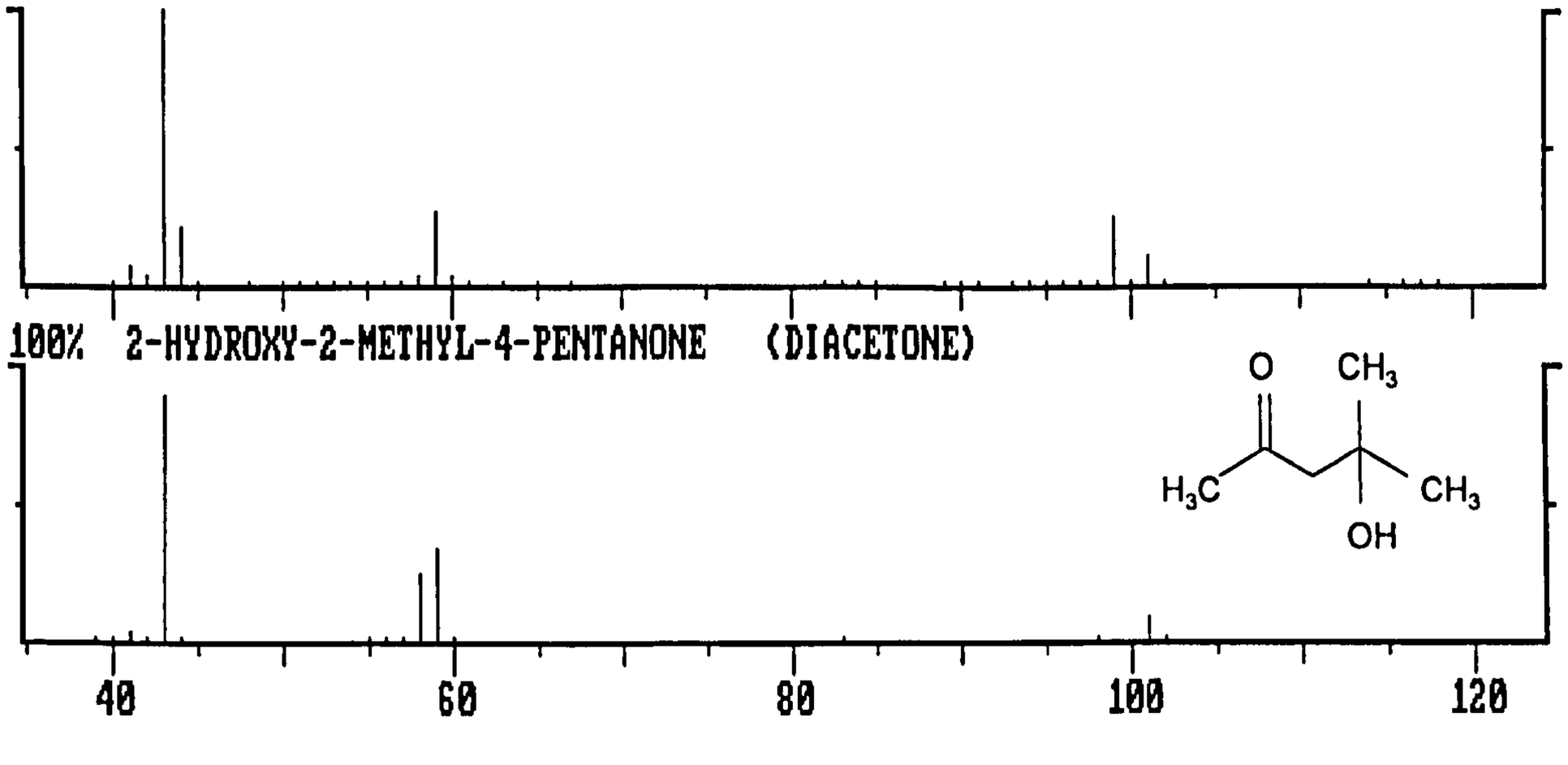

Fig 5.11 Electron impact mass spectrum of *L. odouratus* EAG-active compound L1 (top) and best library match, diacetone: 4-hydroxy-4-methyl-2-pentanone (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

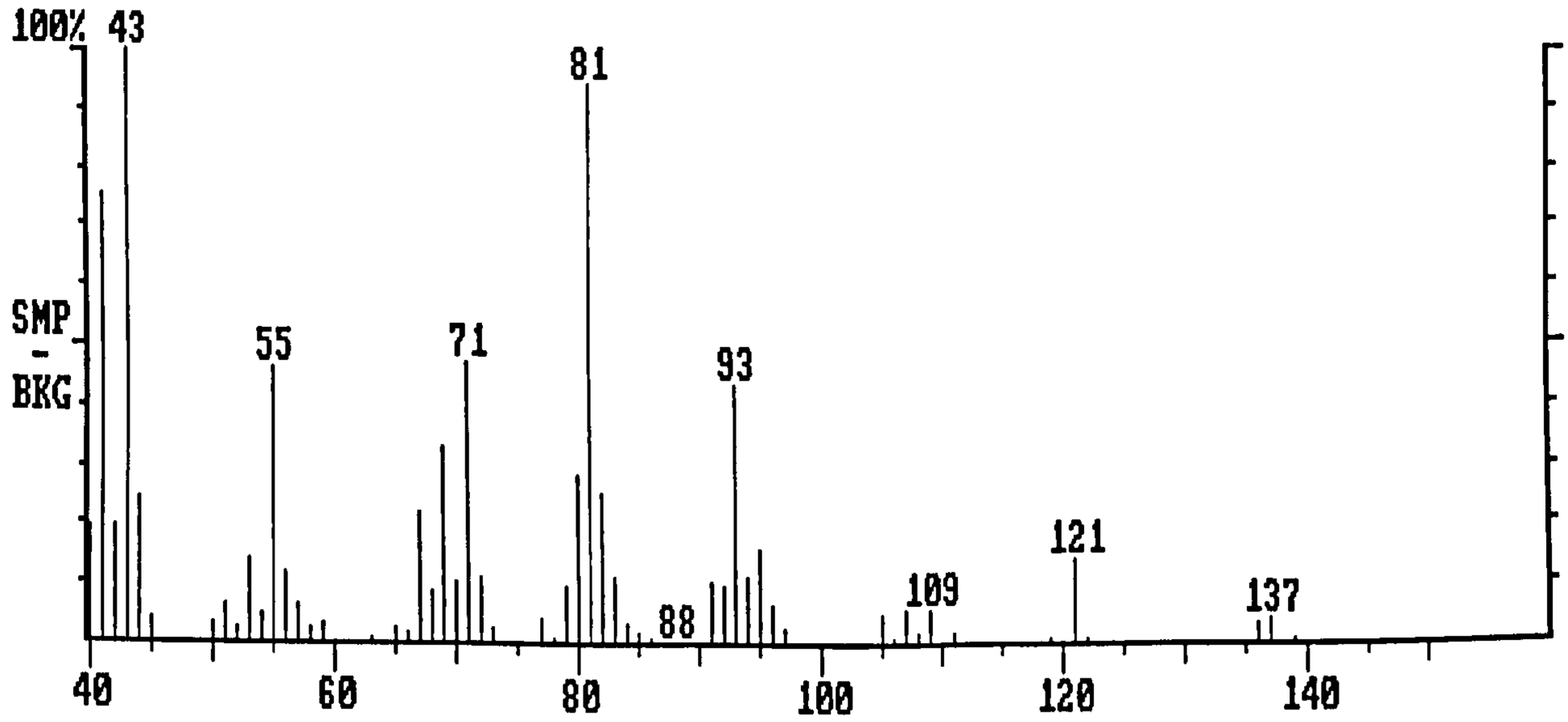

Fig 5.12 Electron impact mass spectrum of *L. odouratus* EAG-active compound L2. 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

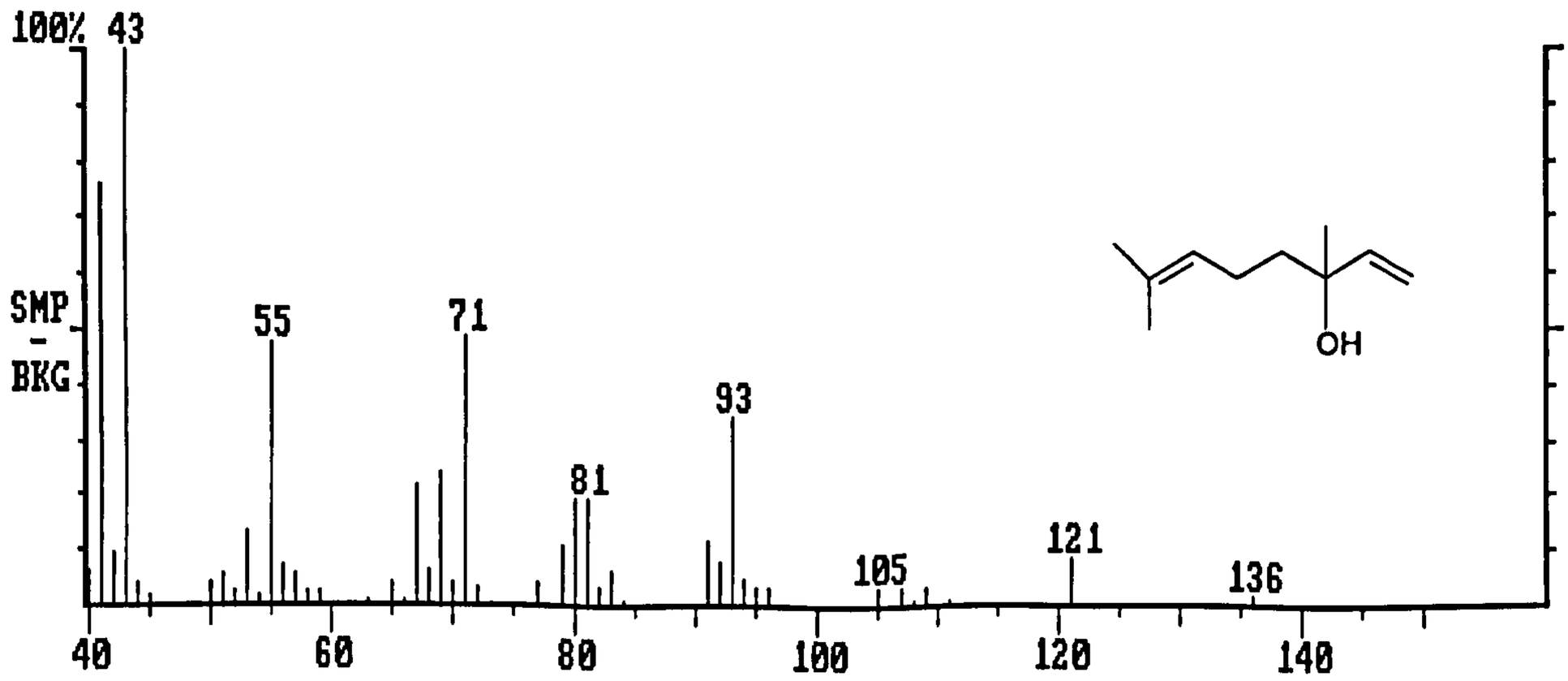

Fig 5.13 Electron impact mass spectrum of synthetic linalool: 3,7–dimethyl-1,6–octadien–3-ol which was the best library match. 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

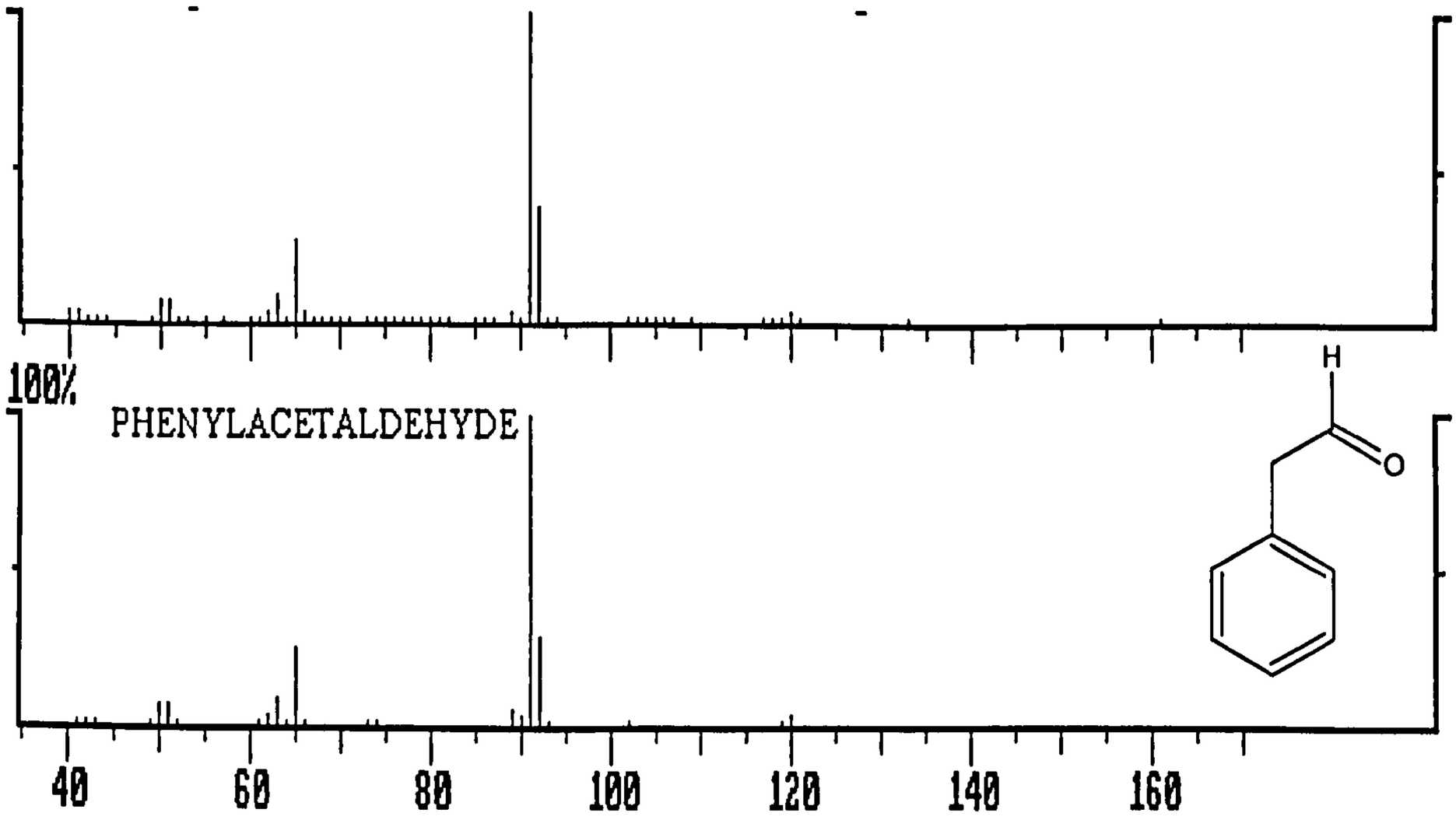

Fig 5.14 Electron impact mass spectrum of *L. odouratus* EAG-active compound L3 (top) and best library match, phenylacetaldehyde (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

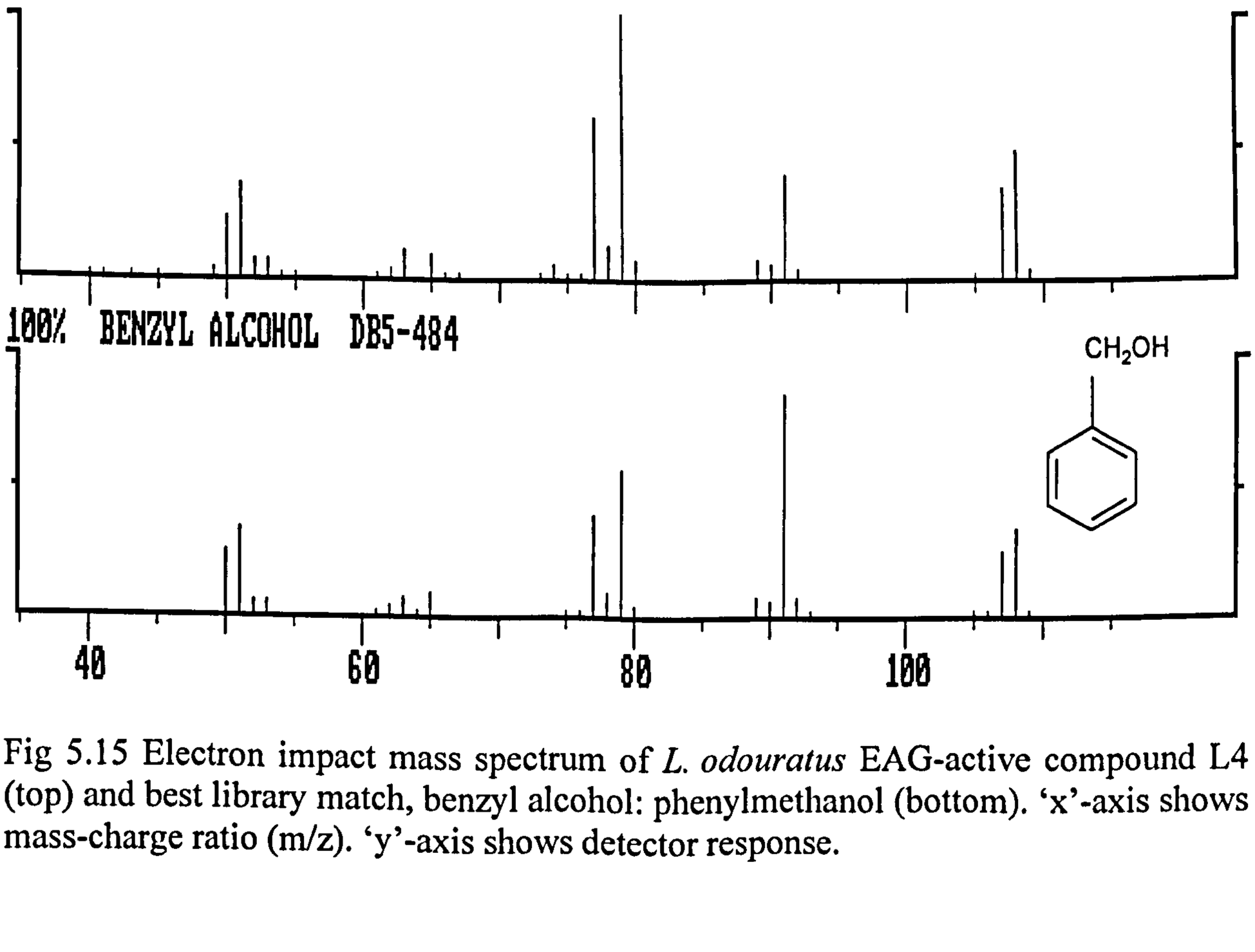

Fig 5.15 Electron impact mass spectrum of *L. odouratus* EAG-active compound L4 (top) and best library match, benzyl alcohol: phenylmethanol (bottom). 'x'-axis shows mass-charge ratio (m/z). 'y'-axis shows detector response.

Table 5.6 EAG-active compounds identified in an extract (L-98-03) prepared by Porapak Q entrainment of volatiles from *L. odouratus* flowers

Compound	Ratio with respect to benzyl alcohol	Retention Index on Polar GC Column		Retention Index on Non-Polar GC Column	
		Natural	Synthetic	Natural	Synthetic
Diacetone (L1)	13.8	1366	1369	821	818
(-)-Linalool (L2)	7.9	1545	1547	1086	1088
Phenylacetaldehyde (L3)	6.3	1652	1654	1014	1015
Benzyl Alcohol (L4)	1.0	1887	1888	1022	1022

GC Analyses of Chiral Compounds

GC analyses were carried out on a β-cyclodextrin column to determine the enantiomeric composition of linalool in the floral volatiles from *L. odouratus*. (-)-Linalool and (+)-linalool eluted at 13.48 and 13.70min respectively. Linalool from the floral volatiles of *L. odouratus* eluted at 13.49 min co-incident with the (-)-enantiomer. [Chiral analyses were carried out at different times for *T. erecta* and *L. odouratus* samples and the chiral β-Cyclodextrin column had been changed inbetween which explains why the retention times of synthetic linalool are different from those reported in Table 5.3.]

5.3.3 Quantification of EAG-active Compounds in Floral volatiles

Comparisons were made between the relative and absolute abundance of the compounds released from freshly cut and live *T. erecta* flowers using GC analysis on a polar GC column.

Porapak Q Entrainments of Cut Flowers

An aliquot (1 µl) of Tag-WT1, a blend of three entrainments (Tag-98-01, Tag-98-03 & Tag-98-04), was coinjected with an internal standard (20ng, 14:Ac) and the results converted to flower-hour equivalents (Table 5.7). Tag-WT1 was used in bioassay series 3 (Chapter 7).

Table 5.7 Quantitative GC analysis of floral volatiles from cut flowers of *T. erecta* collected on a Porapak Q filter (sample Tag-WT1)

Compound	Concentration of Compound (ng / µl)	Emission Rate (µg / flower / h)	Ratio of compounds (benzaldehyde = 1)
Myrcene	27.0	0.14	2.7
Limonene	171.3	0.86	17.0
(*Z*)–Ocimene	88.6	0.44	8.8
(*E*)–Myroxide	73.5	0.37	7.3
Benzaldehyde	10.0	0.05	1.0
Linalool	14.5	0.07	1.4
Caryophyllene	77.5	0.39	7.7
Phenylacetaldehyde	4.6	0.02	0.5
Piperitone	288.1	1.44	28.7
14:Ac (IS, 20ng)	20.0		

Porapak Q Entrainments of Intact Live Flowers

An aliquot (1 μl) of Tag-WT2, an entrainment of volatiles from a single intact live *T. erecta* flower, was coinjected with an external standard (10ng, 14:Ac) and the results converted to flower-hour equivalents (Table 5.8). Sample Tag-WT2 was used in bioassay series 7 (Chapter 7).

Table 5.8 Quantitative GC analysis of volatiles collected from live *T. erecta* flowers, collected on Porapak Q (Sample Tag-WT2)

Compound	Concentration of Compound (ng/μl)	Emission Rate (μg/flower/hour)	Ratio of Compounds (benzaldehyde = 1)
(-)-Limonene	16.06	1.72	1.6
(*Z*)-Ocimene	3.53	0.38	0.4
(*E*)-Myroxide	13.74	1.47	1.4
Benzaldehyde	9.90	1.06	1.0
(±)-Linalool	6.77	0.72	0.7
Phenylacetaldehyde	43.10	4.61	4.4
(-)-Piperitone	26.69	2.86	2.7
14:Ac (IS, 10ng)	10.00		

Air entrainments were carried out on three other headspace samples of live *T. erecta* flowers, the results of which are shown in Table 5.9.

Table 5.9 Summary of release rates of behaviourally active components from entrainments of live *T. erecta* flowers

Compound	Release Rate (μg /flower/h)			Mean	
	Sample TB-99-18	Sample TB-99-40	Sample TB-99-59	(μg/flower/h)	S.E.
(-)-Limonene	0.49	0.18	0.12	0.26	0.11
Benzaldehyde	0.09	0.18	0.13	0.13	0.03
(±)-Linalool	0.04	0.13	0.03	0.06	0.03
Phenylacetaldehyde	0.34	0.61	0.23	0.39	0.11
(-)-Piperitone	1.90	2.55	0.10	1.52	0.73

Emission rates from live and cut flowers were not the same for all compounds due to differences in the blend ratio emitted by cut (Table 5.7) and live flowers (Table 5.9). Release rates from cut flowers (Table 5.7) were lower for benzaldehyde, (±)-linalool and phenylacetaldehyde (0.05, 0.07 and 0.02 μg / flower / h respectively); higher for (-)-limonene (0.86 μg / flower / h) and almost exactly the same for (-)-piperitone (1.44 μg / flower / h).

Table 5.10 Comparison of proportions of compounds emitted from live and cut *T. erecta* flowers

Chemical	Cut Mean[a] (%)	Cut S.E. (%)	Live Mean[a] (%)	Live S.E. (%)	P Value[b]
(-)-Limonene	28.0	5.7	16.5	4.6	0.12
(*Z*)-Ocimene	14.9	3.5	3.8	1.0	0.01
(*E*)-Myroxide	8.5	1.2	5.0	2.0	0.13
Benzaldehyde	5.3	2.9	9.0	2.2	0.04
(+/-)-Linalool	1.2	0.3	2.0	1.3	0.52
Phenylacetaldehyde	8.5	5.9	48.2	8.3	0.01
(-)-Piperitone	33.7	8.2	15.4	4.2	0.057

[a] Amounts are expressed as a percentage of the total area under the GC peaks for the 7 compounds.

[b] Unpaired *t*-test comparing means of cut and live, n = 5 for both cut and live flower entrainments.

As shown in Table 5.10, significantly more phenylacetaldehyde and benzaldehyde were present in the live flower headspace samples than in the cut flower ones. The cut flower blends contained significantly more (*Z*)-ocimene and more (-)-piperitone.

5.4 Discussion

Identification of EAG-active Compounds

The identification of EAG-active compounds in *T. erecta* and *L. odouratus* headspace samples was accomplished by a combination of GC-MS and GC analyses. It was important to compare retention times with authentic synthetic compounds because many terpenes have similar mass spectra (Davies, 1990). A higher degree of confidence in identifications was obtained when analyses were carried out on two GC columns. With known compounds this approach of comparing retention times on two columns of different polarity and mass spectra with authenitic standards provides reliable identifications of compounds. However if the compounds had been unknown (never identified previously in other contexts) then isolation of relatively pure material (>1μg) and use of additional analytical tools such as NMR would have been needed. The EAG-active compounds identified in linked GC-EAG analyses of floral headspace samples of *T. erecta* were (*E*)-myroxide, benzaldehyde, linalool, phenylacetaldehyde, and piperitone (Table 5.2). In *L. odouratus* samples they were diacetone, linalool, phenylacetaldehdyde and benzyl alcohol (Table 5.6).

A cyclodextrin enantioselective GC column was used to measure retention times of separated enantiomers. Whereas a normal GC column gives only one peak for a racemic mixture a cyclodextrin column can resolve it into separate peaks for each enantiomer (König, 1990). Three of the EAG active compounds from the *T. erecta* samples and one from *L. odouratus* samples had chiral centres and therefore existed as enantiomers. It was necessary to identify which of the enantiomers were present in the natural samples in case there were differences in behavioural responses to different enantiomers. Thus samples of enantiomerically pure limonene, linalool and piperitone were obtained and compounds in *T. erecta* headspace were identified as (-)-linalool and (+)-linalool in a 1:1 ratio, (-)-limonene and (-)-piperitone (Table 5.3). (-)-Linalool only was found in *L. odouratus* headspace.

Release Rate of Volatiles from T. erecta *Compared with Other Flowers*

Dobson (1994) found that the quantity of volatiles released from flowers varied widely, with measured amounts ranging from 0.0042 to 50μg/h. The release

rate from live *T. erecta* flowers was relatively high (Table 5.9) with 2.36 µg / flower / h of (-)-limonene, (±)-linalool, benzaldehyde, phenylacetaldehyde and (-)-piperitone. Cut flowers produced similar amounts of these five compounds (2.44 µg / flower / h, Table 5.7) but the ratio emitted was significantly different (Table 5.10).

Comparison of Odours from Live and Freshly Cut T. erecta *Flowers*

The ratio of the EAG-active compounds in headspace entrainments of live and cut *T. erecta* flowers was compared by GC analysis (Table 5.10). Significant differences were found in the proportions of aromatic compounds benzaldehyde and phenylacetaldehyde (Table 5.10) with amounts released from cut flowers being lower (P = 0.035 and P = 0.002 respectively). The proportion of (*Z*)-ocimene in the cut flower volatiles was higher (P = 0.010) and the proportion of piperitone was increased although it was not significantly higher (P = 0.057)

Previous Identifications of T. erecta *and* L. odouratus *Volatiles*

β-Caryophyllene, limonene, linalool (enantiomers unspecified) and (Z)-ocimene are well known constituents of *Tagetes* essential oil (Baslas & Singh 1980, Baser & Malyer 1996, Bicchi *et al.* 1997, Gupta & Bhandari 1974, Hethelyi *et al.* 1986, Singh *et al.* 1992). Enantiomers of limonene and linalool were unspecified except by Baslas & Singh (1980) who specified *d*-limonene occuring in *T. erecta* but did not mention which linalool enantiomers were present. Myrcene has been recorded in *T. lucida* by Bicchi *et al.* (1997) and in *T. minuta* by Baser & Malyer (1996). Chalchat *et al.* (1995) found (*E*)-myroxide (ocimene epoxide) in *T. minuta*. Tucker & Maciarello (1996) found germacrene D in *T. lemmonii*. Piperitone (enantiomer unspecified) has been recorded by Hethelyi *et al.* (1986) in *T. erecta* by Singh *et al.* (1992) in *T. minuta*.

Benzaldehyde and phenylacetaldehyde have not been previously described in *Tagetes* essential oil which is typically prepared from steam distillates of air-dried plants. They are prone to oxidation and thus might not occur in samples collected by steam distillation. It was noted that larger quantities of these two compounds were collected in air entrainments with live flowers than in entrainments carried out with cut flowers. Even under air entrainment conditions the sample is exposed to oxygen and it is possible for oxidation of compounds in the sample to occur.

(*E*)-Ocimene, linalool (enantiomer unspecified), (*Z*)-α-bergamotene, phenylacetaldehyde, benzyl alcohol, nerol and geraniol have all been identified previously as components of *L. odouratus* floral headspace (Porter *et al.* 1990), although it appears that diacetone has not been previously reported.

Wider Occurrence of the Compounds Identified

Knudsen *et al.* (1993) reviewed 700 floral scent compounds identified from 441 taxa in 174 genera in 60 families of plants. Some of the behaviourally active compounds identified in *T. erecta* and *L. odouratus* floral headspace (benzaldehyde, benzyl alcohol, phenylacetaldehyde, limonene and linalool) occur widely in floral odour bouquets (Table 5.11) which suggests that any specificity in the signal received by the insect is perhaps due to the ratio of compounds rather than their presence or absence in the odour bouquet.

Since *H. armigera* is highly polyphagous it is of interest to note that volatiles identified from flowers of *T. erecta* also occur in some of its other host plants albeit in different blends, although this does not necessarily mean that they are important in host location. Thus limonene was the only monoterpene found by Flath *et al.* (1978) in maize silks, Buttery *et al.* (1980) also found benzaldehyde and phenylacetaldehyde in maize silks, and Buttery & Ling (1984) identified limonene and linalool in maize leaf volatiles. Limonene, benzaldehyde and phenylacetaldehyde were identified in tobacco (Loughrin *et al.*, 1990) and benzaldehyde and (+)-limonene were identified in chickpea seed volatiles (Rembold *et al.*, 1989). Limonene has also been found in the flowers of sunflower (Eteviant *et al.*, 1984) and as a tomato leaf volatile (Buttery *et al.*, 1987).

Table 5.11 Other species in which compounds identified in *T. erecta* and *L. odouratus* floral scents also occur (Knudsen *et al.*, 1993)

Benzaldehyde:
Abelia, Actaea, Actinidia, Aglaia, Agraecum, Aspasia, Camellia, Catasetum, Cestrum, Cichorium, Cirsium, Convallaria, Coryanthes, Cymbidium, Cypripedium, Dolichothele, Encephalartos, Epidendrum, Fragaria, Gongora, Hyacinthus, Hydnora, Hypecoum, Jasminum, Medicago, Moneses, Nicotinia, Odontoglossium, Ophyrs, Platanthera, Polycycnis, Pyrola, Rebutia, Selenicereus, Sievekingia, Spathiphyllum, Stanhopea, Stephanotis, Sulcorebutia, Syringa, Tillandsia, Trichopilia, Victoria, Zygopetalum.
Benzyl Alcohol:
Abelia, Actaea, Angraecum, Camellia, Catasetum, Cestrum, Chimonanthus, Coeliopsis, Convallaria, Coryanthes, Cucurbita, Cycnoches, Cymbidium, Cypripedium, Dolichothele, Encephalartos, Fragaria, Gardenia, Gongora, Hyacinthus, Hypecoum, Jasminium, Lonicera, Magnolia, Malus, Masdevallia, Medicago, Moneses, Narciscus, Nicotiana, Nymphaea, Pittosporum, Platanthera, Pyrola, Robinia, Rosa, Salix, Selenicerus, Stanhopea, Stephanotis, Sulcorebutia, Trevorai, Victoria
Phenylacetaldehyde:
Abelia, Bartsia, Cestrum, Cirsium, Hypecoum, Ligustrum, Lilium, Ophyrs, Pyrola, Syringa, Zygopetalum.
Limonene:
Actaea, Actinidia, Anguloa, Anthyllis, Aspasia, Belliolum, Bubbia, Catasetum, Chaubardia, Chimonanthus, Chloranthus, Cichorium, Cimicifuga, Citrus, Clowesia, Convallaria, Coryanthes, Cycnoches, Cymbidium, Cypripedium, Dactylorhiza, Dalechampia, Dendrobium, Dolicothele, Dressleria, Encephalertos, Epidendrum, Exospermum, Fragaria, Galeottia, Gardenia, Gloxinia, Gongora, Helianthus, Herminium, Jasminum, Knautia, Lacaena, Larix, Liriodendron, Listera, Lycaste, Lycomormium, Macrozamia, Magnolia, Masdevallia, Maxillaria, Medicago, Mormodes, Nelumbo, Nicotiana, Ophyrs, Orchis, Osmanthus, Peristeria, Picea, Pinus, Platanthera, Polianthes, Polycycnis, Primula, Rebutia, Rosa, Salix, Selenicerus, Sievekingia, Spathiphyllum, Stanhopea, Sulcorebutia, Syringia, Tillandsia, Vanilla, Victoria, Zamia, Zygogynum
Linalool:
Actaea, Ailanthus, Albizia, Allium, Angraecum, Anthyllis, Belliolum, Bifrenaria, Brassavola, Camellia, Cananga, Catasetum, Cestrum, Chaubardiella, Chimonanthus, Cimicifuga, Citrus, Cochleanthes, Convallaria, Cycnoches, Cymbidium, Cypripedium, Dendroium, Dolichothele, Encephalartus, Freesia, Fritillaria, Gardenia, Gongora, Hoya, Hyacinthus, Jasminium, Ligustrum, Lilium, Listera, Lonicera, Macrozamia, Magnolia, Malus, Medicago, Narcissus, Nelumbo, Neofinetia, Nicotiana, Oenothera, Ophrys, Orchis, Osmanthus, Paphinia, Pittosporum, Platanthera, Polycycnis, Primula, Rebutia, Robinia, Rosa, Salix, Sambucus, Selenicereus, Stanhopea, Stephanotis, Sulcorebutia, Syringa, Wistaria, Zamia, Zygogynum.

Chapter 6

EAG ANALYSIS OF SYNTHETIC COMPOUNDS

6.1 Introduction

The objective of these analyses was to ascertain whether the identified *T. erecta* and *L. odouratus* floral headspace compounds could elicit electrophysiological responses from the antennae of *H. armigera* providing an indication of whether *H. armigera* could perceive the compounds at the peripheral level. Any ability of the compounds to evoke depolarisations in the antennal haemolymph, as measured by an electroantennogram, would provide evidence for the possible significance of the compounds in host finding behaviour (Raguso *et al.*, 1996).

The analyses provided an independent means of confirming the structural assignments of the electrophysiologically-active compounds tentatively identified by GC and GC-MS and of confirming EAG-activity of compounds identified in the GC-MS analyses. Other compounds identified in air-entrained samples that did not elicit EAG responses in linked GC-EAG analyses were also tested since the dose that insects could be exposed to in the linked GC-EAG analyses was limited by the capacity of the bonded capillary GC columns (ca 100 ng). This was important because the threshold concentration of compounds to elicit an EAG response from the antennae of Lepidoptera is higher for plant volatiles than for sex pheromones (Topazzini *et al.*, 1990). The additional compounds tested were chosen on the basis of reports of their eliciting behavioural responses from Lepidoptera recorded in the literature (see Section 2.4.3). Electrophysiological responses to the host-plant volatiles were tested at a range of concentrations using female *H. armigera*. Compounds that elicited an EAG response were subsequently tested against male *H. armigera* to determine whether there was a sex-linked difference in their sensitivity to the compounds.

6.2 Methods and Materials

6.2.1 Comparison of relative EAG activity of synthetic host odour volatiles at a single dose.

The EAG preparation was set up essentially as described in Chapter 4. However, in this case the EAG preparation was not linked to a GC but exposed to the odours of candidate compounds in a stream of nitrogen released for pre-determined times at pre-determined intervals and concentrations from filter paper held in a Pasteur pipette.

The electrophysiological activity of synthetic host plant volatiles was tested by placing a 1 µl aliquot of a 1mg/ml solution of test compound in dichloromethane (except in the case of (+)-limonene for which 1 µl of a 10mg/ml solution was used) onto a 5 x 15 mm Whatman No. 4 filter paper strip and placing it inside a Pasteur pipette. The solvent was allowed to evaporate for approximately 1 min and then after coupling the pipette to a nitrogen supply the solvent was removed by a 3 sec pulse of nitrogen at 100ml/min. The pipette was then positioned ca. 1 cm above the EAG preparation at the mid point of the recording antenna to maximise the exposure of the antenna to the test compound when released.

Nitrogen was subsequently pulsed (100 ml/min) through the pipette for 3 s over the EAG preparation and the electrophysiological response recorded. The EAG preparation was exposed to five replicate pulses from each test sample at 1 min intervals during each EAG analysis. EAG responses were measured and compared with those generated by the same volume of solvent on filter paper. The depolarisation in response to the first nitrogen pulse of a sample was found to be significantly larger but also inconsistent and less reliable than subsequent responses and so was discarded from the analysis (Burguière *et al.*, in press). Thus, averaged EAG responses for samples and solvent control were calculated for each EAG preparation using replicates 2 – 5.

All the compounds were tested on nine female *H. armigera* (9 replicates). Test compounds were numbered and presented in a randomised order using a calculator or numbered cards. A different randomisation was carried out for each insect. EAG

recordings from the solvent control were made at the beginning and end of each sequence of compounds being tested and their mean calculated. A 1mV standard pulse generated by the amplifier was applied after each set of replicates for a single compound. This allowed calibration of the on screen output and made clear which output belonged to which test compound. A record of the order of compounds, dose, sex and time of day was kept. EAG responses were measured manually as the difference between the top of the EAG response peak and the baseline as described by Burguière *et al.* (in press).

The baseline of the electroantennograph changed over time because of random "long-term drift" (Marion Poll & Thièry, 1996) (Figure 6.2). Data from EAG preparations where the baseline changed by ± 1mV over 10 sec were discarded. EAG responses were converted into mV by dividing the on-screen reading by the response elicited by the 1mV standard from the high impedance AC/DC amplifier. Mean EAG responses to dichloromethane solvent control and test compounds on different insects were compared using a paired Student's *t*-test. A significant EAG response was taken to indicate that a particular volatile was perceived by the insect.

6.2.2 Effect of dose on the magnitude of EAG responses elicited by synthetic host volatiles

Dilution series of benzaldehyde, (±)-linalool and phenyacetaldehyde were made up so that EAG dose-response characteristics could be investigated. (+)-Limonene was also investigated to see whether higher concentrations than the dose used in EAG tests with the synthetic at a single dose (1µg) could elicit a significant EAG response. A semi-log scale of doses was tested: 0.1, 0.3, 1, 3, 10 and 30 µg of the various compounds against the solvent control (1µl). The various test solutions of different concentrations of different compounds were numbered and then the order of presentation was randomised using random numbers. Control treatments were carried out at the beginning and end of each series of compounds tested and also after every three test samples. There were 10 replicates in all consisting of 10 different insects for which all six concentrations of the four different compounds were tested. Each test sample and solvent control was replicated five times over the EAG preparation with a one minute interval between pulses. A standard -1 mV spike was recorded between

samples to enable EAG responses to be calibrated. EAG responses were calculated first by subtracting the mean of the control EAG responses measured at the start and end of each group of three test samples and then converted into mV using the -1 mV standard. Series numbers relate to compounds which were tested at the same time and hence are compared with the same solvent control treatments.

6.3 Results

6.3.1 Comparison of relative EAG activity of synthetic host odour volatiles at a single dose.

Magnitude of EAG Responses

At the dose tested (1μg), the mean EAG responses elicited by all the compounds tested were less than 200% of the mean control responses (Tables 6.1 - 6.4). Fig 6.2 shows a typical EAG trace. There was a large variation in EAG responsiveness from insect to insect to the floral volatiles (e.g. benzaldehyde, -0.20 to –0.97mV) and to the solvent control (-0.12 to –0.77 mV) possibly due to the differing physiological condition of different insects (Topazzini *et al.*, 1990) or genetic variation in responsiveness. Responsiveness could also have been altered by subtle differences in positioning of the electrodes although they were inserted in a similar way for all insects tested. When an insect gave a higher than usual response to the dichloromethane control (e.g. Rep 6 in Series 2) it also gave a higher than usual response to the test chemicals. This variation was eliminated when paired Student's *t*-tests were carried out because the fact that control and test responses were measured on the same insect is allowed for in the statistical analysis.

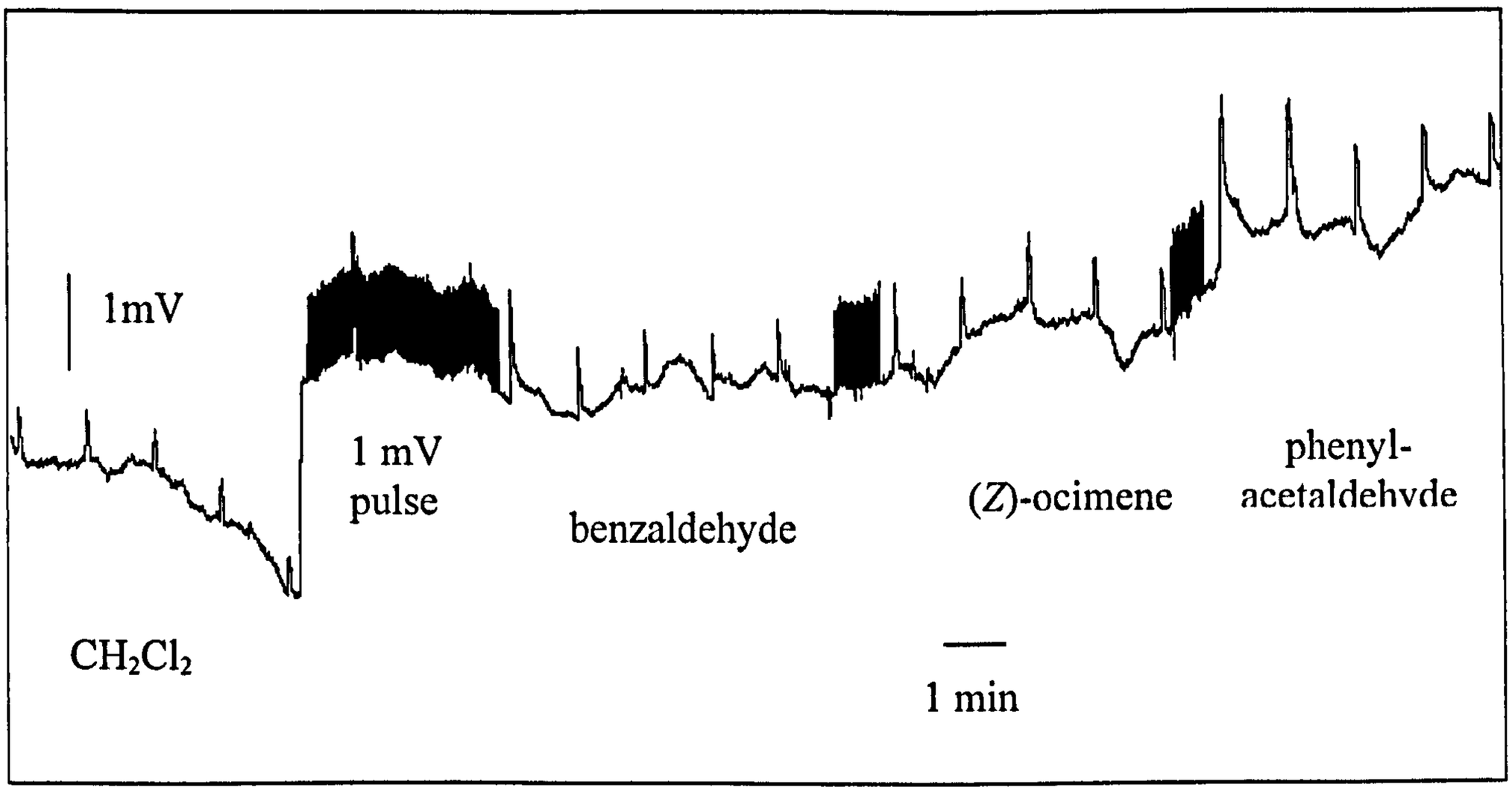

Fig 6.2 EAG responses elicited from female *H. armigera* by synthetic compounds identified in volatiles of floral odours. Figure shows one replicate. Mean responses to pulses two to five were –0.49 mV, -0.77 mV, -0.76 mV and –1.05 mV respectively for dichloromethane (1μl), benzaldehyde (1 μg), ocimene (1 μg) and phenylacetaldehyde (1 μg).

EAG Responses of Female H. armigera *to Compounds Identified from* T. erecta *and* L. odouratus*, Series 1, 2 & 3*

The compounds observed to have EAG-activity in GC-EAG tests, myroxide, benzaldehyde, (±)-linalool, phenylacetaldehyde, and (-)-piperitone were shown to elicit significant EAG responses ($P<0.05$) (Table 6.1 & 6.2). Four other compounds present in the *T. erecta* air entrained samples were also tested at a 1μg dose, of these (+)-limonene (unnatural enantiomer), β-caryophyllene and myrcene were not EAG-active but ocimene (mixture of isomers) was (Table 6.2). The correct geometric isomers of ocimene and myroxide were obtained and tested in Series 3. (*Z*)-ocimene and (*E*)-myroxide both elicited significant EAG responses (Table 6.3).

EAG Responses of Female H. armigera *to Compounds Identified from* T. erecta *and* L. odouratus, *Series 4*

Series 4 was carried out after obtaining enantiomerically pure (-)-limonene, which was the optical configuration present in the natural *T. erecta* samples. Benzyl alcohol and diacetone were also included in this series so as to obtain EAG data for the compounds identified from *L. odouratus* which did not occur in the floral volatiles produced by *T. erecta*.

A significant EAG response was elicited from female *H. armigera* on exposure to (-)-limonene (1μg) (Table 6.4) but the same dose of (+)-limonene did not elicit a significant EAG response (Table 6.2). Significant EAG responses were elicited by the *L. odouratus* compounds, benzyl alcohol and diacetone, (Table 6.4) with female *H. armigera*.

EAG Responses of Male H. armigera *to Compounds Identified from* T. erecta, *Series 5*

T. erecta compounds were tested with male *H. armigera* in Series 5 (Table 6.5). The only difference in EAG responsiveness of male and female *H. armigera* was with benzaldehyde which elicited significant responses from female (-0.61 ± 0.08 mV, Table 6.1) but not male (0.46 ± 0.06 mV, Table 6.5) EAG preparations.

Table 6.1 EAG responses elicited from female *H. armigera* by synthetic compounds, (1µg dose), Series 1

Chemical	Mean EAG response (-mV) elicited from each EAG preparation									Mean Response (-mV)	Standard Error	*P*-value *
	1	2	3	4	5	6	7	8	9			
(*Z*)-Ocimene	0.48	0.65	0.30	0.38	0.54	0.56	0.76	0.12	0.53	0.48	0.06	0.002
Benzaldehyde	0.50	0.97	0.45	0.61	0.68	0.80	0.77	0.20	0.53	0.61	0.08	0.001
Phenylacetaldehyde	0.78	1.07	0.64	0.61	0.88	0.77	1.05	0.22	0.74	0.75	0.08	<0.001
(±)-Linalool	0.45	1.19	0.47	0.52	0.47	0.55	1.06	0.11	0.56	0.60	0.11	0.032
Control (CH_2Cl_2)	0.36	0.77	0.30	0.49	0.46	0.42	0.52	0.12	0.46	0.43	0.06	

* Paired *t*-test comparing treatments and dichloromethane control means.

Table 6.2 EAG responses elicited from female *H. armigera* by synthetic compounds, (1µg dose), Series 2

Chemical	Mean EAG response (-mV) elicited from each EAG preparation									Mean Response (-mV)	Standard Error	*P*-value *
	1	2	3	4	5	6	7	8	9			
(-)-Piperitone	0.27	0.52	1.58	0.55	1.01	1.68	0.62	0.79	0.66	0.85	0.16	0.001
Myroxide (mixture of (*E*)- & (*Z*)- isomers)	0.59	0.38	1.20	0.49	0.82	1.51	0.42	0.70	0.56	0.74	0.13	<0.001
Myrcene	0.28	0.34	1.00	0.47	0.61	1.15	0.51	0.49	0.48	0.59	0.10	0.434
(+)-Limonene	0.23	0.41	0.94	0.39	0.64	1.31	0.30	0.47	0.33	0.56	0.12	0.645
Ocimene (mixture of isomers)	0.50	0.38	1.15	0.45	0.70	1.32	0.45	0.67	0.47	0.68	0.11	0.002
β-Caryophyllene	0.29	0.37	1.12	0.53		1.15	0.43	0.43	0.56	0.61	0.11	0.291
Control (CH_2Cl_2)	0.29	0.34	1.01	0.41	0.58	1.32	0.31	0.47	0.36	0.56	0.12	

* Paired *t*-test comparing treatments and dichloromethane control means.

Table 6.3 EAG responses elicited from female *H. armigera* by synthetic compounds, (1µg dose), Series 3. Isomerically Pure *T. erecta* Compounds

Chemical	Mean EAG response (-mV) elicited from each EAG preparation									Mean Response (-mV)	Standard Error	*P*-value *
	1	2	3	4	5	6	7	8	9			
(*E*)-Myroxide	0.28	0.57	0.38	0.60	0.36	0.34	0.63	0.56	0.35	0.45	0.04	<0.001
(*Z*)-Ocimene	0.20	0.33	0.64	0.54	0.55	0.61	0.41	0.73	0.19	0.47	0.06	0.003
Control (CH_2Cl_2)	0.15	0.26	0.17	0.48	0.54	0.45	0.15	0.19	0.22	0.29	0.05	

* Paired *t*-test comparing treatments and dichloromethane control means.

Table 6.4 EAG responses elicited from female *H. armigera* by synthetic compounds, Series 4

Chemical	Mean EAG response (-mV) elicited from each EAG preparation								Mean Response (-mV)	Standard Error	*P*-value *
	1	2	3	4	5	6	7	8			
1µg Benzyl alcohol	0.58	0.68	0.83	0.51	0.59	0.53	0.62	0.62	0.62	0.03	<0.001
1µg Diacetone	0.48	0.38	0.50	0.63	0.46	0.50	0.58	0.66	0.48	0.03	0.001
1µg (-)-Limonene	0.47	0.42	0.39	0.47	0.35	0.39	0.36	0.33	0.39	0.03	0.012
10µg (-)-Limonene	0.60	0.48	0.51	0.49	0.39	0.47	0.53	0.25	0.48	0.02	<0.001
Control (CH_2Cl_2)	0.41	0.33	0.25	0.34	0.33	0.25	0.37	0.39	0.31	0.02	

* Paired *t*-test comparing treatments and dichloromethane control means.

Table 6.5 EAG responses elicited from male *H. armigera* by synthetic compounds, (1µg dose), Series 5

Chemical	Mean EAG response (-mV) elicited from each EAG preparation									Mean Response (-mV)	Standard Error	*P*-value *
	1	2	3	4	5	6	7	8	9			
(-)-Piperitone	1.11	0.68	0.85	0.86	0.20	0.42	0.90	0.49	0.92	0.72	0.10	0.001
(±)-Linalool	1.38	0.40	0.83	0.67	0.42	0.22	0.91	0.35	0.66	0.65	0.12	0.011
(+)-Limonene	0.98	0.40	0.54	0.41	0.17	0.29	0.43	0.31	0.44	0.44	0.08	0.649
Benzaldehyde	0.73	0.39	0.71	0.41	0.17	0.31	0.53	0.38	0.56	0.46	0.06	0.227
Phenylacetaldehyde	0.82	0.81	1.01	0.64	0.34	0.22	0.66	0.51	0.91	0.66	0.09	0.011
Control (CH_2Cl_2)	0.63	0.23	0.60	0.38	0.18	0.23	0.57	0.28	0.41	0.39	0.06	

* Paired *t*-test comparing treatments and dichloromethane control means.

6.3.2 Effect of dose on the magnitude of the EAG response elicited from female *H. armigera* by synthetic floral volatiles

EAG responses elicited by different doses of compound on filter paper were analysed by ANOVA and compared using Duncan's Multiple Range test (Duncan, 1955). Benzaldehyde, (±)-linalool, (+)-limonene and phenylacetaldehyde dose-response analyses gave response curves as shown in the figures below:

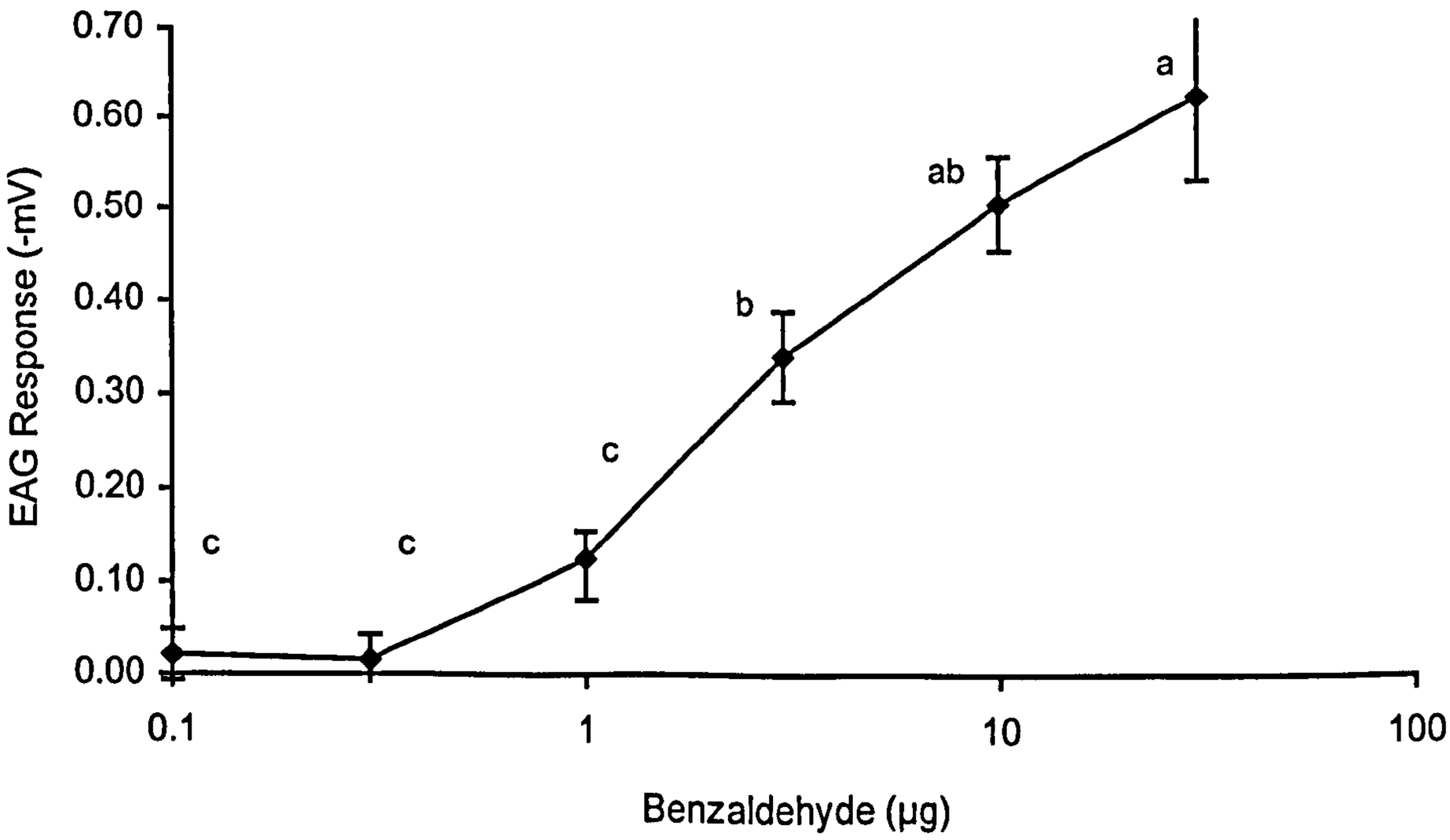

Fig. 6.1 Corrected EAG-responses elicited from female *H. armigera* to benzaldehyde. EAG responses with the same letter were not significantly different at the 5% level (Duncan's Multiple Range test).

The EAG responses elicited from female *H. armigera* to different doses of benzaldehyde after correction for the solvent control are shown in Fig. 6.1. The 3µg dose was the minimum dose that elicited an EAG response that was significantly higher than the control response. However, the EAG responses elicited by doses of 1µg and above increased in an almost linear scale with no apparent levelling off at the 30µg dose.

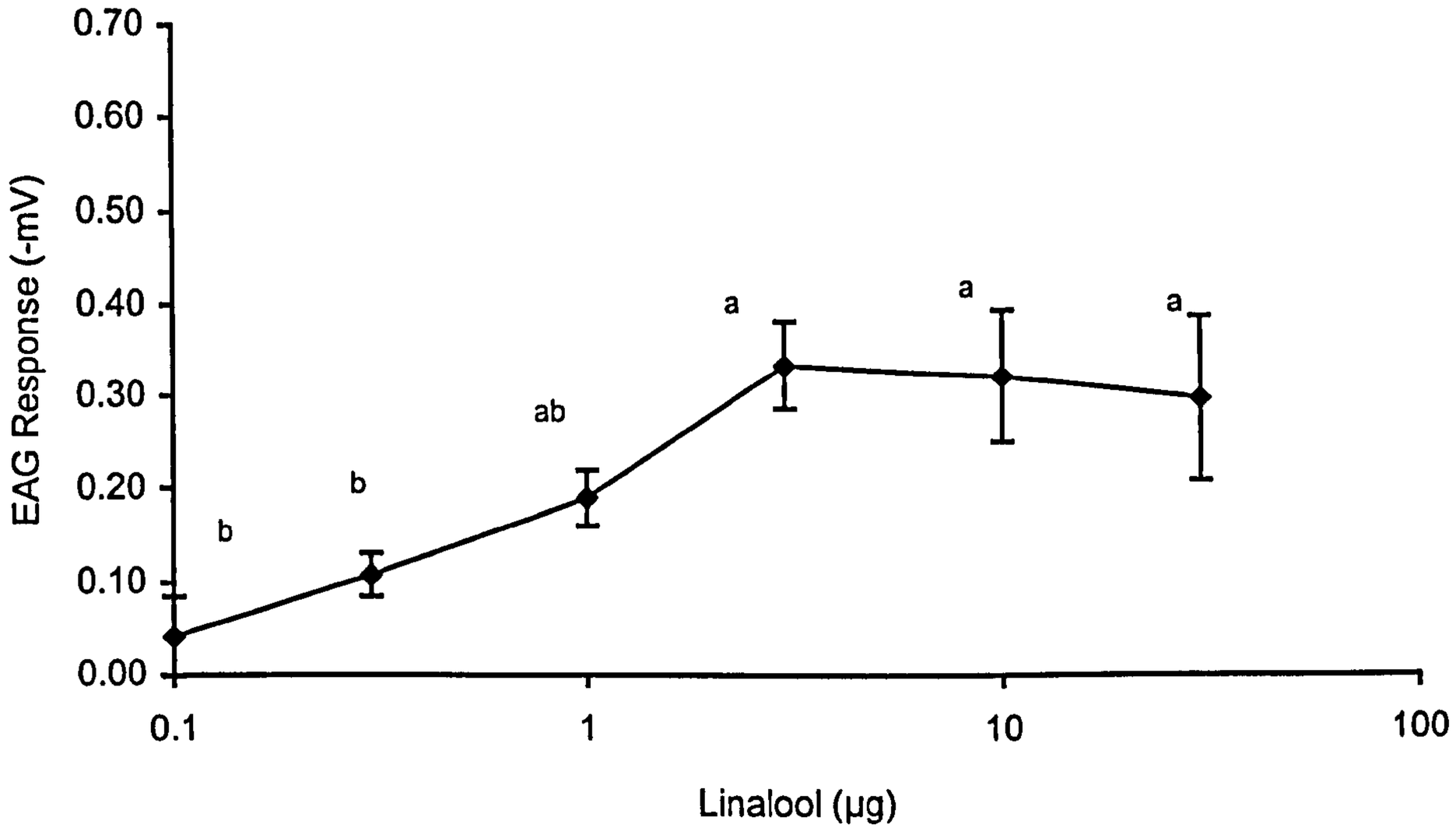

Fig. 6.2 Corrected EAG-responses elicited from female *H. armigera* to (±)-linalool. EAG responses with the same letter were not significantly different at the 5% level (Duncan's Multiple Range test).

The EAG responses elicited from female *H. armigera* to different doses of linalool after correction for the solvent control are shown in Fig. 6.2. The 1µg dose was the minimum dose that elicited an EAG response that was significantly higher than the control response. However, unlike benzaldehyde the EAG response did not increase in a linear manner at doses above 1 µg but levelled off at 3 µg The maximum amplitude of response elicited by linalool (-0.35 mV) was approximately half that elicited by benzaldehyde (-0.65 mV) over the dose range tested.

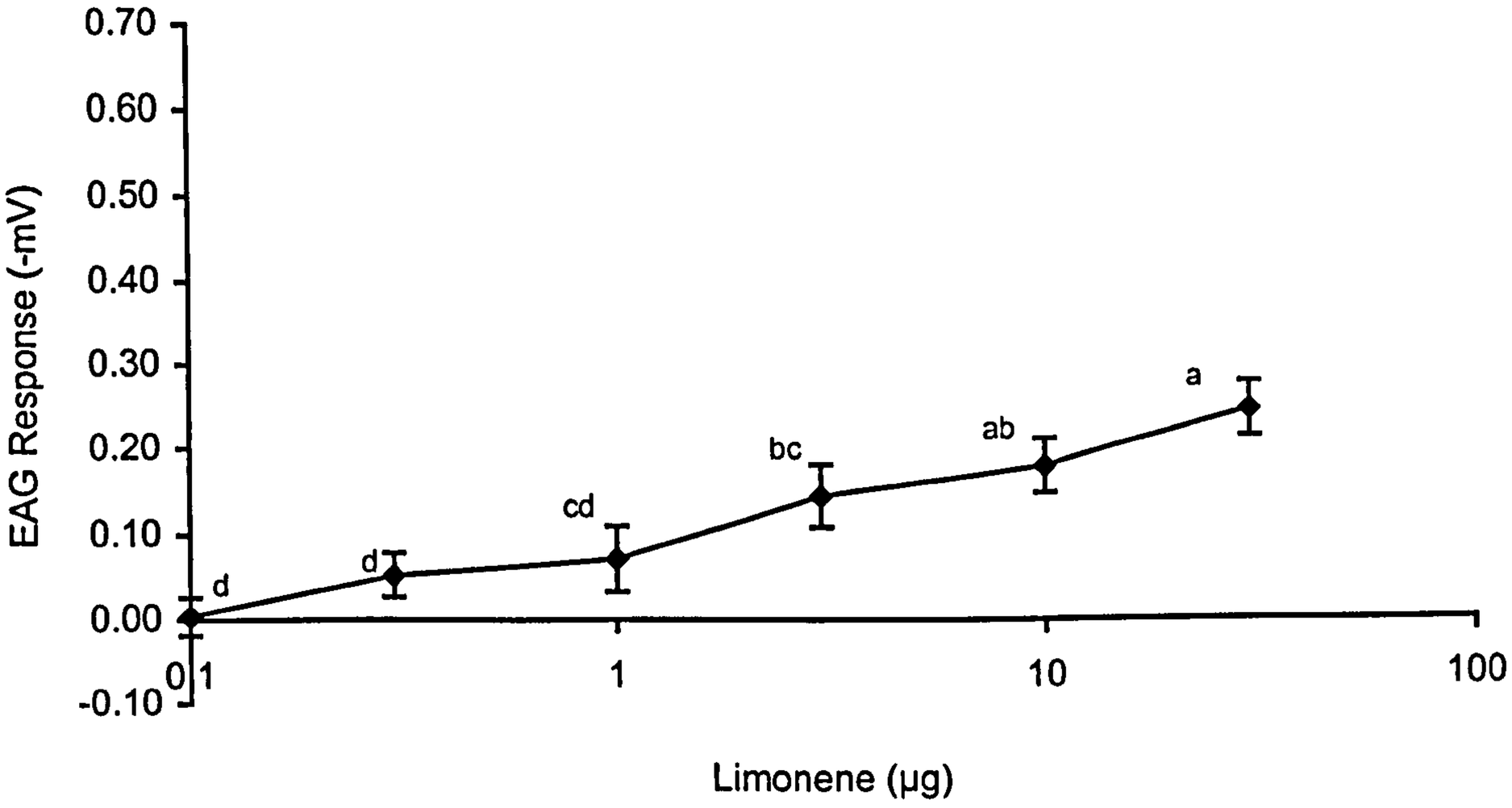

Fig. 6.3 Corrected EAG-responses elicited from female *H. armigera* to (+)-limonene. EAG responses with the same letter were not significantly different at the 5% level (Duncan's Multiple Range test).

The EAG responses elicited from female *H. armigera* to different doses of limonene after correction for the solvent control are shown in Fig. 6.3. The direct EAG response to 3µg (+)-limonene was significantly higher than the solvent control response (P=0.004, paired *t*-test) although there was no significant difference with the 1µg dose. The 3µg dose was the minimum dose that gave a response which was significantly higher than the control response. Response to the 30µg (+)-limonene was significantly higher than the response to 3µg. The increase in EAG response with dose had not yet levelled off even at 30µg. The maximum EAG response elicited by (+)-limonene was comparable to that elicited by (±)-linalool although unlike linalool the EAG response did not level off at 30 µg suggesting that female *H. armigera* could discern a greater range of doses of limonene than linalool.

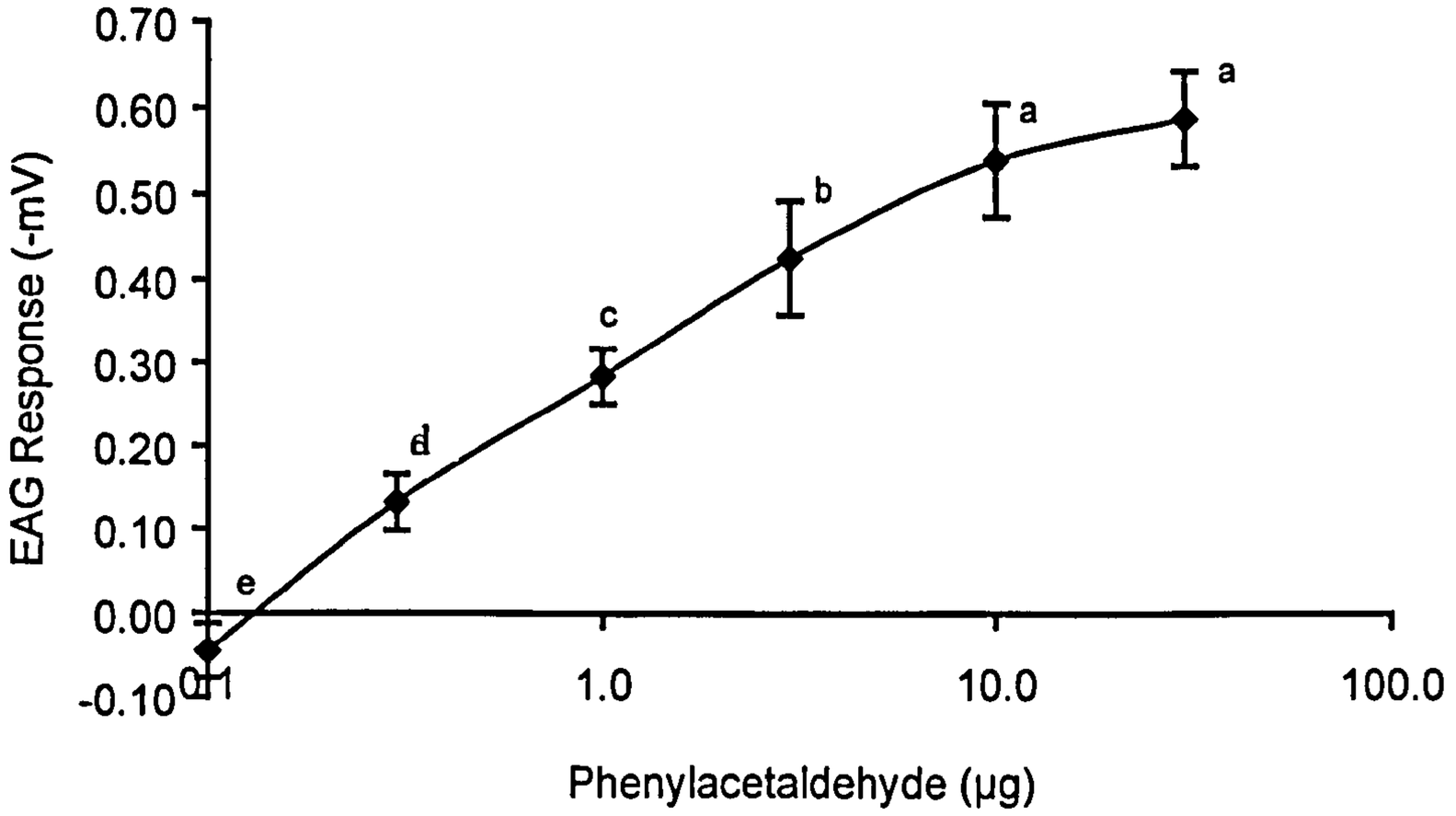

Fig. 6.4 Corrected EAG-responses elicited from female *H. armigera* to phenylacetaldehyde. EAG responses with the same letter were not significantly different at the 5% level (Duncan's Multiple Range test).

The EAG responses elicited from female *H. armigera* to different doses of phenylacetaldehyde after correction for the solvent control are shown in Fig. 6.4. Phenylacetaldehyde had the lowest threshold of response of the compounds tested, eliciting a significant EAG response from female *H. armigera* at a dose of 0.3µg. The EAG response increased in an almost linear manner from 0.3 to 10 µg as observed for benzaldehyde but unlike benzaldehyde appeared to level off at the 30 µg dose.

6.4 Discussion

Confirmation of EAG-Activity of Components of Floral Volatiles

The question of whether or not EAG responses could be elicited from *H. armigera* on exposure to specific floral compounds (Chapter 1) was answered by EAG analyses as the floral compounds did indeed generate significant EAG responses from the antennae of female and male *H. armigera*. A summary of these responses is given in Table 6.6.

Table 6.6 Summary of EAG responses of *H. armigera* to floral volatiles (dose of 1μg on filter paper) from *T. erecta* and *L. odouratus*. '+' indicates a significant response; '-' indicates a non-significant response; 'n.t.' is when a compound was not tested.

Compound	Present in *T. erecta* ?	Present in *L. odouratus* ?	Female EAG response	Male EAG response
(E/Z)-myroxide	(*E*)-isomer	n	+	n.t.
(E)-myroxide	y	n	+	n.t.
benzaldehdye	y	n	+	-
(±)-linalool	y	y	+	+
phenylacetaldehyde	y	y	+	+
(-)-piperitone	y	n	+	+
β-caryophyllene	y	n	-	n.t.
myrcene	y	n	-	n.t.
(Z)-ocimene	y	(*E*)-isomer	+	n.t.
(-)-limonene	y	n	+	n.t.
benzyl alcohol	n	y	+	n.t.
diacetone	n	y	+	n.t.
(+)-limonene	(-)-enantiomer	n	_	_

The five *T. erecta* compounds (*E*)-myroxide, benzaldehyde, (±)-linalool, phenylacetaldehyde and (-)-piperitone which were suspected to have EAG activity in linked GC-EAG analyses were confirmed as being EAG-active at a dose of 1μg on filter paper. Significantly higher EAG responses than with the solvent control were

obtained. Four other compounds present in the *T. erecta* air entrained samples were also tested. (+)-Limonene, β-caryophyllene and myrcene were not EAG-active but ocimene was. (+)-Limonene was EAG-active when a larger dose (3μg) was used. Significant EAG responses to the sweet pea, *L. odouratus*, compounds diacetone and benzyl alcohol were obtained at a dose of 1μg on filter paper.

Burguière *et al.* (in press) carried out EAG testing of synthetic compounds selected on the basis of literature reports of occurrence in *H. armigera* host plants as a prelude to the current study. A dose of 5×10^{-2} μmoles was used and (±)-linalool, α-pinene, myrcene, β-pinene, limonene, β-caryophyllene, 1,8-cineole, (*Z*)-ocimene, β-phellandrene, acetophenone, methyl salicylate, benzyl alcohol, 2-phenylethanol, phenylacetaldehyde and benzaldehyde were found to elicit significant EAG responses from female *H. armigera*. β-Caryophyllene was not significantly EAG-active at the dose used in the current study ($P>0.05$). Myroxide, piperitone and diacetone, identified from entrained samples, were additional compounds found to be significantly EAG-active in the present tests.

Magnitude of EAG Responses Elicited from H. armigera

EAG responses ranged from -0.60 ± 0.11 mV for (±)-linalool to -0.85 ± 0.15mV for (-)-piperitone (1 μg dose on filter paper) compared with a mean control response of –0.43 ± 0.06 mV. The control EAG test using only solvent was carried out to ensure that any apparent response was in fact bigger than the response to solvent only. The control response was high compared with other insects because the mechanoreceptors of *H. armigera* are very sensitive to the pulse of nitrogen which is passed over the antennae. Examples with other Lepidoptera are mean solvent control responses of –0.24 ± 0.03 mV with *Hyles lineata* (Raguso *et al.*, 1996) and –0.18 ± 0.02 mV with cabbage moth, *Mamestra brassicae*, (Rojas, 1999). The EAG responses elicited from *H. armigera* by floral compounds were similar in magnitude to the EAG responses elicited by floral volatiles from *Hyles lineata* and *Sphinx pereglans* (Raguso *et al.*, 1996, Raguso & Light, 1998), who also delivered the compounds from filter paper into a pulsed airstream.

Rojas (1999) commented that isothiocyanates elicited only weak EAG responses from the antennae of *Mamestra brassicae* and Omura *et al.* (1999) also

found weak responses to floral volatiles by cabbage butterfly, *Pieris rapae*, but these authors expressed responses as percentages of the response to a standard rather than in millivolts. The sizes of EAG responses to host-plant odours in Lepidoptera were typically lower than their EAG responses to pheromone compounds indicating a lower sensitivity to plant volatiles (Topazzini *et al.*, 1990) which means that larger doses are needed to obtain significant EAG responses. Of the more abundant compounds identified in the *T. erecta* entrainments that did not elicit EAG responses in the linked GC-EAG analyses only (*Z*)-ocimene was found to elicit a significant response in these tests, possibly due to limited capacity of capillary columns in GC-EAG analyses.

EAG Dose-Response Studies

In dose-response studies, the lowest dose tested for all compounds (0.1μg on filter paper) did not give an EAG response that was significantly different from the control response. As the dose was increased so did the EAG response for all compounds tested. It was decided to use a standard weight of test compound (1μg) rather than using equal concentrations (numbers of moles) of compounds. For a compound with a molecular weight of 150, 1μg represents 0.7×10^{-2} μmoles. Using an equal number of moles for each compound would have been affected by the different volatilities of the different compounds from the filter paper (Brockerhoff & Grant, 1999) and so no attempt at equalising the doses of different compounds was made in the current study. The four compounds investigated in dose-response EAG studies, benzaldehyde, (±)-linalool, (+)-limonene and phenylacetaldehyde had a range of molecular weights from 106 for benzaldehyde to 154 for linalool, and a range of boiling points (volatility) from 175°C for (+)-limonene to 197°C for (±)-linalool. The different molecular weights and volatilities of different compounds meant that the dose received by the EAG preparations were not identical for each compound. Nevertheless, the general trends of the dose-response curves could be compared: For benzaldehyde and (+)-limonene, the response continued to increase up to the highest dose tested (30μg on filter paper), indicating that the upper threshold was above the range tested. With phenylacetaldehyde the response only began levelling off from 10 to 30μg. In the case of (±)-linalool the EAG response levelled off at 3μg. This indicated that there was a lower threshold for maximum EAG response with linalool

possibly because there were fewer receptors for this compound on the antennae of *H. armigera.*

The EAG dose-response study helped to explain an apparent discrepancy observed between the results of the linked GC-EAG studies and subsequent wind-tunnel bioassay experiments. There was no significant EAG response either in linked GC-EAG or EAG studies to (+)-limonene at a dose of 1μg, but (+)-limonene (and (-)-limonene) enhanced attraction to the synthetic blends tested in wind-tunnel experiments. Thus, when the dose of (+)-limonene was increased from 1μg to 3μg a significant EAG response was obtained (P = 0.004). This indicated that there were receptors for (+)-limonene on the antennae of female *H. armigera* albeit less sensitive than those for benzaldehdye, (±)-linalool and phenylacetaldehyde. To elicit an EAG response they required a higher threshold concentration of (+)-limonene than for the other compounds tested, including (-)-limonene which elicited a significant EAG response at the 1μg dose (Table 6.4).

In Chapter 4 it was found that the dose of whole sample on filter paper required to elicit an EAG response was approximately one flower-hour worth of emission (Table 4.1 and comments beneath) which was 0.26μg limonene, 0.13μg benzaldehyde, 0.06μg linalool and 0.39μg phenylacetaldehyde presented simultaneously (Table 5.9). However the doses on filter paper do not represent the actual dose the antennae was exposed to.

Comparison of EAG Responses Elicited by Male and Female H. armigera

Male *H. armigera* responded to (±)-linalool, phenylacetaldehyde and (-)-piperitone, as female insects, but they did not respond to benzaldehyde (1ug dose). Benzaldehyde did not elicit a significant EAG response from male *H. armigera* but did elicit a significant EAG response from female *H. armigera* (P = 0.001). This could possibly be because benzaldehyde is a component secreted by the male scent glands of Noctuidae (Aplin & Birch, 1970). Raguso *et al.* (1996) observed a general trend of higher EAG responses in female *Hyles lineata* (Sphingidae) than males although sex was not a significant factor in the ANOVA analysis they carried out. Similarly, larger EAG responses were observed to be elicited from female (Tables 6.1

& 6.2) than male (Table 6.5) *H. armigera* to the floral volatiles in the current study although they were not significantly higher. However, Suckling *et al.* (1996) found that male lightbrown apple moth, *Epiphyas postvittana*, actually gave higher EAG responses to a range of plant volatiles they tested. In contrast Topazzini *et al.* (1990) did not observe any significant differences between the responses of male and female *Bombyx mori* and *Lymantria dispar* EAG responses to floral volatiles.

EAG Responses to Host-Plant Volatiles and their Possible Role in Host Location

Other authors have also reported EAG responses by polyphagous moth species to plant volatiles, often finding responses to a large number of compounds (Raguso *et al.*, 1996, Suckling *et al.*, 1996, Topazzini *et al.*, 1990). In addition Raguso & Light (1998) found that the oligophagous species *Sphinx perelegans* also responded to a large number of plant derived chemicals and Topazzini *et al.* (1990) found that *Bombyx mori* gave EAG responses to many compounds. This broad olfactory receptivity indicates that plant odour perception does not simply depend on a few compounds but encompasses a large range of compounds. This raises important questions about the nature of the olfactory coding associated with the host-plant such as whether there is preference for specific compounds or just a weak, generalist response to a large variety of plant volatiles. The blend of volatiles associated with host plants also requires investigation because it seems unlikely that individual host-plant volatiles act in isolation in eliciting behavioural responses. A flower, which is under selection pressure to attract pollinators, may use other means to obtain species-specificity if the fragrance emitted attracts a variety of different types of insects. Dodson *et al.* (1969) wrote, considering bio-active compounds in orchid fragrances and attraction of specific pollinators:

> "*Fragrances may attract a broad spectrum of pollinators; in these cases, structural modifications* (to the shape of the flower), *which exclude all but one or a few kinds of pollinators, become critical.*"

Significant EAG responses to the compounds tested indicated that *H. armigera* could perceive them via chemo-receptors on the antennae. However, the size of an EAG response is related to the number of chemo-receptors for the test compound on the antennae which is not necessarily directly related to the effect the compound has

on an insect behaviour, such as upwind flight. Mori *et al.* (1991) carried out EAG analyses and behavioural bioassays with stereoisomers of the sex pheromone of the rice moth, *Corcyra cephalonica*. They found the same order of attractiveness in the behavioural assay as the order of the compounds ranked by the size of the EAG response they elicited. Suckling *et al.* (1996) investigated *Epiphyas postvittana* (Lepidoptera: Tortricidae) responses to plant volatiles and found that EAG was a poor predictor of oviposition attractancy or repellency.

EAG-activity means that compounds can be detected by the insect but it does not tell us anything about how the moths behave upon perception of them and in particular whether they are cues for attraction. An insect's response to a compound or blend of compounds may be influenced not only by the composition of the odour source but also the physiological state of the insect and abiotic factors such as temperature, light intensity and humidity and may be moderated by previous experience (Dethier, 1982; Dent & Pawar, 1988). Indeed repellent volatiles would also elicit EAG responses. Behavioural experiments using a wind-tunnel bioassay therefore were necessary to find out if the identified compounds actually elicited attraction of adult *H. armigera*.

Chapter 7

WIND TUNNEL BIOASSAY WITH AIR ENTRAINED SAMPLES AND PUTATIVE SYNTHETIC KAIROMONE BLENDS

7.1 Introduction

As the objective of this study was to investigate the olfactory basis of attraction to flowers a bioassay was needed in which flight behaviour of female *H. armigera* exposed to floral odours could be observed. A wind-tunnel was chosen because free flying moths could be observed under controlled conditions in the laboratory with it. After having identified floral volatiles in air entrained samples to which there was an electrophysiological response it was necessary to investigate whether a behavioural response to these compounds could be obtained.

Flowers, aliquots of natural headspace samples on filter paper or solutions of synthetic mimics on filter paper were used as odour sources. The behavioural response tested for was an increase in upwind flight towards a source emitting volatiles in the wind-tunnel compared with the level of upwind flight in their absence. Sustained upwind anemotactic flight is observed in response to attractants in a wind-tunnel (Miller & Roelofs, 1978).

7.2 Methods and Materials

Wind-tunnel Bioassay

The wind-tunnel (Fig. 7.1) used to investigate the behavioural responses of female *H. armigera* to natural and synthetic blends of putative kairomone components was without a moving floor, but otherwise similar to that of Miller & Roelofs (1978). The wind-tunnel was made from transparent polyethylene sheet (500 gauge thickness) placed over a metal frame (225 x 60 x 60 cm). The polyethylene sheet was periodically replaced to ensure that it was uncontaminated. Air from the room in

which the wind-tunnel was housed was blown through the tunnel using a fan. To avoid contamination the room was used solely for *H. armigera* rearing and bioassays and no plants were grown in it. Air entered and exited through gauze covers that prevented moths escaping. Airspeed, as measured by a smoke test (Baker & Linn, 1984), was 50 cm / sec. All bioassays were conducted in reduced lighting (0.8 lux) provided by a series of three bulbs (60 watt, fitted with red filter) with variable power output positioned at 40 cm intervals, 1.0 m above the wind-tunnel and angled to provide even coverage of the whole tunnel.

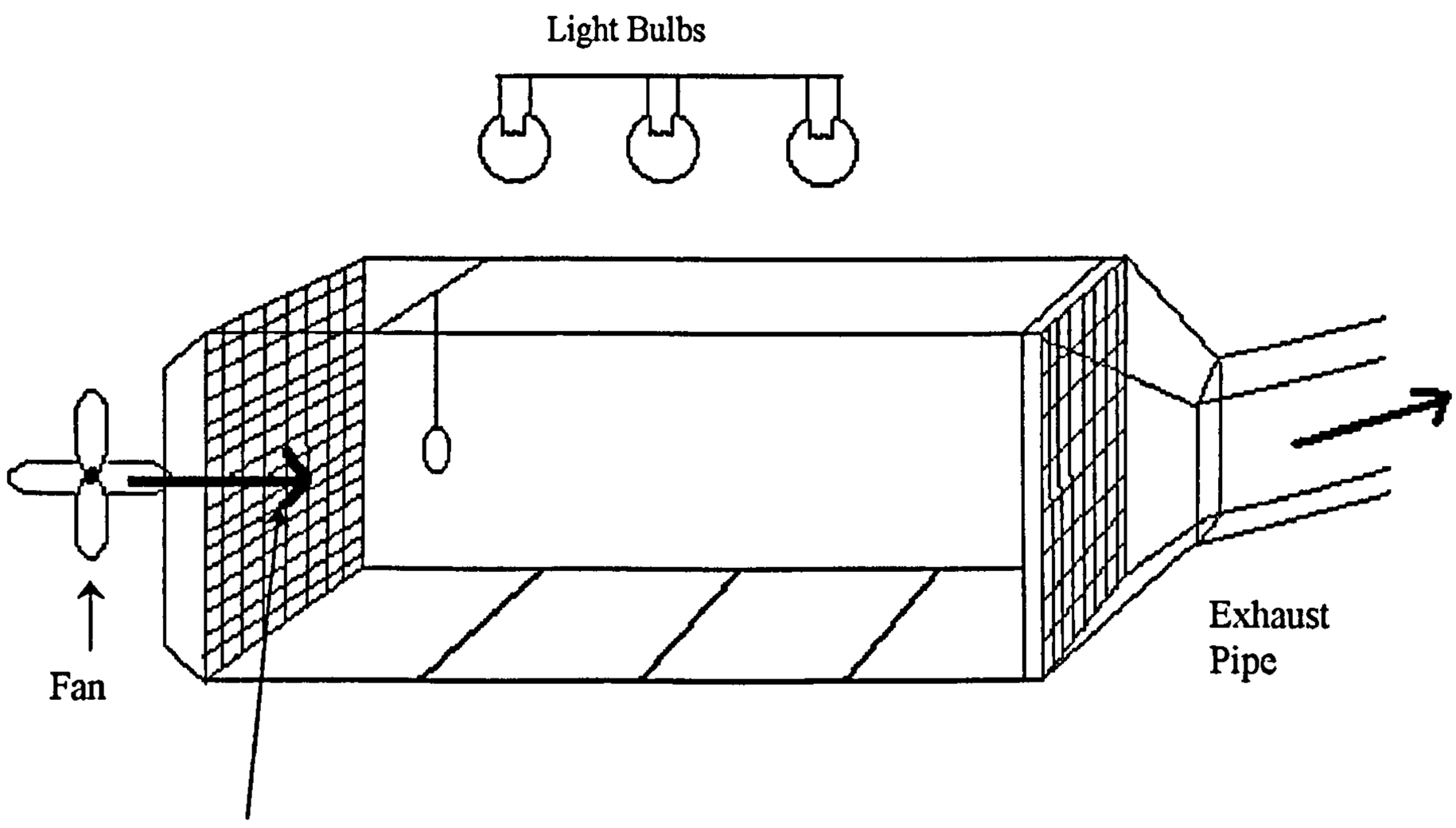

Fig. 7.1 Diagram of the Wind-Tunnel (225 x 60 x 60 cm). Airflow (50 cm/sec) was provided by a fan blowing air in and air was ducted out by an extractor fan. Lighting (0.8 lux) was provided by a series of overhead bulbs. The lure was positioned 200cm upwind and insects were released at the downwind end.

Experimental Insects

Test moths were removed from mixed sex groups during the photophase, at least 2 hours prior to bioassay, and placed in individual holding containers without sucrose solution. The room in which the wind-tunnel was sited was kept under the same environmental conditions as that used for rearing (25 ± 1 °C, 50% relative humidity). All bioassay experiments except one with a field collected Pakistani strain used *H. armigera* moths from the NRI laboratory culture (Chapter 3). Male moths were used in bioassay series 1 (tested with sex pheromone) but female moths were used in all subsequent bioassays. All moths were assumed to be mated as they were

taken from mixed sex cultures within 7 days of emergence, although this was not confirmed by disection because they were required to maintain the insectary culture. Moths were used once and then returned to the culture into separate cages from newly emerged moths to ensure each replicate used a different insect.

Procedure used in Bioassay

Bioassays were conducted during the first three hours of the scotophase which corresponded with the natural time of nectar foraging and oviposition of female *H. armigera* (Roome, 1975). Bioassays were conducted with individual insects introduced into the wind-tunnel through a side-panel at the downwind end of the tunnel. Insects were kept in the holding containers until the test sample had been introduced. Test samples were put in through another side panel 200 cm upwind and attached, using a bulldog clip, to a vertical support at the centre of the cross section in the wind-tunnel. The holding container was immediately opened and the insect allowed to emerge.

The control treatment was tested first to ensure that there was no contamination of the wind-tunnel from previous treatments. Test treatments were bioassayed in a randomised order. Samples for use in the wind-tunnel bioassay were applied to filter paper discs (Whatman No. 1PS, 7.0 cm diameter) in aliquots (50 µl) of dichloromethane. Solvent was allowed to evaporate from the filter paper discs for 2 min before they were placed in the wind-tunnel. Samples were renewed for each individual insect bioassayed. The polyethylene sheet was changed periodically (every 2 months) to ensure it did not become contaminated.

Since the male *H. armigera* response to the synthetic sex pheromone is well established (Nesbitt *et al.*, 1979) it was used as a positive control to test that the wind-tunnel provided an adequate arena for investigating the responses of *H. armigera* to semiochemicals (Bioassay series 1). Subsequent bioassays were carried out with Porapak Q collected floral volatiles of African marigold, *Tagetes erecta*, sweet pea, *Lathyrus odouratus*, and buddleia, *Buddleia davidii*, and synthetic blends of volatiles from *T. erecta* and *L. odouratus* with compounds in the same ratio and at the same concentration as in the natural samples. Details of treatments are given in the results section.

In bioassay series 10, odours from a live *T. erecta* flower and a synthetic blend were compared using additional apparatus blowing odours from a flask outside the wind-tunnel. A round bottomed glass flask with three necks, screened from the wind-tunnel, was used in which either a piece of filter paper with an aliquot of synthetic *T. erecta* floral blend or one live *T. erecta* flower (with a bung around the stem) was inserted. Charcoal filtered incoming air was blown through the flask into a tube exiting into the wind-tunnel (attached at the same position as where filter papers were clipped in previous bioassays) using a pump (CAPEX L2C, Fisher Scientific, Loughborough, UK, 500ml/min flow rate). This method of presentation provided a means of presenting natural and synthetic olfactory stimuli to female *H. armigera* in the absence of any visual cues (such as filter papers or flowers) or tactile cues.

Measurement of Behavioural Responses

Behavioural responses of moths in the wind-tunnel were observed and recorded manually during a 12 min bioassay period. Behaviours preceding initiation of upwind flight - wing fanning, walking and whether or not the insect took flight - were recorded together with three criteria associated with upwind flight: the number of upwind flights or approaches made to the lure, the furthest distance flown upwind and the number of contacts made with the lure. For counting approaches a vertical line 20 cm downwind of the odour source was used as an objective reference point to aid quantification. Each upwind flight that crossed the line was counted as an approach. The number of approaches made by a moth to a lure provided a relative measure of the amount of chemo-anemotactic flight elicited by the lure. Number of upwind approaches to the lure has been used previously as a scoring method in wind-tunnel studies of *H. armigera* responses to host-plant odours (Hartlieb & Rembold, 1996) and in observations of redbanded leafroller moth, *Argyrotaenia velutinana*, responses to sex pheromone (Miller & Roelofs, 1978).

Statistical Analysis

The wind-tunnel data were analysed using non-parametric tests since the assumptions for parametric tests of equal variance and normal distribution of data from all treatments were not valid (Mead *et al.*, 1993). For each series of wind-tunnel bioassays a Kruskal-Wallis test was first carried out to see whether there was a significant difference overall between treatments for the whole series. After this was validated, a Mann Whitney 'U' test was used to determine whether the medians of individual treatments and control were significantly different.

Statistical analyses were carried out for scores of behavioural responses: furthest distance flown upwind, number of upwind approaches and number of source contacts. Data were analysed using Statistical Package for Social Scientists for Windows (Release 9.0.0, SPSS Inc. 1998).

7.3 Results

7.3.1 Bioassay Series 1: Behavioural responses of Male *H. armigera* to the Female Sex Pheromone

The sex pheromone was a positive control to which a behavioural response was expected. Treatments used are shown in Table 7.1

Table 7.1 Treatments in Bioassay Series 1: Female sex pheromone

Source	Material tested (50µl CH_2Cl_2 on filter paper)	No. of Replicates
Control	50µl CH_2Cl_2	48
Treatment	2µg of a 1 : 33 blend of (*Z*)-9-hexadecenal and (*Z*)-11-hexadecenal	47

Table 7.2 Responses of male *H. armigera* to sex pheromone (P value is for comparision of treatment and control medians by Mann Whitney 'U' test).

	No. of Upwind Approaches		Furthest Flown Upwind (cm)		Source Contacts	
Treatment	Control	Pheromone	Control	Pheromone	Control	Pheromone
Mean	0.81	2.43	116	150	0	0
S.E.	0.17	0.47	12.1	11.5	0	0
P-value		0.018		0.0023		1

Bioassay data (Table 7.2) confirmed that male *H. armigera* were able to respond to the synthetic sex pheromone in the wind-tunnel and that the apparatus and experimental design were such that significant responses were measured. Thus, male moths made more upwind approaches to the source when the sex pheromone was present than when the solvent alone was present ($P = 0.018$) and the median distance flown upwind was significantly greater ($P = 0.002$). However, no source contacts were made with the sex pheromone, possibly because the concentration was too high.

7.3.2 Bioassay Series 2: Behavioural responses of female *H. armigera* to cut *T. erecta* flowers

This series of bioassays was conducted to observe the responses of female *H. armigera* to whole cut flowers of *T. erecta* before tests were carried out with headspace samples. Two cut flowers were positioned at the upwind end (Table 7.3).

Table 7.3 Treatments in Bioassay Series 2: Cut flowers of *T. erecta*

Source	Material tested	No. of Replicates	Mean No. of Source Contacts
Control	Blank filter paper	20	0
Treatment	Two cut flowers of *T. erecta*	20	0.30

Table 7.4 Responses of female *H. armigera* to *T. erecta* flowers (P value is for comparision of treatment and control medians by Mann Whitney 'U' test).

	No. of Upwind Approaches		Furthest Flown Upwind (cm)		Source Contacts	
Treatment	Control	Cut Flower	Control	Cut Flower	Control	Cut Flower
Mean	1.05	2.15	101	131	0	0.3
S.E.	0.48	1.03	22.9	22.1	0	1.13
P-value		0.48		0.35		0.018

Cut flowers of *T. erecta* did not elicit a significant increase in the number of upwind approaches or the furthest distance flown by female *H. armigera* compared to the filter paper control (Table 7.4). Nevertheless, they did elicit a significant increase in the number of source contacts ($P = 0.018$) compared to the control (Table 7.4). The mean time spent on the flower during source contact was 2 min 41 sec and one female moth remained on a cut flower for 6 min 35 sec. The longer periods of time spent settled, in source contact with flowers, reduced the time available for upwind flight. The moths were observed to extend their proboscises and imbibe nectar which would have further reduced their motivation to fly to the flower.

7.3.3 Bioassay Series 3: Behavioural responses of female *H. armigera* to entrained floral volatiles from cut flowers of *T. erecta*

The behavioural responses of female *H. armigera* to an aliquot of floral volatiles collected by air entrainment of freshly cut *T. erecta* flowers was investigated. The sample of floral volatiles (Tag-WT1) used was prepared by combining the most EAG-active *T. erecta* samples available (Tag-98-01, Tag-98-03 and TB-98-04). A 50µl aliquot of the extract was used for the wind-tunnel bioassay, a dose that elicited an EAG response twice that of the solvent control. This dose represented approximately 1.5 flower-hours of emission. Treatments used are shown in Table 7.5.

Table 7.5 Treatments in Bioassay Series 3: Extract of entrained volatiles from cut flowers of *T. erecta*

Source	Material tested (50µl CH_2Cl_2 on filter paper)	No. of Replicates	Mean No. of Source Contacts
Control	50µl CH_2Cl_2	69	0.014
Treatment	50µl of extract of entrained floral volatiles from cut flowers of *T. erecta* (Tag-WT1)	63	0.159

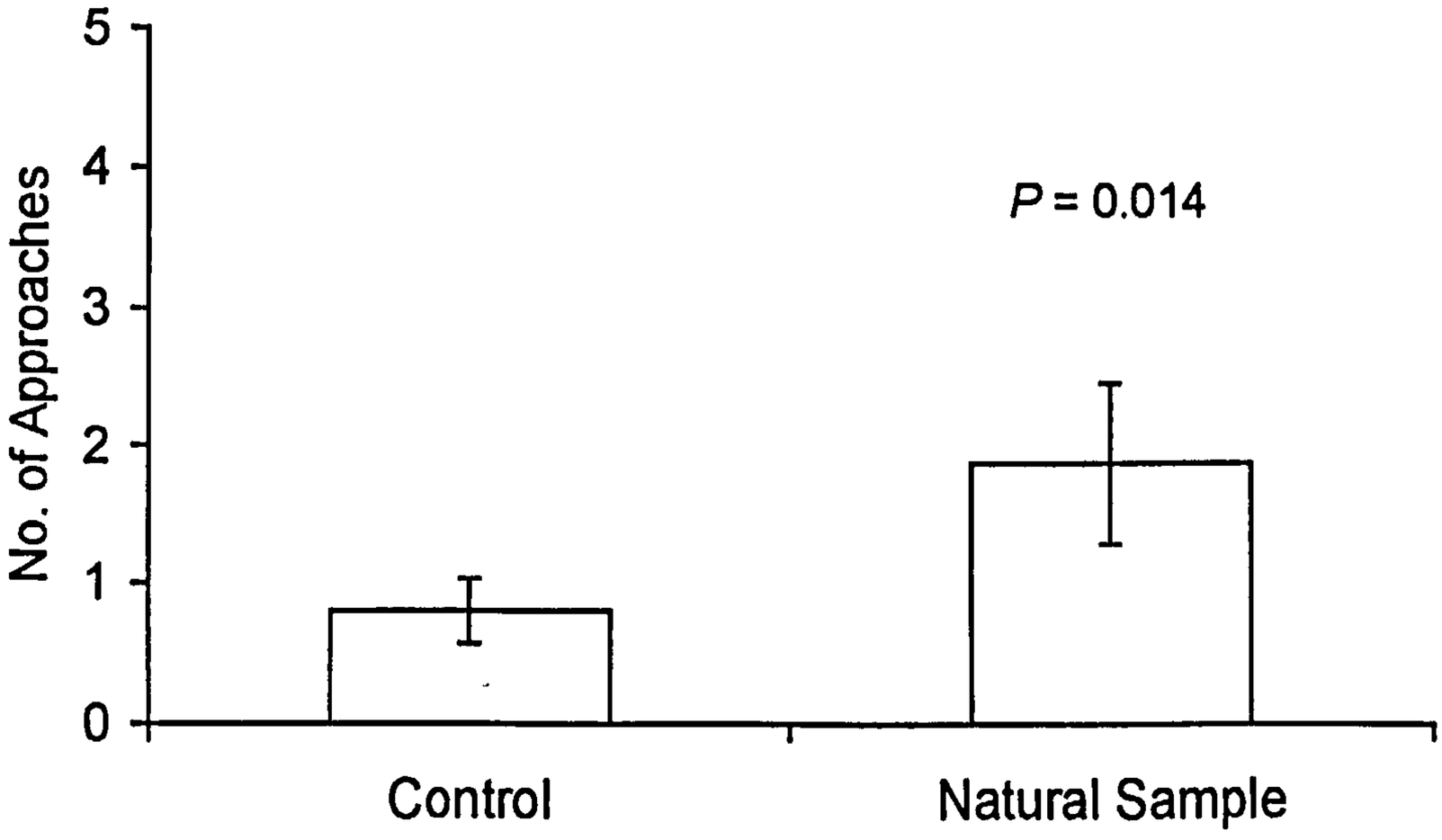

Fig. 7.2 Mean upwind approaches of female *H. armigera* to floral volatiles from cut flowers of *T. erecta* (P value is for comparision of treatment and control medians by Mann Whitney 'U' test).

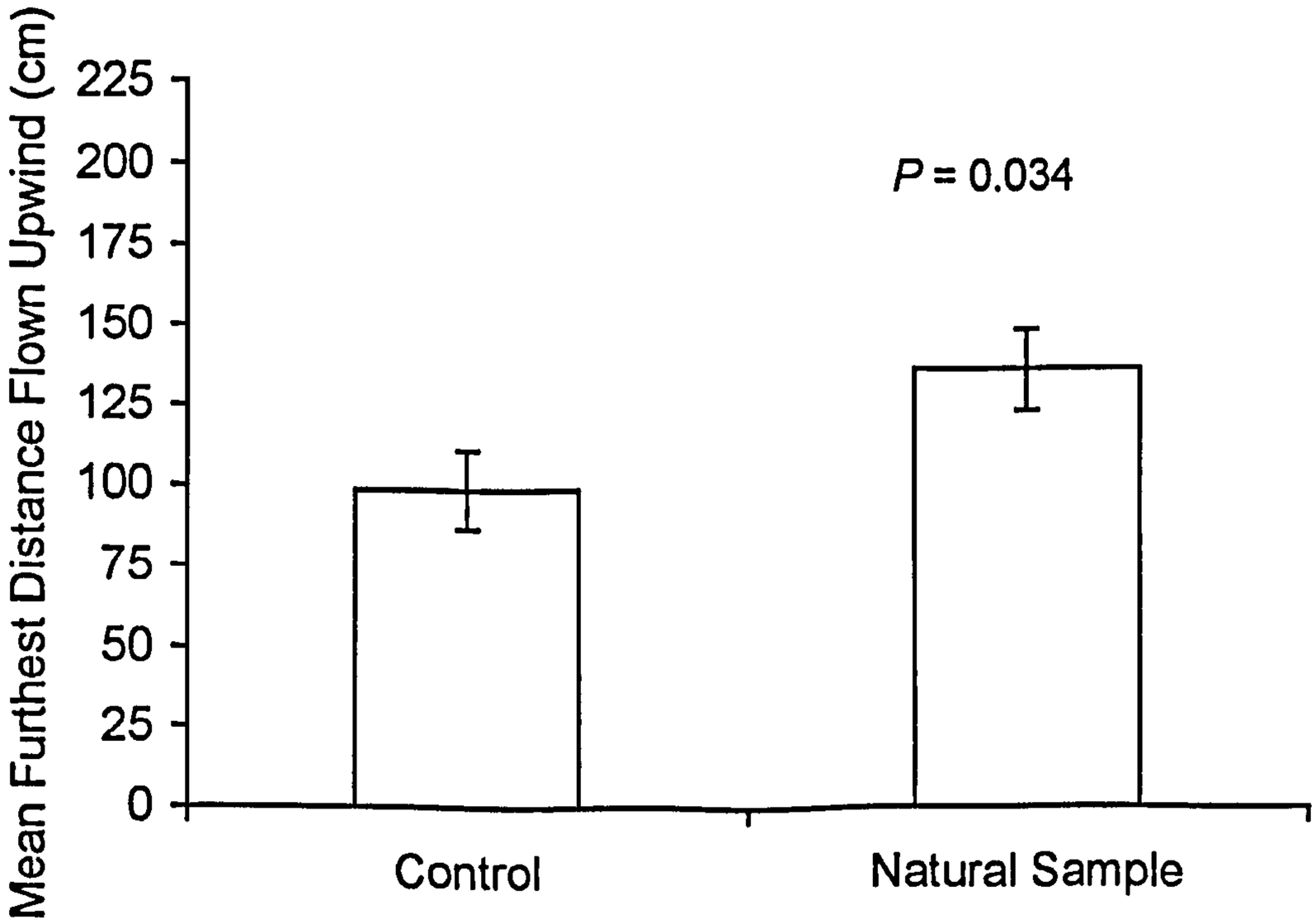

Fig. 7.3 Furthest flown upwind by female *H. armigera* to floral volatiles from cut flowers of *T. erecta* (P value is for comparision of treatment and control medians by Mann Whitney 'U' test).

Floral volatiles from cut flowers of *T. erecta* elicited significantly more upwind approaches ($P = 0.014$) (Fig 7.2), a significant increase in the furthest distance flown upwind ($P = 0.034$) (Fig. 7.3) and a significantly greater number of source contacts ($P = 0.005$) (Table 7.5) than the control treatment. Those moths that flew upwind beyond the control source tended not to fly downwind again or make any further upwind forays although those that were presented with natural floral odours from cut flowers made numerous attempts to reorient towards the source, exhibiting 'seeking' type behaviour (Brantjes, 1976).

7.3.4 Bioassay Series 4: Behavioural responses of female *H. armigera* to blends of synthetic compounds based on the composition of floral volatiles isolated from cut flowers of *T. erecta*

Blends of synthetic compounds identified in linked GC-EAG analyses of volatiles collected from *T. erecta* floral headspace were tested. The compositions of blends were based on the relative proportions of the five EAG-active compounds present in the sample of floral volatiles from *T. erecta* bioassayed in Series 3 (Tag-WT1). Details of treatments are shown in Table 7.6.

Table 7.6 Treatments in Bioassay Series 4

Treatment	Material tested (µg) (50µl CH_2Cl_2 on filter paper)					No. of Reps	Mean No. of Source Contacts
	Phenylacetalde-hyde	Benzaldehyde	(±)-Linalool	(*E*/*Z*)-Myroxide	(-)-Piperitone		
Control	0	0	0	0	0	38	0
3C+Myroxide [a]	0.23	0.5	0.73	3.7	0	15	0
3C+Piperitone [a]	0.23	0.5	0.73	0	14.4	22	0.13
5-comp blend	0.23	0.5	0.73	3.7	14.4	16	0

[a] 3C = 3 component blend (phenylacetaldehyde, benzaldehyde, linalool)

Table 7.7 Upwind approaches of female *H. armigera* to synthetic blends of compounds identified in *T. erecta* flowers (P value is for comparison of medians of all treatments in the series by Kruskal-Wallis test).

Treatment	Control	3C+Myroxide	3C+Piperitone	5-comp blend
Mean	0.66	0.93	1.59	0.63
S.E.	0.27	0.93	0.85	0.30
P-value	0.54 (N.S.)			

Table 7.8 Furthest flown upwind by female *H. armigera* to synthetic blends of compounds identified in *T. erecta* flowers (P value is for comparison of medians of all treatments in the series by Kruskal-Wallis test). Units in cm.

Treatment	Control	3C+Myroxide	3C+Piperitone	5-comp blend
Mean	74.6	35.7	83.6	71.9
S.E.	15.0	19.5	21.5	24.5
P-value	0.37 (N.S.)			

A 5-component blend containing all the EAG-active compounds identified in GC-EAG analyses of sample Tag-WT1 (used in bioassay Series 3) of *T. erecta* floral volatiles (benzaldehyde, (±)-linalool, myroxide, phenylacetaldehyde and (-)-piperitone), in the natural ratio and concentration, did not elicit a behavioural response that was significantly different from the control response (Table 7.7 & 7.8).

Removal of either piperitone or myroxide from the 5-component blend did not make a significant difference to behavioural responses elicited although a 4-component blend, in which myroxide was excluded (3C/piperitone), elicited more than twice as many upwind approaches as the solvent control (Table 7.7) and 2 of the 15 moths tested made source contact. These data suggested the synthetic myroxide used, which was a mixture of (*E*)- and (*Z*)-isomers, was inhibitory.

7.3.5 Bioassay Series 5: Behavioural responses of female *H. armigera* to blends of synthetic compounds based on the composition of floral volatiles isolated from cut flowers of *T. erecta*

Table 7.9 Treatments in Bioassay Series 5

Treatment	Material tested (µg) (50µl CH_2Cl_2 on filter paper)								No. of Reps.	Mean No. of Source Contac
	Phenylacetaldehyde	Benzaldehyde	(±)-Linalool	Myrcene	β-Caryophyllene	Ocimene	(+)-Limonene	(-)-Piperitone		
Control	0	0	0	0	0	0	0	0	41	0
3-Components	0.23	0.5	0.73	0	0	0	0	0	22	0
3C/Myrcene	0.23	0.5	0.73	1.4	0	0	0	0	12	0.08
3C/Caryophyllene	0.23	0.5	0.73	0	3.9	0	0	0	9	0
3C/Ocimene	0.23	0.5	0.73	0	0	4.4	0	0	9	0
3C/Limonene	0.23	0.5	0.73	0	0	0	8.6	0	35	0.03
3C/Lim/Piperitone	0.23	0.5	0.73	0	0	0	8.6	14.4	13	0.15

Bioassay Series 5 included four components which were present in *T. erecta* volatiles (Tag-WT1) but did not cause an EAG response in GC-EAG analyses. They were chosen due to reports in the literature of their behavioural activity with other moth species (Section 2.4.3). They were myrcene, β-caryophyllene, (*Z*)-ocimene and (+)-limonene, added in the naturally occuring amount to the three-component blend of benzaldehyde, (±)-linalool and phenylacetaldehyde (Table 7.9). The three EAG-active compounds (benzaldehyde, (±)-linalool, phenylacetaldehyde) that were used are characteristic of the floral odour of moth pollinated flowers (Haynes *et al.*, 1991, Heath *et al.* 1992, Raguso & Pichersky, 1995).

In Series 5 the first two synthetic *T. erecta* blends which elicited significant upwind attraction (Fig. 7.4 and 7.5) were bioassayed: a 4-component blend including (+)-limonene and a 5-component blend including (+)-limonene and (-)-piperitone. However, addition of myrcene, β-caryophyllene or (*Z*)-ocimene to the 3-component blend had no significant effect on activity (number of upwind approaches, furthest distance flown upwind or source contacts) compared to the 3-component blend alone.

Addition of (+)-limonene to the 3-component blend (10 μg total dose) gave a significant increase in number of upwind approaches relative to the solvent control ($P = 0.0008$; 9 times more upwind approaches). There were significantly more upwind approaches than with the 3-component blend alone ($P = 0.036$). Insects flew significantly further upwind than with the solvent control ($P = 0.0011$) and one of the 35 insects tested made source contact.

With addition of (+)-limonene and (-)-piperitone to the 3-component blend in the natural ratio (24.5 μg dose) the number of source contacts increased to two out of 13 insects tested. This was significantly more than with the control treatment ($P = 0.011$). The number of approaches was significantly greater than the control ($P = 0.0001$) and comparable with the 3-component blend plus (+)-limonene.

7.3.6 Bioassay Series 6: Behavioural responses of female *H. armigera* to different doses of a synthetic blend of volatiles identified from cut flowers of *T. erecta*

The 5-component blend containing benzaldehyde, linalool, phenylacetaldehyde, limonene and piperitone in the same ratio as the air entrained natural sample used in Series 3 was tested at the same concentration as in the natural sample, at a tenth and at ten times the concentration (Table 7.10).

Table 7.10 Treatments in Bioassay Series 6.

Treatment	Material tested (µg) (50µl CH_2Cl_2 on filter paper)					No. of Replicates	Mean No. of Source Contacts
	Phenyl-acetaldehyde	Benzaldehyde	(±)-Linalool	(+)-Limonene	(-)-Piperitone		
Control	0	0	0	0	0	14	0.07
0.1x dose	0.023	0.05	0.073	0.86	1.44	18	0.06
1x dose	0.23	0.5	0.73	8.6	14.4	15	0.13
10x dose	2.3	5.0	7.3	86.0	144.0	16	0

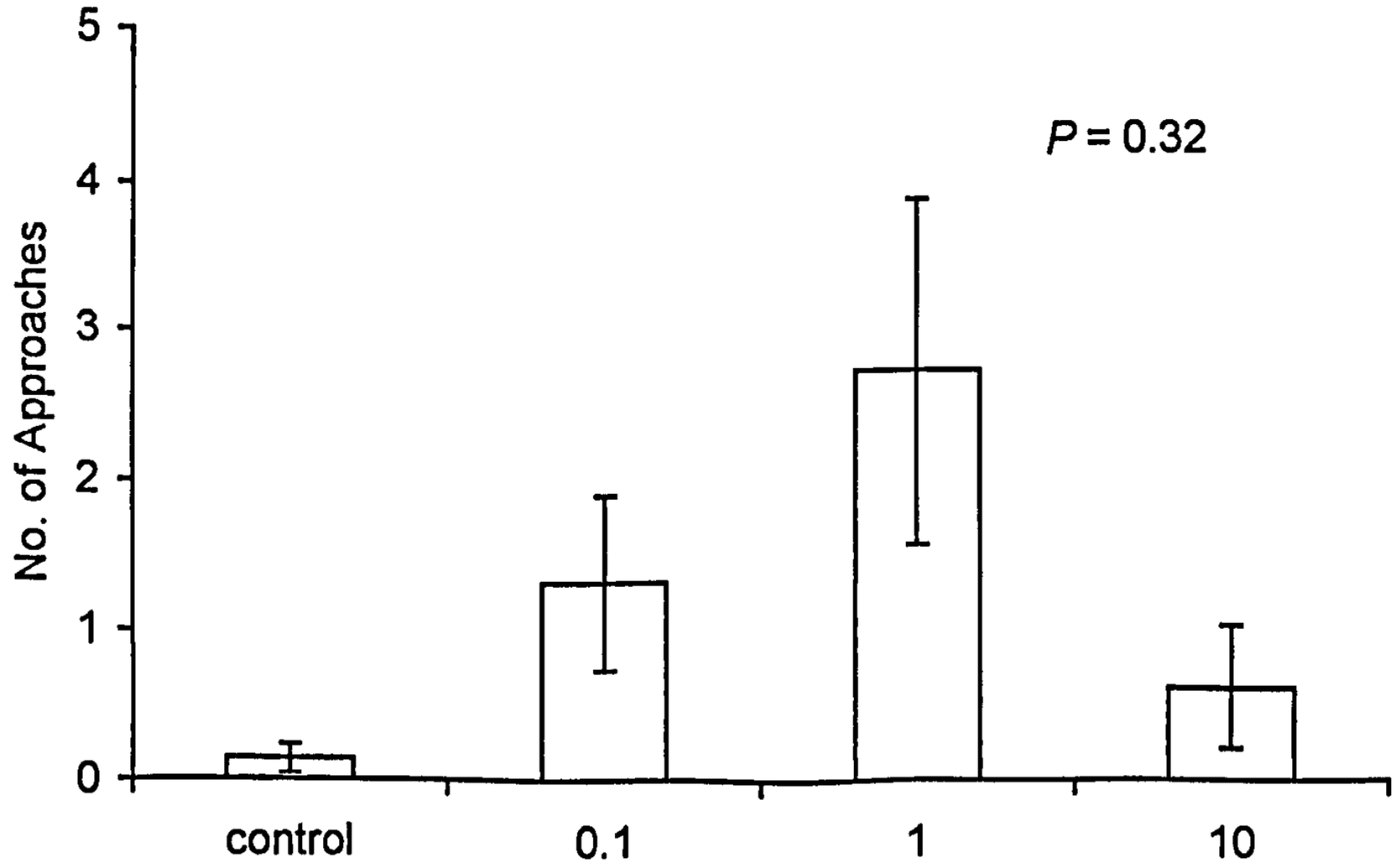

Fig. 7.6 Mean upwind approaches of female *H. armigera* to different doses of a standard synthetic blend of *T. erecta* volatiles (P value is for comparison of medians of all treatments in the series by Kruskal-Wallis test).

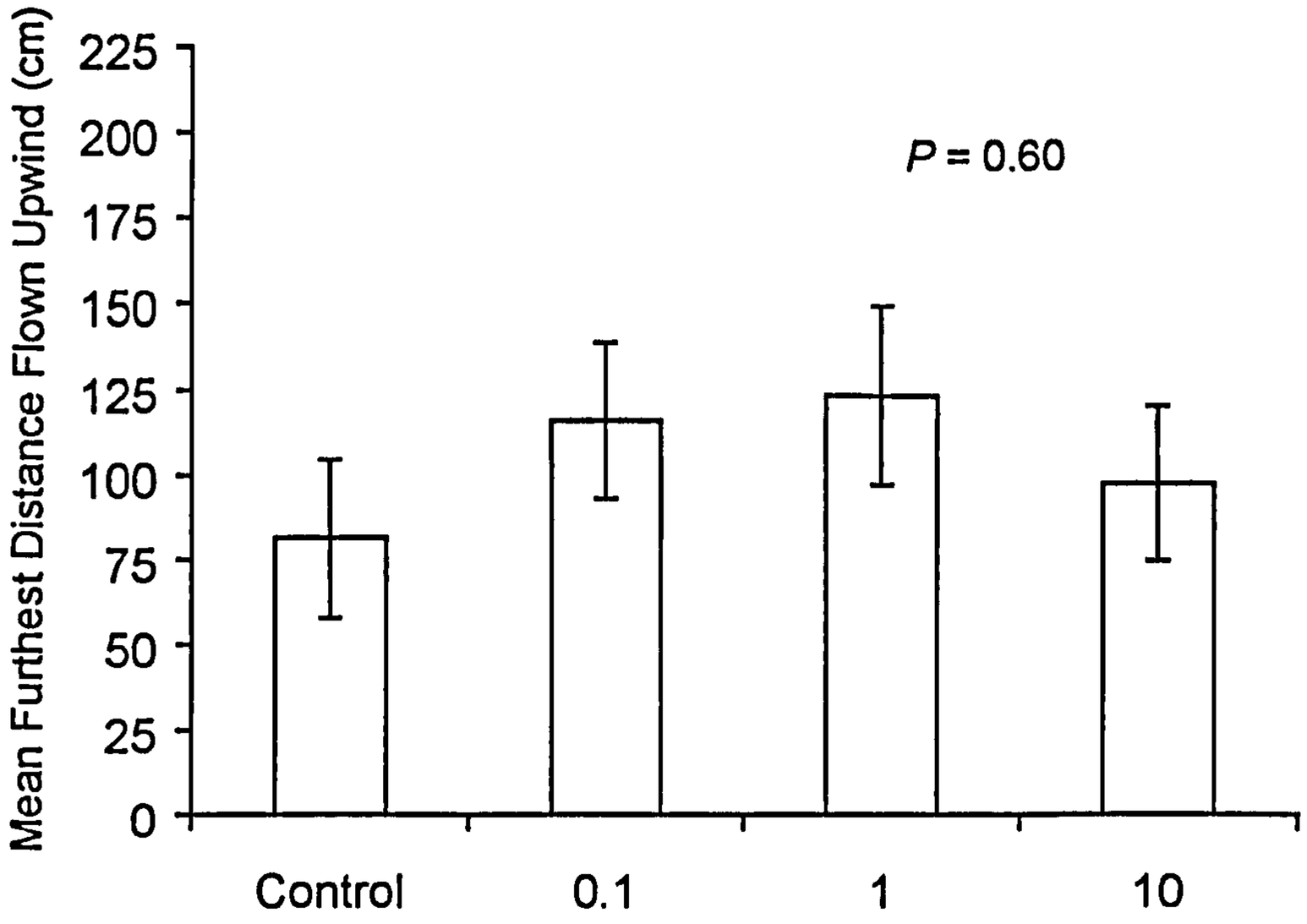

Fig. 7.7 Furthest flown upwind by female *H. armigera* to different doses of a standard synthetic blend of *T. erecta* volatiles (P value is for comparison of medians of all treatments in the series by Kruskal-Wallis test).

The dose of the 5-component blend containing (+)-limonene and (-)-piperitone used in Series 5 elicited more upwind approaches to the source than either one-tenth or ten times the dose in Series 6 although differences were not significant (Fig. 7.6). There was little difference between doses in furthest distance flown upwind (Fig. 7.7). Thus, it appeared that increasing dose might have reduced rather than improved attraction to the lure. Indeed Cardé & Elkinton (1984) suggested that semiochemical release rates that were higher than natural levels could cause arrestment of upwind flight near to an odour source.

7.3.7 Bioassay Series 7: Behavioural responses of female *H. armigera* to floral volatiles from an uncut live flower of *T. erecta* and synthetic blends of floral volatiles identified from flowers of *T. erecta*

This series was conducted using the ratio of components occuring in an air entrainment (Tag-WT2) of live *T. erecta* flowers which were still attached to the plant. Thus, the dose used in the wind-tunnel could be expressed in terms of flower equivalents because the release rate from live (uncut) flowers was known. Since the enantiomeric composition of compounds in the natural samples had been established the compounds used in the synthetic blends were enantiomerically correct. Also the natural (*E*)-myroxide had been purified (Section 5.2.1) and was available for bioassay without contamination from the (*Z*)- geometric isomer.

A sample of *T. erecta* floral headspace volatiles collected by air entrainment of a live uncut flower was tested along with 6- and 7-component synthetic blends containing compounds in the same ratio and the same enantiomeric and isomeric composition (Table 7.11). As the relative proportions of *T. erecta* floral volatiles were found to vary in the collections made from live flowers, that were analysed by GC, one entrainment sample was selected for bioassay that contained a typical ratio of constituent compounds. An aliquot equivalent to 0.4 flower hours (60µl) was tested against the same volume of a 7-component blend of synthetic compounds containing the same ratio and concentration of EAG-active components and a 6-component blend in which (*E*)-myroxide was omitted was also tested.

Table 7.11 Treatments in Bioassay Series 7

Treatment	Material tested (µg) (in 60µl CH_2Cl_2 on filter paper)							No. of Replicates	Mean No. of Source Contacts
	phenylacetaldehyde	benzaldehyde	(±)-linalool	(-)-limonene	(Z)-ocimene	(E)-myroxide	(-)-piperitone		
Control	0	0	0	0	0	0	0	30	0.033
Natural Porapak Q Sample (Tag-WT2)	60 µl of natural air entrained sample (0.4 flower hours)							30	0
6-component synthetic blend	2.59	0.59	0.41	0.96	0.21	0	1.6	30	0.13
7-component synthetic blend	2.59	0.59	0.41	0.96	0.21	0.82	1.6	30	0.10

$P = 0.006$

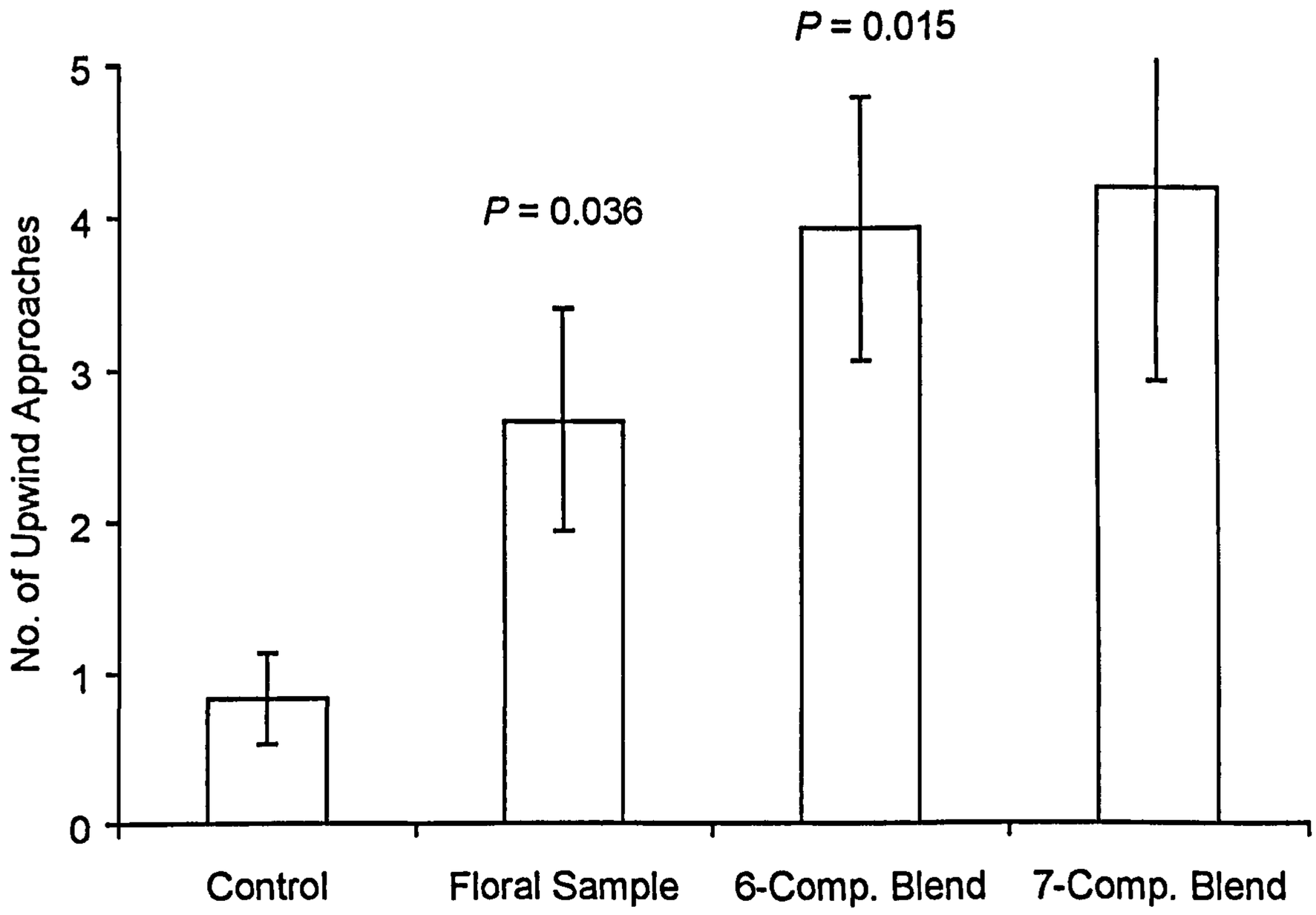

Fig. 7.8 Mean upwind approaches of female *H. armigera* to a natural *T. erecta* sample and synthetic blends of *T. erecta* volatiles, with enantiomerically correct composition and ratio as emitted by live flowers (P values are for comparison of treatment and control medians by Mann Whitney 'U' test)

The extract prepared from floral volatiles collected from a live flower of *T. erecta* elicited significantly more upwind approaches to within 20 cm of the lure from female *H. armigera* compared to the solvent control ($P = 0.036$) (Fig. 7.8). The 6- and 7-component blends of synthetic EAG-active compounds also gave increases in number of upwind approaches ($P = 0.015$ and $P = 0.006$ respectively) to the natural floral volatiles (Fig. 7.14). There was no significant difference between the number of upwind approaches elicited by the natural floral collections and the 6-component and the 7-component synthetic blends. Both synthetic blends gave more than one source contact although none were observed with the natural sample.

The mean furthest distance flown upwind to the solvent control, natural volatile collection, 6-component and 7-component blend were 143cm, 171cm, 171cm and 173cm respectively, although these were not significantly different (Kruskal-Wallis test, $P = 0.25$). The dose used was equivalent to 0.4 flower-hours emission. Since the bioassay lasted 12 minutes, the dose represented the emission from 2 *T. erecta* flowers during that time, assuming all volatiles were released from the filter paper.

7.3.8 Bioassay Series 8: Behavioural responses of female *H. armigera* to synthetic blends of volatiles based on *T. erecta* flowers

Series 8 used the same 6-component blend as in Series 7. The 7-component blend with myroxide was not used because there was no significant difference in the level of attraction to it compared with the 6-component blend in Series 7. Also tested were six 5-component blends with one compound from the 6-component blend removed from each (Table 7.12).

Table 7.12 Treatments in Bioassay Series 8

Treatment	Material tested (µg) (in 60µl CH_2Cl_2 on filter paper)						No. of replicates	Mean No. of Source Contacts
	Phenylacetaldehyde	Benzaldehyde	(±)-Linalool	(-)-Limonene	(Z)-Ocimene	(-)-Piperitone		
Control	0	0	0	0	0	0	33	0
6-component synthetic blend	2.59	0.59	0.41	0.96	0.21	1.6	32	0.06
6-component minus limonene	2.59	0.59	0.41	0	0.21	1.6	32	0
6-component minus ocimene	2.59	0.59	0.41	0.96	0	1.6	33	0.09
6-component Minus piperitone	2.59	0.59	0.41	0.96	0.21	0	33	0.10
6-component minus benzaldehyde	2.59	0	0.41	0.96	0.21	1.6	33	0
6-component minus Phenylacetaldehyde	0	0.59	0.41	0.96	0.21	1.6	34	0.03
6-component minus linalool	2.59	0.59	0	0.96	0.21	1.6	29	0

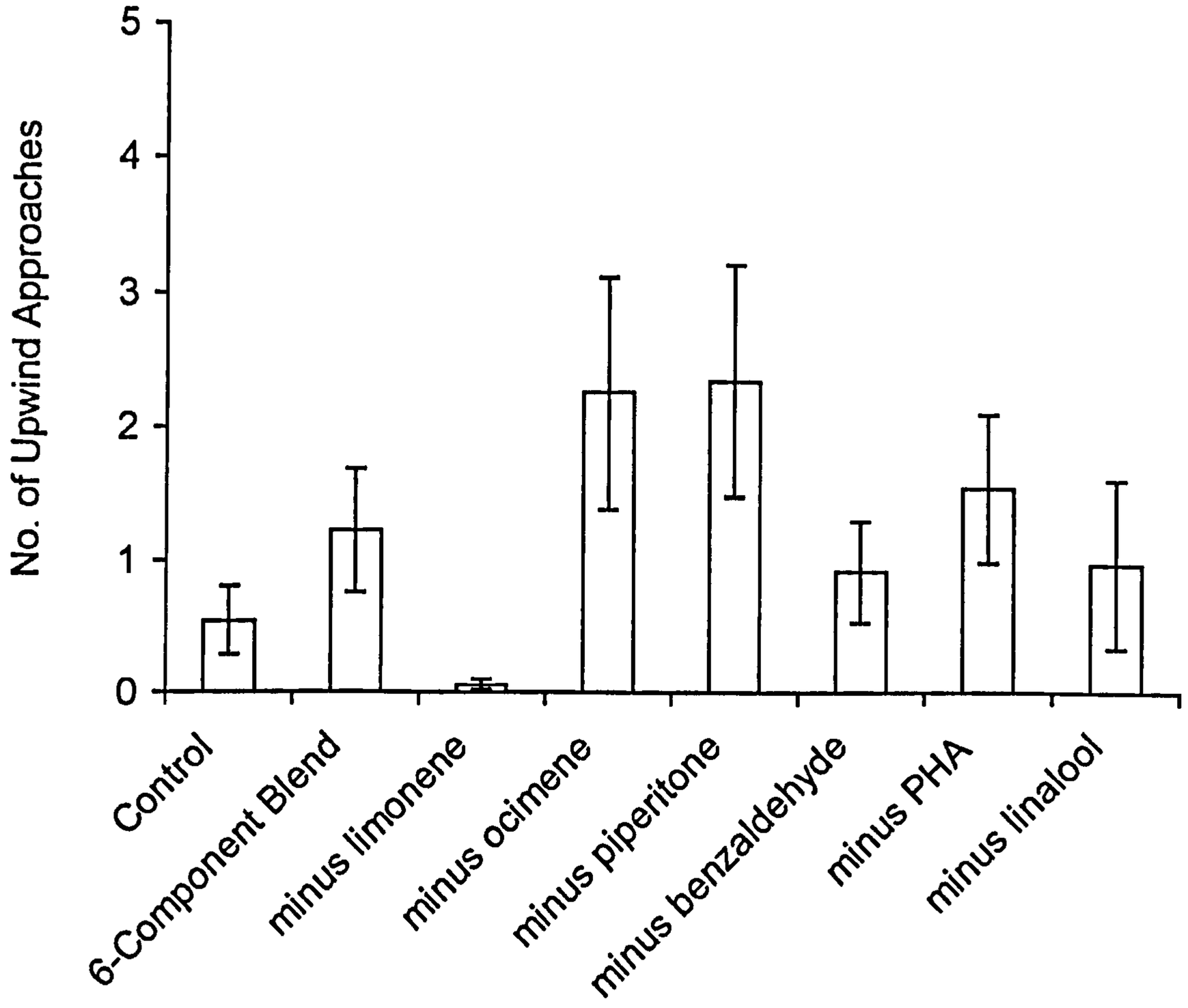

Fig. 7.9 Mean upwind approaches of female *H. armigera* to a full and to reduced component synthetic blends of *T. erecta* volatiles

It was shown (Fig 7.9) that when (-)-limonene was omitted from the 6-component blend (based on live flowers) there was a significant reduction in number of approaches compared to the 6-component blend (P=0.030, Mann Whitney 'U' test). However, any one of the other five components could be omitted without significantly affecting the number of upwind flight approaches (P>0.25) (Fig. 7.25). This implied that (-)-limonene was an essential component of the 6-component synthetic blend of compounds identified from floral volatiles of *T. erecta*.

7.3.9 Bioassay Series 9: Behavioural responses of female *H. armigera* to a 3-component synthetic blend based on floral volatiles identified from flowers of *T. erecta* and phenylacetaldehyde presented alone

A simplified 3-component synthetic blend of volatiles from *T. erecta* was tested. (-)-Limonene was used because it had been shown to be important in previous bioassays (Series 8). Phenylacetaldehyde and benzaldehyde were included because of reports in the literature of their attractiveness to moth species (Cantelo & Jacobson, 1979; Haynes *et al.*, 1991; Heath *et al.* 1992). The three compounds were presented to female *H. armigera* in the same ratio as they were found in the entrainments of floral volatiles from live flowers of *T. erecta* used in Series 7 (Table 7.13). The dose of phenylacetaldehyde used on its own was the same as that as the total dose of the 3 components. It was tested to investigate whether the mixture of three compounds was more effective than one compound presented alone.

Table 7.13 Treatments in Bioassay Series 9

Treatment	Material tested (μg) ($60\mu l$ CH_2Cl_2 on filter paper)			No. of Replicates	Mean No. of Source Contacts
	Phenylacetaldehyde	Benzaldehyde	(-)-Limonene		
Control	0	0	0	21	0
3-component blend	2.59	0.59	0.96	21	0.07
Phenylacetaldehyde	5.0	0	0	11	0

The three-component blend of compounds identified in floral volatiles identified from live flowers of *T. erecta*, benzaldehyde, phenylacetaldehyde, (-)-limonene, elicited significant increases in upwind flight of female *H. armigera* ($P = 0.0009$, Mann Whitney 'U' test, Fig. 7.10). There was no significant attraction to one of these components, phenylacetaldehyde, when presented on its own. Differences

between treatments in terms of furthest distance flown upwind were not significant (P = 0.10, Kruskal-Wallis test, Fig. 7.11) although the 3-component blend gave the highest value.

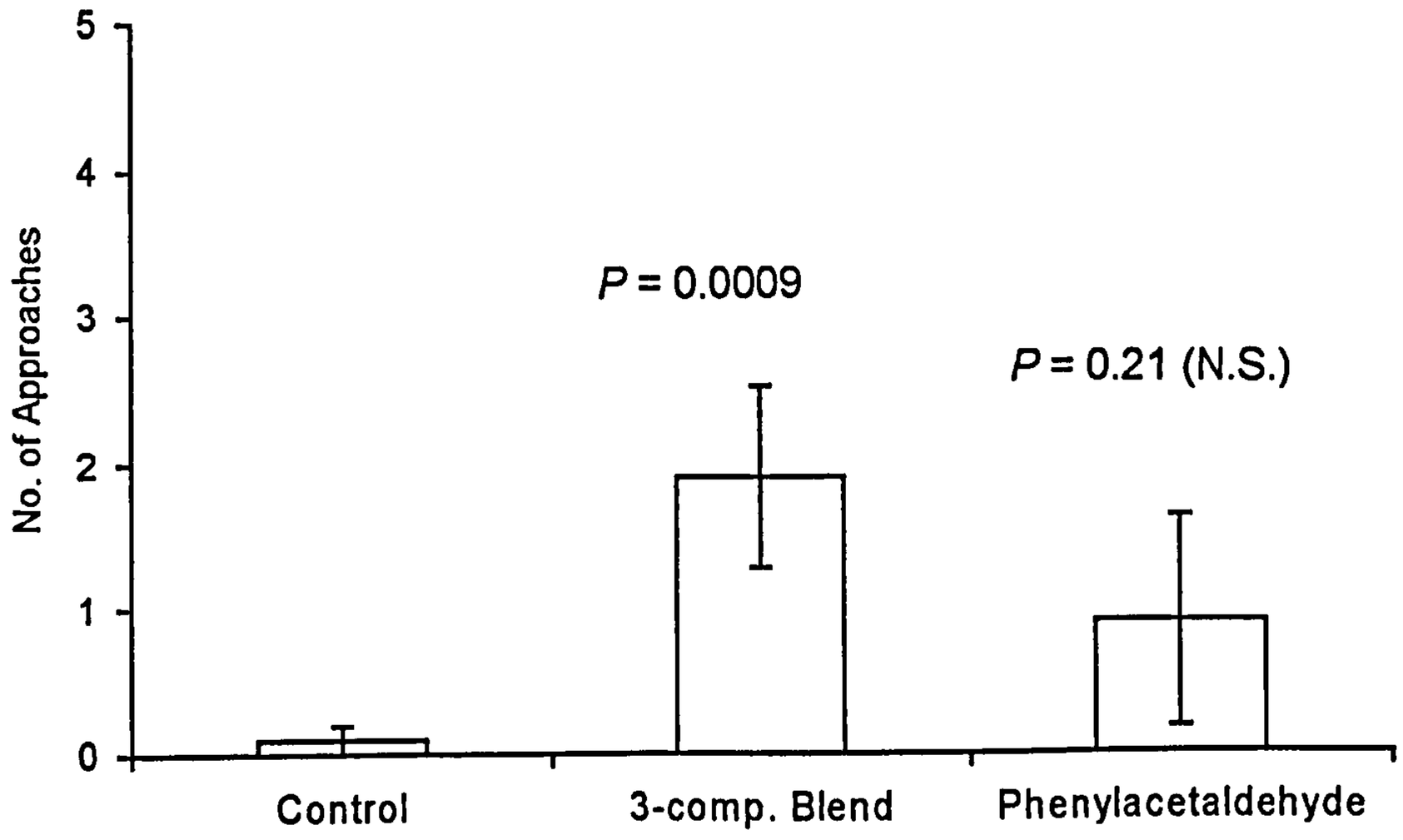

Fig. 7.10 Mean upwind approaches of female *H. armigera* to a 3-component *T. erecta* synthetic blend and phenylacetaldehyde (P values are for comparison of treatment and control medians by Mann Whitney 'U' test)

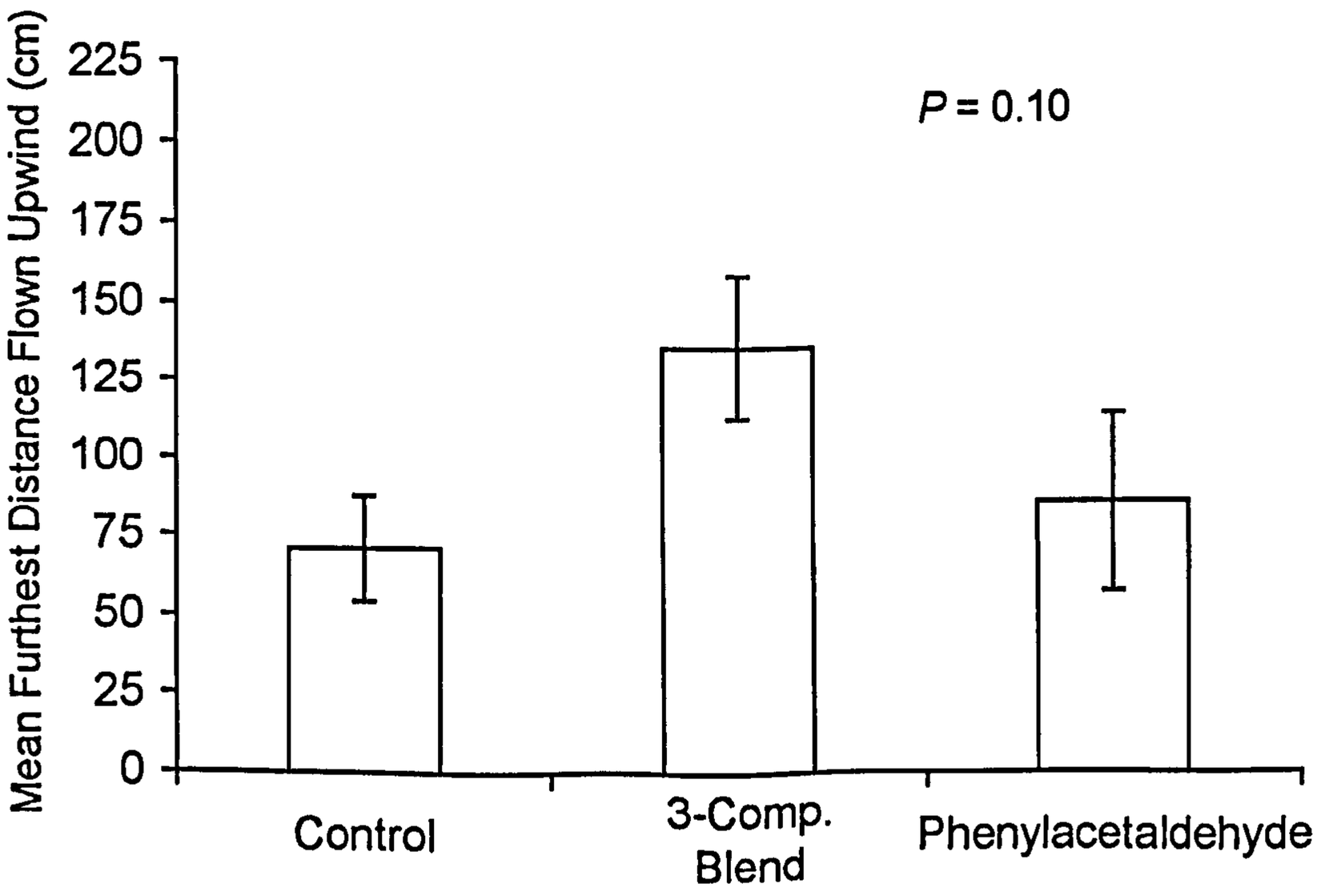

Fig. 7.11 Furthest flown upwind by female *H. armigera* to a 3-component *T. erecta* synthetic blend and phenylacetaldehyde (P value is for comparison of medians of all treatments in the series by Kruskal-Wallis test).

7.3.10 Bioassay Series 10: Behavioural responses of female *H. armigera* to odours ducted from a live *T. erecta* flower and synthetic blends of volatiles based on *T. erecta* flowers

After having carried out bioassays with synthetic blends of floral headspace volatiles on filter paper in the wind-tunnel, attraction to volatiles from a *T. erecta* synthetic blend was compared with attraction to volatiles emitted from a live *T. erecta* flower. Treatments were in a separate flask and volatile odours were piped into the wind-tunnel. The synthetic blend contained approximately the same amount of floral volatiles that would be released from one live flower over the 12 minute bioassay period.

Table 7.14 Treatments in Bioassay Series 10

Treatment	Material tested (µg) ($60\mu l$ CH_2Cl_2 on filter paper)						No. of Replicates
	Phenyl-acetaldehyde	Benzaldehyde	(±)-Linalool	(Z)-Ocimene	(-)-Limonene	(-)-piperitone	
Synthetic	2.59	0.59	0.41	0.21	0.96	1.6	43
Natural	One live *T. erecta* flower						61

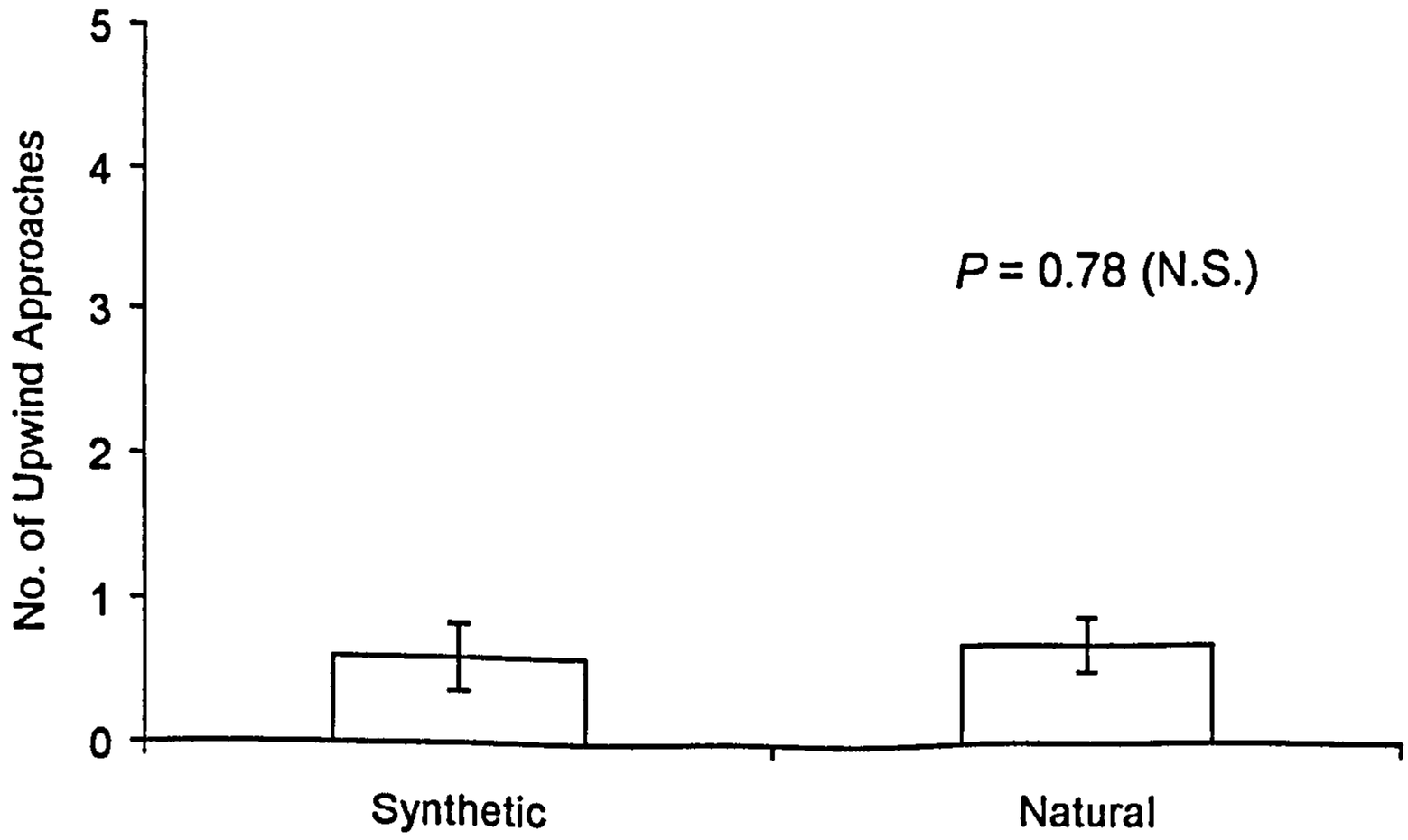

Fig. 7.12 Mean upwind approaches of female *H. armigera* to a 6-component blend of *T. erecta* volatiles and to odours blown from a live *T. erecta* flower (P value is for comparison of treatment and control medians by Mann Whitney 'U' test)

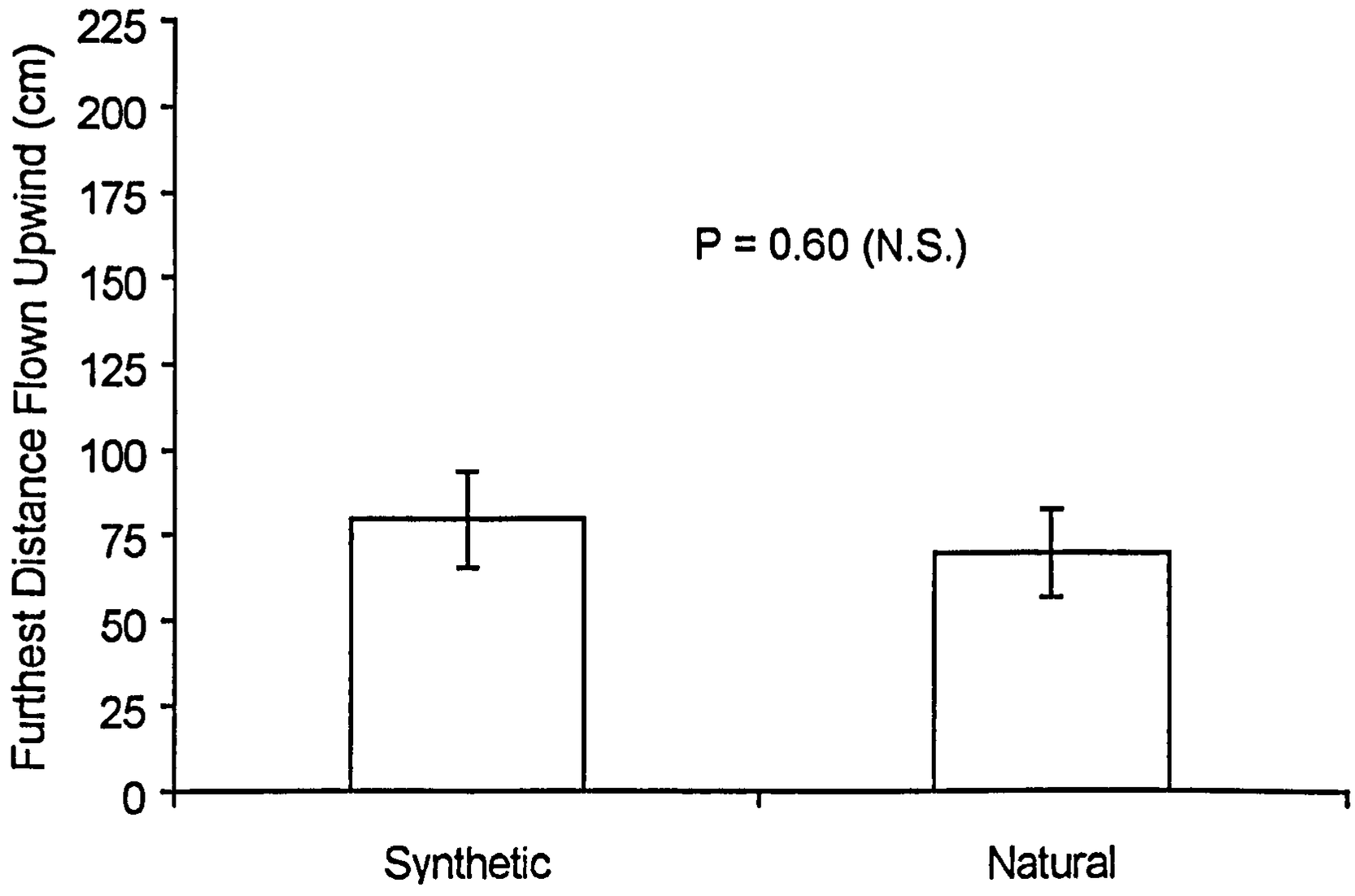

Fig. 7.13 Furthest flown upwind by female *H. armigera* to a 6-component blend of *T. erecta* volatiles and to odours blown from a live *T. erecta* flower (P value is for comparison of treatment and control medians by Mann Whitney 'U' test).

There was no significant difference in the level of attraction elicited from female *H. armigera* between the 6-component synthetic blend of chemicals and the natural floral odour released by the live *T. erecta* flower in the absence of visual cues (Figs. 7.12 and 7.13). This confirmed that the synthetic blend was an effective mimic of the natural in terms of *H. armigera* flight response to the olfactory stimuli.

7.3.11 Bioassay Series 11: Behavioural responses elicited from a Pakistani field collected strain of *H. armigera* to a synthetic blend of volatiles based on *T. erecta* flowers

Wind-tunnel bioassays Series 1 to 10 involved female *H. armigera* from the laboratory strain at NRI, originating from ICRISAT, India. In order to ascertain whether these insects had similar responses to those of field-collected insects from a location where field trials were subsequently carried out, Series 11 was undertaken with *H. armigera* collected from Multan, Pakistan. Thus, the behavioural response elicited from the synthetic blend which had elicited the highest level of upwind flight with laboratory insects was recorded. This 5-component blend contained benzaldehyde, linalool, phenylacetaldehyde, limonene and piperitone in the same ratio as the air entrained floral volatiles from *T. erecta* used in Series 3 (Table 7.15).

Table 7.15 Treatments in Bioassay Series 11

Treatment	Material tested (µg) (60µl CH_2Cl_2 on filter paper)					No. of Replicates
	Phenylacetaldehyde	Benzaldehyde	(±)-Linalool	(+)-Limonene	(-)-Piperitone	
Control	0	0	0	0	0	9
5-component blend	0.23	0.5	0.73	8.6	14.4	15

Table 7.16 Responses of female *H. armigera* (field-collected insects from Pakistan) to a standard synthetic blend of *T. erecta* volatiles (P value is for comparison of treatment and control medians by Mann Whitney 'U' test)

	No. of Upwind Approaches		Furthest Flown Upwind (cm)		Source Contacts	
Treatment	Control	5-Comp	Control	5-Comp	Control	5-Comp
Mean	0.44	2.33	109	184	0	0.07
S.E.	0.18	0.67	37.7	19.1	0	0.07
P-value		0.02		0.08		0.44

Significantly more upwind approaches were obtained with the field collected insects from Pakistan when presented with the 5-component synthetic blend compared with the solvent control (P=0.02; Table 7.16). However, the furthest distance flown upwind and the number of source contacts although apparently greater than the control were not significantly different from the control treatment ($P = 0.08$ and $P = 0.44$ respectively, Mann Whitney 'U' test)

7.3.12 Bioassay Series 12: Behavioural responses of female *H. armigera* to natural cut *L. odouratus* and *Buddleia davidii* floral headspace volatiles

The behavioural responses to volatile collections from cut *L. odouratus* and *B. davidii* flowers with female *H. armigera* were investigated. Treatments are shown in Table 7.17.

Table 7.17 Treatments in Bioassay Series 12

Treatment	Material tested (50µl CH_2Cl_2 on filter paper)	No. of Replicates	Mean No. of Source Contacts
Control	50µl CH_2Cl_2	17	0
L-98-03	50µl air entrained sweet pea, *Lathyrus odouratus.*	17	0.29
TB-98-54	50µl air entrained *Buddleia davidii.*	5	0

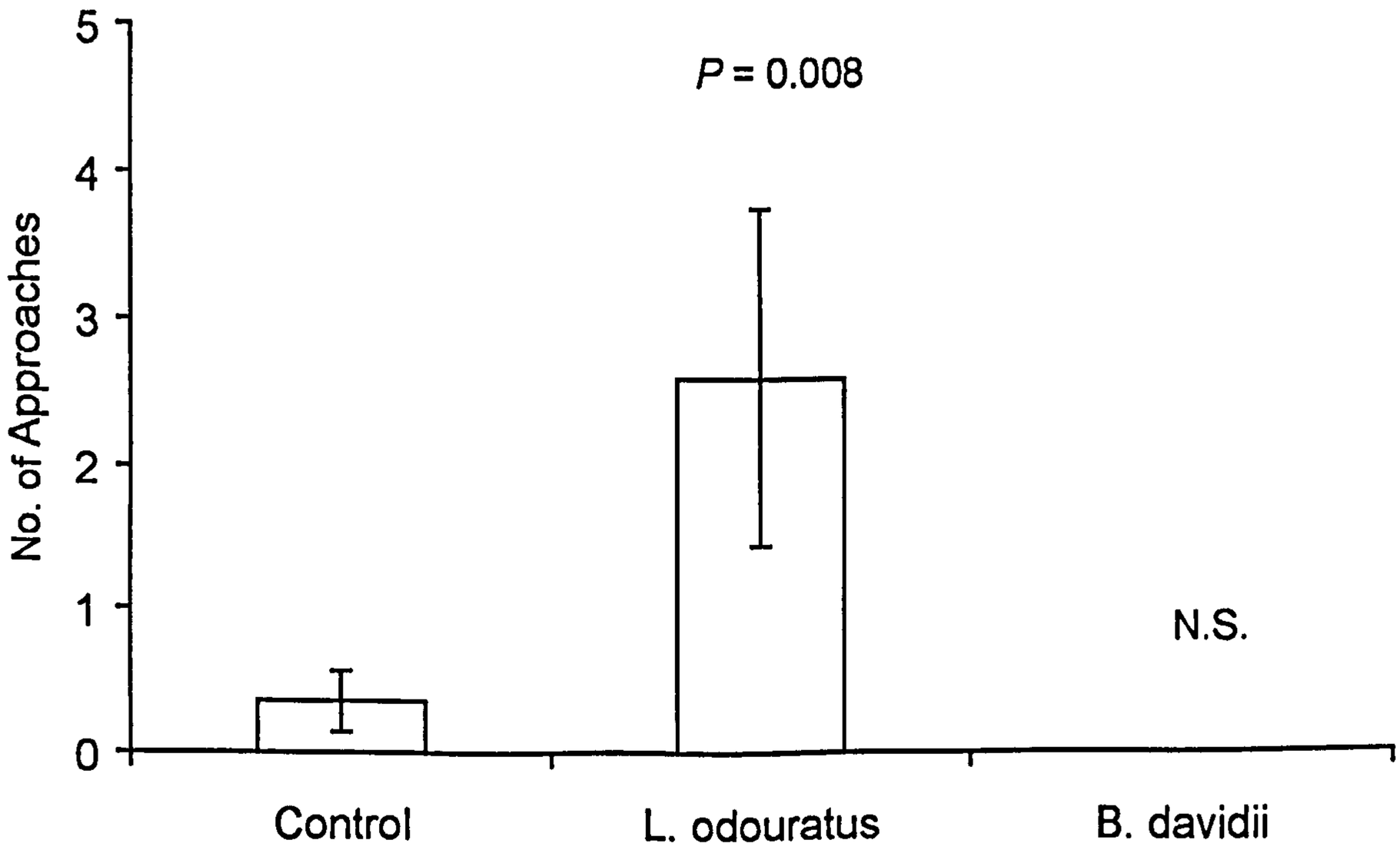

Fig. 7.14 Mean upwind approaches of female *H. armigera* to *Lathyrus odouratus* and *Buddleia davidii* floral headspace samples (P values are for comparison of treatment and control medians by Mann Whitney 'U' test)

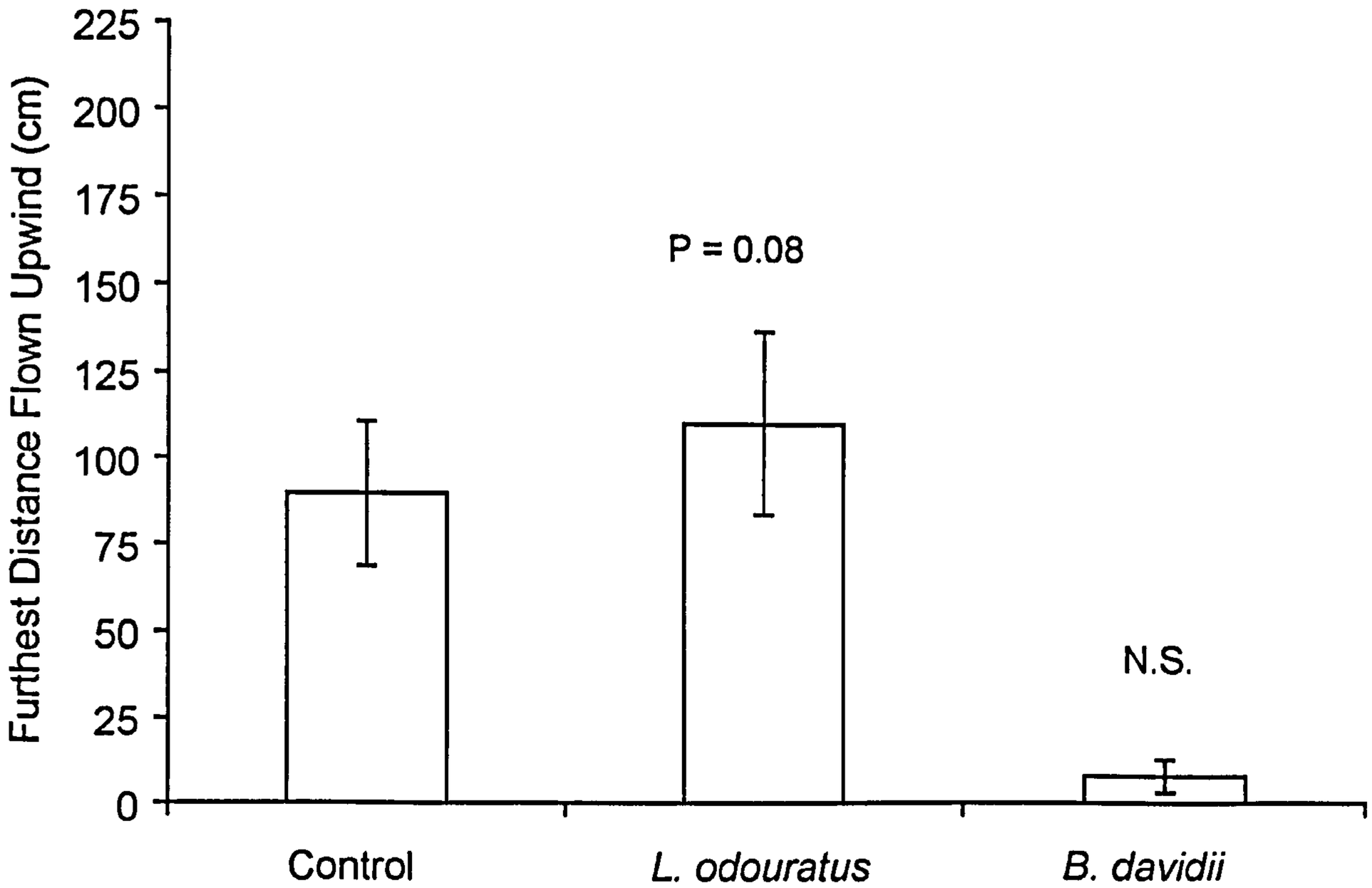

Fig. 7.15 Furthest flown upwind by female *H. armigera* to *Lathyrus odouratus* and *Buddleia davidii* floral heaspace samples (P values are for comparison of treatment and control medians by Mann Whitney 'U' test).

With the *L. odouratus* headspace sample a significant increase in number of upwind flight approaches was found, compared with the solvent control ($P = 0.036$, Fig 7.14). Furthest distance flown upwind was greater but not significantly so (Fig. 7.15). The air entrained *B. davidii* headspace sample was not attractive.

7.3.13 Bioassay Series 13: Behavioural responses of female *H. armigera* to a synthetic blend of volatiles based on *L. odouratus* flowers

A blend of EAG-active compounds identified in linked GC-EAG analyses of the floral volaties collected from *L. odouratus* were tested on female *H. armigera* in Series 13. The sources used contained the same ratio and concentration of compounds as in the natural sample (L-98-03). Treatments used are shown in Table 7.18.

Table 7.18 Treatments in Bioassay Series 13

Treatment	Material tested (µg) (60µl CH_2Cl_2 on filter paper)				No. of Replicates	Mean No. of Source Contacts
	Phenyl-acetaldehyde	Benzyl alcohol	(±)-Linalool	Diaceotne		
Control	0	0	0	0	17	0
4-component blend	3.1	0.44	3.3	5.9	18	0.08

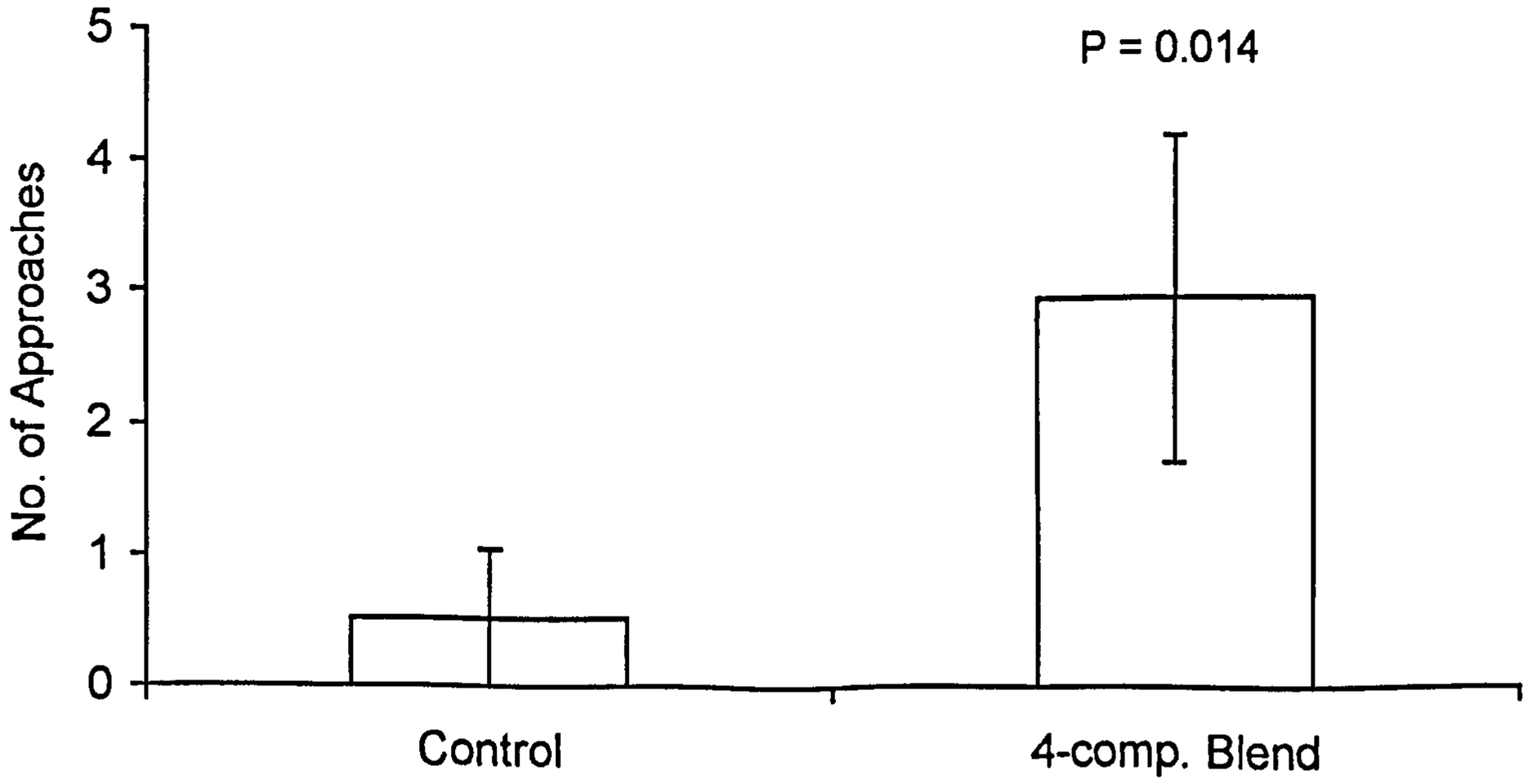

Fig. 7.16 Mean upwind approaches of female *H. armigera* to a synthetic blend of EAG-active compounds identified in *L. odouratus* flowers (P value is for comparison of treatment and control medians by Mann Whitney 'U' test)

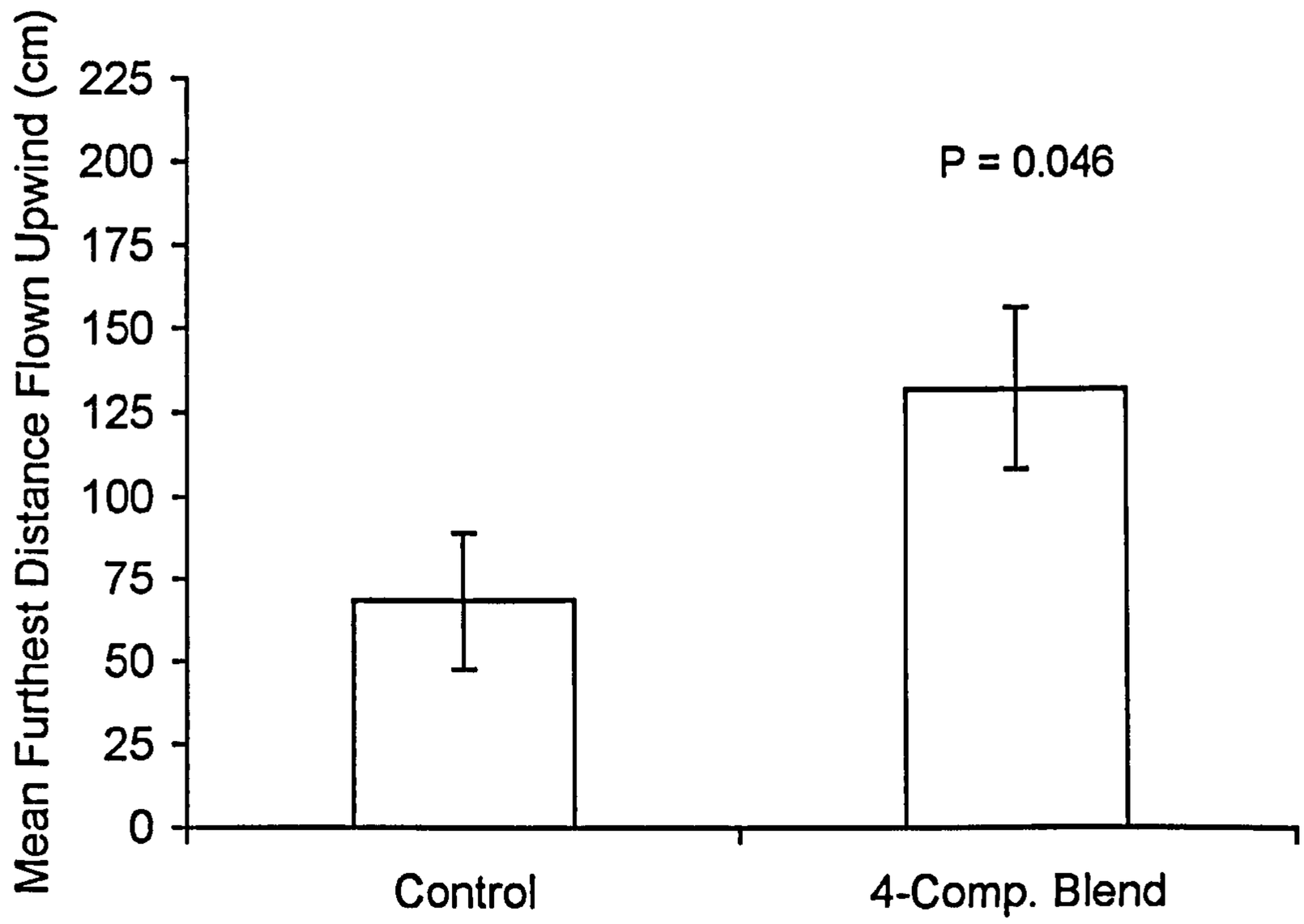

Fig. 7.17 Furthest flown upwind by female *H. armigera* to a synthetic blend of EAG-active compounds identified in *L. odouratus* flowers (P value is for comparison of treatment and control medians by Mann Whitney 'U' test).

Significant attraction in terms of number of upwind approaches in the wind-tunnel (P = 0.014, Fig. 7.16) and furthest distance flown upwind (P = 0.046, Fig. 7.17) was obtained when insects were presented with the four component synthetic blend of benzyl alcohol, (±)-linalool, phenylacetaldehyde and diacetone.

7.4 Discussion

Significant attraction to air entrained headspace samples of floral volatiles was observed indicating that these cues caused attraction when presented to female *H. armigera* in the absence of any visual cues under the conditions of the wind-tunnel experiments. This finding supported the hypothesis that volatile floral compounds play a role in the attraction of *H. armigera* to the flowers of its host plants. Collections of odours of African marigold, *T. erecta*, in Series 3 (Fig. 7.2 & 7.3), and sweet pea, *L. odouratus*, in Series 12 (Fig. 7.14 & 7.15), both contained attractive floral volatile compounds. However, a *B. davidii* headspace sample tested in Series 12 was not attractive. Unlike the other two species, *B. davidii* is not a recorded host *H. armigera* (Zalucki *et al.*, 1986).

Synthetic blends of compounds identified from the natural *T. erecta* and *L. odouratus* samples elicited significant increases in upwind flight compared with a solvent control treatment. The attractive *T. erecta* blend contained phenylacetaldehdye, benzaldehyde, (±)-linalool, (-)-piperitone and either (+)-limonene (Series 5, Table 7.9, Fig. 7.4) or (-)-limonene (Series 7, Table 7.11 and Fig. 7.8). The attractive *L. odouratus* blend contained phenylacetaldehyde, benzyl alcohol, (±)-linalool and diacetone (Series 13, Table 7.18, Fig. 7.16).

Using different doses of the 5-component *T. erecta* blend in Series 6 did not improve attractiveness. Significant increases in number of upwind flight approaches relative to the control were obtained with a reduced 3-component *T. erecta* blend comprising phenylacetaldehyde, benzaldehyde and (-)-limonene in Series 9 (Fig. 7.10). No difference was observed in upwind flight response to odours ducted from a live *T. erecta* flower and a 6-component synthetic *T. erecta* blend on filter paper in Series 10 (Fig. 7.12). Like the NRI laboratory strain of *H. armigera*, field collected *H. armigera* from Pakistan were attracted to the 5-component *T. erecta* blend (Series 11, Table 7.16).

Comparison of Response to T. erecta *Whole Cut Flowers and Headspace Samples*

There were more upwind approaches in the wind-tunnel to the volatiles in the headspace samples of *T. erecta* flowers in Series 3 (Fig. 7.2) than to the actual flowers themselves in Series 2 (Table 7.4). This appeared to be because the moths settled on the flowers in Series 2 and remained on them once they had landed. On several

occasions they were observed feeding on the flowers and they would remain on the flower for up to 6.5 minutes. With the real flower, once the insect had made source contact, it remained on the flower and initiated feeding. Because the moth was resting on the flower there was no significant increase in number of upwind flight approaches. The only significant behavioural response to actual cut flowers was for source contacts ($P = 0.018$, Table 7.4). The median distance flown upwind would have been expected to be more with the cut flowers present. A possible explanation for this could be that the emission rate of volatiles was lower from the cut flower than with the headspace sample spotted onto filter paper. With the headspace samples of floral volatiles moths flew upwind and when they failed to reach a flower and had flown past the odour source they turned around, flew back downwind and attempted to fly upwind to the floral odour again. With volatiles on filter paper source contacts were brief, typically 2 seconds, and insects resumed a seeking flight after contacting the odour source.

Relationship Between EAG Activity and Behavioural Activity

In Series 5 (Fig. 7.4 & 7.5) (+)-limonene enhanced the response to the *T. erecta* synthetic blend and in Series 8 (Fig. 7.9) (-)-limonene was found to be an essential component of the blend. This was surprising because no EAG response was observed to a 1μg dose of (+)-limonene in direct EAG tests (Table 6.2) or in linked GC-EAG analyses (Chapter 4). However, in dose-repsonse EAG studies (Fig 6.3) it was found that higher doses of (+)-limonene (in excess of 3 μg) gave a significant EAG response. Enantiospecific GC analyses carried out subsequently (Table 5.4) showed that only (-)-limonene not (+)-limonene was present in the natural *T. erecta* samples. (-)-Limonene was EAG-active at a dose of 1μg (Table 6.4), although less so than most other synthetic floral compounds tested.

Rojas (1999) carried out an investigation of cabbage moth, *Mamestra brassicae*, electrophysiological and behavioural responses to plant volatiles in which they found that allyl isothiocyanate elicited upwind flight and landing despite not having stimulated EAG responses at the doses tested. Conversely Rojas (1999) found that the EAG-active compound 4-pentyl isothiocyanate was a less effective attractant in the wind-tunnel. Thus, it appears that for plant volatiles EAG responses are not directly correlated with the behavioural activity of the compounds eliciting them. The

explanation offered by Rojas (1999) was that there could be smaller numbers of chemo-receptors, and thus smaller EAG responses, for some specialised olfactory cells which are behaviourally important. If this is the case then the subsequent processing of stimuli from chemo-receptors in the nervous system becomes an important factor in determining the behavioural response. Another explanation is that the low EAG reponse observed with (-)-limonene was due to its higher volatility than the other compounds used in synthetic blends of floral volatiles (pers. com., Prof David Hall; see Chapter 8, Table 8.3). Blaney and Simmonds (1990) found a correlation between presence of limonene (enantiomer unspecified), amongst other volatile compounds, and favourability for oviposition by *H. armigera* with crop legumes and their wild predecessors (see p.41).

Use of Wind-Tunnel Bioassays to Test Insect Responses to Host Odours

Wind-tunnels enable investigation of insect behavioural responses to host-plant odours under controlled conditions. Haynes & Baker (1989) compared anemotactic flight in female navel orangeworm, *Amyelois transitella* moths stimulated by host odour to males stimulated by the sex pheromone. The females showed similar upwind anemotactic flight and casting behaviour after plume loss but female responses were slower than male ones. Baker & Linn (1987) used a wind-tunnel to demonstrate upwind flight of *A. transitella*, in response to almond oil. Tingle & Mitchell (1992) showed a preference of *Heliothis virescens* for landing on cloth dispensers treated with floral extracts of cotton compared with control dispensers treated with solvent using a wind-tunnel bioassay. Haynes *et al.* (1991) carried out wind-tunnel bioassays which showed attraction of cabbage looper moths, *Trichoplusia ni*, to a synthetic blend of volatiles, phenylacetaldehyde, benzaldehyde, 2-phenyethanol and benzyl alcohol, identified from flowers of *Abelia grandifloria*. Heath *et al.* (1992) also used a wind-tunnel to show attraction of *T. ni* to a synthetic blend of volatiles identified from flowers of night-blooming jessamine, *Cestrum nocturnum*. Rembold *et al.* (1991) found increased upwind flight compared with the solvent control by *H. armigera* in response to volatiles identified from chickpea, *Cicer arietinum*, seed, pentan-1-ol, Δ^3-carene, myrcene and α-pinene. Hartlieb & Rembold (1996) found similar increases in upwind flight in response to pigeonpea, *Cajanus*

cajan, volatiles, β-caryophyllene, α-humulene, α-guajene, α-muurole, γ-muurole and α-bulnesene.

Comparison with the blank solvent control treatments typically used in wind-tunnel bioassays might be unrealistic and statistically significant behavioural responses obtained in the laboratory cannot be assumed to occur in the field. Despite this the positive results in wind-tunnel bioassays such as the ones described above provide evidence for the recognition of the presence of host-plants using olfactory cues is possible. Field trapping experiments were conducted (see Chapter 9) to corroborate wind-tunnel bioassay results.

Chapter 8

DEVELOPMENT OF A CONTROLLED RELEASE FORMULATION

8.1 Introduction

Having elicited significant increases in upwind flight response from female *H. armigera* in wind-tunnel bioassays with natural and synthetic blends of *T. erecta* and *L. odouratus* floral volatiles, the next step in a behavioural context was to test whether they could be used as the basis for a lure in traps. However, before that could be done it was necessary to develop controlled release formulations that would protect and release the synthetic compounds in a predictable manner under field conditions that would be suitable for use in traps.

Release rates from live flowers was established by air entrainment of the floral volatiles. Release rate data from synthetic blends of bio-active compounds on filter paper used in wind-tunnel bioassays was obtained by GC analysis of residues and by collection of volatiles by air entrainment.

A suitable formulation that would give a sustained high release rate of the identified volatile compounds was required. Polyethylene sachets were chosen because they provided a constant controlled release rate of the volatiles contained in them (Torr *et al.*, 1997). They are a type of formulation described as a reservoir system (Leonhardt, 1990) in which the release rate is controlled by the outer membrane. Release rate is determined by the diffusivity and solubility of the compound in the membrane and the surface area and thickness of the membrane (Weatherston, 1989). Release rate is initially zero order being independent of the concentration of compounds remaining in the sachet although this changes as the sachet is exhausted.

It was considered that a higher release rate than with pheromone lures would be needed in the field because the moth is less sensitive to floral volatiles than sex

pheromones (Hsiao, 1985). The release rate from sachets is higher than from the natural rubber septa and polyethylene vials typically used for female sex pheromones due to their thin, permeable walls. Weight losses from polyethylene sachets of various sizes and wall thickness hung in a wind-tunnel were recorded allowing calculation of their release. Thus, performance of sachet formulations loaded with *T. erecta* and *L. odouratus* floral volatiles in terms of release rate and longevity of release were characterised under controlled laboratory conditions prior to use in the field.

8.2 Methods and Materials

8.2.1 Determination of Release Rates from Filter Papers used in Bioassays

Air entrainment and solvent extraction methods were used to measure the release rates of floral volatiles from filter papers (Whatman No. 1PS, 7.0cm diameter) used in the wind-tunnel bioassay. Release rates calculated by these two methods were then compared.

Air Entrainment

Firstly, filter papers were air entrained using the apparatus described in Chapter 3. A 50 µl aliquot of a four-component synthetic blend, containing benzaldehyde, linalool, phenylacetaldehyde and limonene (125µg of each) was spotted onto a filter paper and the filter paper air entrained for 12 min. at 500 ml/min and 25 °C. Tetradecyl acetate (14:Ac 1µg) was added to the extract prepared from the Porapak Q filter as an internal standard. GC analysis was carried out to determine the amount of compound released on a polar GC column (CPWax52CB, 30 m x 0.32 mm ID; temperature held at 50°C for 2min, then programmed to 250°C at 7°C per min).

Solvent Extraction

Secondly, filter papers were exposed in the wind-tunnel that was used for the bioassays, extracted in solvent and analysed by GC with an internal standard to determine the proportion of starting material remaining and hence the amount lost and the release rate. This method served as a check on the efficiency of the air entrainment

method for calculating release rates to ensure that the differences in conditions between the air-entrainment apparatus and the wind-tunnel did not substantially affect release rates. Higher doses of synthetic compounds than in the bioassays were used for this release rate study to enable accurate determination of compounds in the sample by GC. Benzaldehyde, (±)-linalool, phenylacetaldehyde, (-)-limonene and (-)-piperitone in a 1 : 1 : 1 : 1 : 1 ratio (by weight) were added to make a solution in dichloromethane of total concentration 10 mg/ml. Aliquots (50µl) of this solution were then used which was equivalent to 100µg of each component.

The filter papers dosed with 50µl aliquots of the synthetic mixture were exposed in the same wind-tunnel and under the same conditions as was used for the bioassays for 10 min. Filter papers were immediately collected and placed individually in sample tubes with a screw top (Fisher, TUL 520-071V, 21cm^3 specimen tube). Dichloromethane (4 ml) was added, containing 14:Ac (100µg) as an internal standard and the papers were extracted overnight. The resulting extract was assayed by GC analysis using conditions described above in order to calculate the amount lost in the wind-tunnel per unit time. Four replicates of each treatment were carried out for both the exposed and unexposed filter papers, as controls.

8.2.2 Characterisation of Release Rates from Polyethylene Sachets

To characterise the influence of sachet size and wall thickness on release rate of the putative synthetic kairomone components from polyethylene sachets a range of sachets of various dimensions were prepared. Sachets were made from rolls of 'lay-flat' polyethylene tubing (Transatlantic Plastics, Southampton, UK) of 5 cm width and three different thicknesses (37.5µ, 135µ and 255µ). The size required was measured, marked out and cut with scissors. A measured dose of pure compound was pipetted into the sachet after sealing the bottom with a heat sealing device (Audion Elektro, The Netherlands) after which the top was similarly sealed.

Recording Release Rates by Weight Loss

The release of chemicals was recorded by weighing after 1, 2, 3, 7, 14, 21 and 28 days exposure in wind-tunnels at 22 °C and 27°C (2.22 m/sec wind-speed). Dispensers were left to equilibrate overnight before the first weighing. Mass was

recorded in grams to an accuracy of 4 decimal places (Sartorius analytic balance, Model A2008, Gottingen, Germany). The exact time at which the sachet was weighed was also recorded. Graphs of weight loss against time were plotted. The time period over which release rate was constant was noted and the release rate in mg/h calculated.

Use of Dioctyl Phthalate to Slow the Release Rate of (-)-Limonene

Due to the high volatility of (-)-limonene, a series of sachets containing 0, 10, 40, 70, 85, 95 and 97.5 % dioctyl phthalate as a non-volatile solvent was prepared. It was anticipated that dilution of (-)-limonene with dioctyl phthalate would have the effect of reducing release rate (Torr *et al.*, 1997). Release rate was calculated as described above by periodic weighing of sachets held at constant temperature under known wind-tunnel conditions.

Release Rates of Combinations of Compounds with Similar Volatilities in a Sachet

The effect of mixing compounds on their release rates from polyethylene sachets was determined by air entrainment of volatiles released from sachets containing blends of compounds. Sachets were air entrained using the apparatus described in Chapter 3 for 12 min. at 500 ml/min and 25 °C. Tetradecyl acetate (1μg) was added to the extract prepared from the Porapak Q filter as an internal standard. GC analysis was carried out to determine the amount of compound released on a polar GC column (CPWax52CB, 30 m x 0.32 mm ID; temperature held at 50°C for 2min, then programmed to 250°C at 7°C per min).

8.3 Results

8.3.1 Determination of Release Rates from Filter Papers used in Bioassays

(i) Air Entrainment

The synthetic blend used in this analysis was significantly attractive to female *H. armigera* in wind-tunnel bioassay series 5 (Fig. 7.10 & Fig. 7.11). A filter paper with the same dose of compounds as used in the bioassay was air-entrained. The sample eluted from the Porapak Q filter was analysed by GC to determine the quantity of each compound released (Table 8.1).

Table 8.1 GC Analysis of Air Entrained Porapak Q sample of Synthetic *T. erecta* Volatiles Released from Filter Paper

Compound	Dose applied to Filter Paper (μg)	Amount of Compound released in 12 min (μg)	Release Rate (μg / h)
(-)-Limonene	8.56	8.17	40.8
Benzaldehyde	0.50	0.48	2.41
(±)-Linalool	0.73	0.31	1.54
Phenylacetaldehyde	0.23	0.17	0.86
14:Ac (1μg) [a]			

[a] Added to sample as internal standard

(ii) Solvent Extraction

The residues left on filter papers exposed in the same wind-tunnel as used for bioassays after 10 min exposure are shown in Table 8.2. The amounts released were expressed as a percentage of the initial dose and provided a relative measure of the volatility of a compound. Thus, the order of volatility was (-)-limonene > benzaldehyde > (±)-linalool > (-)-piperitone > phenylacetaldehyde.

Table 8.2 Percentage loss of volatiles from filter papers analysed by solvent extraction

Compound	Mean Amount of Compound (ng) in unexposed samples [a]	Mean Amount of Compound (ng) in exposed samples [a]	Mean % Released during 10 min exposure
(-)-Limonene	242	0.14	99.9
Benzaldehyde	201	11.04	94.5
(±)-Linalool	198	26.02	86.9
Phenylacetaldehyde	174	85.97	50.5
(-)-Piperitone	173	46.89	72.8

[a] Mean of 4 replicates

Table 8.3 Release rates of compounds from filter paper determined by solvent extraction

Compound	Dose (µg) used in wind-tunnel bioassay	% lost during 10 min exposure	µg released in 10 min	Release rate (µg /h)
(-)-Limonene	8.56	99.94	8.55	51.33
Benzaldehyde	0.50	94.51	0.48	2.85
(±)-Linalool	0.73	86.87	0.63	3.78
Phenylacetaldehyde	0.23	50.46	0.12	0.69
(-)-Piperitone	14.41	72.84	10.49	62.96

To calculate the release rate of volatiles from the 4-component synthetic blend used in bioassay series 5, the doses of compounds on filter papers used in bioassays were multiplied by the proportion lost in 10 min. to calculate the amount of synthetic released. This was converted into a release rate in units of µg / h. Release rates reflect the volatility of the compounds and the dose put on the filter paper. The order of

release rates was (-)-piperitone > (-)-limonene > (±)-linalool > benzaldehyde > phenylacetaldehyde (Table 8.3).

Comparison of Release Rates Measured by Air Entrainment and by Solvent Extraction

The different conditions of the air entrainment vessel compared to those in the wind-tunnel could have been a possible source of error in the air entrainment method. Release rates calculated by solvent extraction could possibly have been affected by the higher doses used. However, Table 8.4 shows that release rates calculated by the two methods were similar, validating the data.

Table 8.4 Comparison of release rates from filter paper determined by air entrainment and solvent extraction and live *T. erecta* flowers

Compound	Release Rate calculated by air entrainment (µg /h)	Release rate calculated by solvent extraction (µg /h)	Release rate from live *T. erecta* flower (µg/flower/h)
(-)-Limonene	40.85	51.33	0.26
Benzaldehyde	2.41	2.85	0.13
(±)-Linalool	1.54	3.78	0.06
Phenylacetaldehyde	0.86	0.69	0.39
(-)-Piperitone	n / a	62.96	1.52

Release rates from live flowers were calculated in Chapter 5 (see Table 5.9). The release rates of compounds in the synthetic blend used in Series 5 wind-tunnel bioassays were on average 65 times higher (Table 8.4). However, there was considerable variation from compound to compound because the ratio of compounds emitted from a live flower was different from the ratio of compounds emitted from cut flowers which the synthetic blend was based on.

8.3.2 Longevity of Release Rates from Polyethylene Sachets

Sachet Experiment 1: Release rates from a standard (50cm^2 surface area, 135 μ thickness) sachet

Aliquots (1 ml) of benzaldehyde, (±)-linalool, phenylacetaldehyde and (-)-piperitone were pipetted into polyethylene sachets of 50cm^2 surface area (5cm x 5cm) and 135 μ thickness. There were 2 replicate sachets for each compound except (-)-piperitone which was not available in a large enough quantity to make 2 sachets. Sachets were hung in a wind-tunnel maintained at 22^{0}C, 2.2 m/sec windspeed.

Table 8.5 Release rates of individual compounds from polyethylene sachets (5cm x 5cm x 135μ thickness, 22^{0}C, 2.2 m/sec windspeed

Compound	Time Release Rate Remained Constant (Days)	Release Rate (mg/h)	Half life (days)
benzaldehyde	15.2	3.62	11
(±)-linalool	34.9	0.42	99
phenylacetaldehyde	28.0	0.62	67
(-)-piperitone	21.0	1.4	30

As shown in Table 8.5 and Fig. 8.1, linalool and phenylacetaldehyde had similar release rates under the standard conditions used in this experiment, (-)-piperitone had an approximately threefold higher release rate, and benzaldehyde had the highest release rate, approximately ten times the release rate of (±)-linalool. Release rates from sachets with benzaldehyde remained uniform for 15 days while release rates from sachets with (±)-linalool, phenylacetaldehyde and (-)-piperitone remained uniform for 35, 28 and 21 days respectively. The graphs (Fig. 8.1) show cumulative release over time and flatten out as the sachets became exhausted. Longevity of release could be changed by using different sachet loadings because it depends on the quantity of material in the sachet, unlike release rate which depends on sachet dimensions, temperature and humidity.

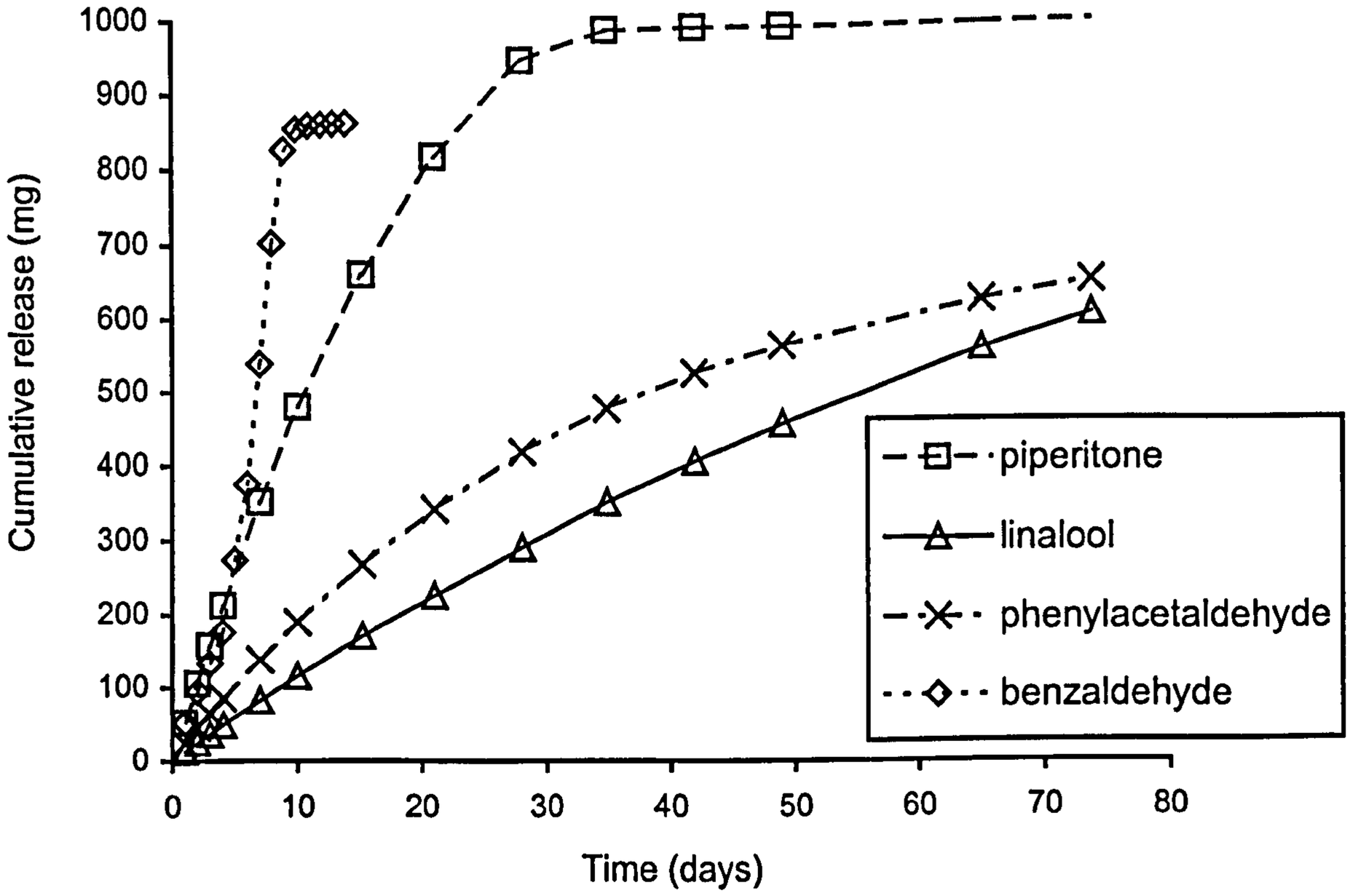

Fig 8.1 Sachet Release Rates in Experiment 1. Polyethylene sachets of 50 cm^2 surface area (5 x 5 cm) and 135 μ thickness loaded with 1 ml of compounds at 22°C.

Sachet Experiment 2: To investigate the effect of varying temperature and sachet dimensions on release rate

Aliquots (1 ml) of four compounds, benzaldehyde, (±)-linalool, phenylacetaldehyde and (-)-limonene, were pipetted individually into polythene sachets of varying surface area and thickness. Sachets were maintained at 22°C and 27°C. At 22°C three different sachet surface areas (25 cm^2, 50 cm^2 and 100cm^2) and at 27°C three thicknesses (37.5μ, 135μ and 255μ) were used. Each of the treatments was replicated twice.

Benzaldehyde

Changing temperature from 22°C to 27°C caused the release rate to almost double for most sachets containing benzaldehyde (Table 8.6), except the 100cm² area, thin sachet which already had the highest release rate and unaffected by the change in temperature. At 22°C doubling sachet surface area resulted in an approximately twofold increase in release rate although the effect was greatest with the 37.5μ sachet thickness. At 27°C doubling surface area had little effect except for the 255μ sachets for which it caused a doubling of release rate. At 27°C sachets maintained a constant release rate for approximately one week except for the thin 37.5μ sachets which had a reduced release rate after only 2 days.

Table 8.6 Release rate data for different dimensions of sachets containing benzaldehyde at different temperatures

Benzaldehyde, 22°C

Sachet Thickness	Sachet Surface Area (cm^2)	Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half Life (days)
Thin (37.5μ)	25	19.0	2.4	8.7
	50	5.1	5.26	4.0
	100	2.0	11.09	1.9
Medium (135μ)	25	12.0	1.46	14.3
	50	19.0	2.11	9.9
	100	12.0	2.72	7.7
Thick (255μ)	25	12.0	0.95	21.9
	50	12.0	1.56	13.4
	100	12.0	2.54	8.2

Benzaldehyde, 27°C

Sachet Thickness	Sachet Surface Area (cm^2)	Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half Life (Days)
Thin (37.5μ)	50	1.8	10.4	2.0
	100	1.8	11.52	1.8
Medium (135μ)	50	8.8	3.96	5.3
	100	6.0	4.86	4.3
Thick (255μ)	50	13.1	2.51	8.3
	100	8.8	4.29	4.9

Linalool

As can be seen in Table 8.7, changing temperature from 22 to 27°C caused release rate to almost double for most sachets containing (±)-linalool, except the 100cm², thin sachet. Doubling sachet surface area resulted in a twofold increase in release rate at 22°C and one third increase in release rate at 27°C in most cases. Longevity of release was better than with benzaldehyde.

Table 8.7 Release rate data for different dimensions of sachets containing (±)-linalool at different temperatures

(±)-Linalool, 22°C

Sachet Thickness	Sachet Surface Area (cm^2)	Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half life (days)
Thin (37.5μ)	25	19.0	0.65	32.1
	50	19.0	0.98	21.3
	100	1.9	2.62	8.0
Medium (135μ)	25	19.0	0.14	148.8
	50	19.0	0.28	74.4
	100	19.0	0.45	46.3
Thick (255μ)	25	25.9	0.13	160.3
	50	25.9	0.22	94.7
	100	19.0	0.42	49.6

(±)-Linalool, 27°C

Sachet Thickness	Sachet Surface Area (cm^2)	Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half Life (days)
Thin (37.5μ)	50	6.0	2.08	10.0
	100	8.8	2.42	8.6
Medium (135μ)	50	26.9	0.52	40.1
	100	20.0	0.72	28.9
Thick (255μ)	50	41.1	0.43	48.4
	100	26.9	0.74	28.2

Phenylacetaldehyde

As shown in Table 8.8, increasing temperature from 22 to 27°C resulted in an approximate doubling of release rates. Increasing sachet surface area only had a small effect on release rate except for the 255μ sachets at 22°C. Longevity of uniform release was at least one week for all sachet dimensions except the 100cm² 37.5μ sachets at 27°C.

Table 8.8 Release rate data for different dimensions of sachets containing phenylacetaldehyde at different temperatures

Phenylacetaldehyde, 22°C

Sachet Thickness	Sachet Surface Area (cm^2)	Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half Life (days)
Thin (37.5μ)	25	8.9	0.53	39.3
	50	8.9	0.79	26.4
	100	12.0	0.87	23.9
Medium (135μ)	25	12.0	0.24	86.8
	50	19.0	0.3	69.4
	100	19.0	0.35	59.5
Thick (255μ)	25	19.0	0.13	160.3
	50	19.0	0.25	83.3
	100	12.0	0.33	63.1

Phenylacetaldehyde, 27°C

Sachet Thickness	Sachet Surface Area (cm^2)	Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half Life (Days)
Thin (37.5μ)	50	8.8	1.05	19.8
	100	6.0	1.61	12.9
Medium (135μ)	50	13.0	0.6	34.7
	100	8.8	0.72	28.9
Thick (255μ)	50	13.0	0.46	45.3
	100	13.0	0.48	43.4

Limonene

The results with (-)-limonene were affected by its very high volatility: the sachets were depleted to below the uniform release rate before the first weighing when they were being left to equilibrate overnight (Table 8.9). Later experiments were conducted in which a dioctyl phthalate was incorporated into the (-)-limonene sachets to retard release rate.

Table 8.9 Release rate data for different dimensions of sachets containing (-)-limonene at different temperatures

(-)-Limonene, 22°C

Sachet Thickness	Sachet Surface Area (cm^2)	Time Release Rate Remained Constant (hours)	Release Rate (mg/h)
Thin (37.5μ)	25	< 24	0.1
	50	< 24	0.17
	100	< 24	0.18
Medium (135μ)	25	< 24	1.61
	50	< 24	9.53
	100	< 24	0.71
Thick (255μ)	25	< 24	4.9
	50	< 24	9.75
	100	< 24	9.38

(-)-Limonene, 27°C

Sachet Thickness	Sachet Surface Area (cm^2)	Time Release Rate Remained Constant (hours)	Release Rate (mg/h)
Thin (37.5μ)	50	< 24	0.23
	100	< 24	0.15
Medium (135μ)	50	< 24	0.31
	100	< 24	0.49
Thick (255μ)	50	< 24	2.86
	100	< 24	1.22

Experiment 3: To investigate the possibility of reducing the release rate of (-)-limonene using dioctyl phthalate

(-)-Limonene was mixed with dioctyl phthalate in varying proportions in an attempt to slow its release from polyethylene sachets. Sachets (50cm^2 surface area, 135 μ wall thickness) were made up containing 0.5ml of 100, 90, 60, 30, 15, 5 and 2.5% (-)-limonene (the remainder of the volume being made up with dioctyl phthalate). There were 3 replicates of each of these treatments. The sachets were hung in the wind-tunnel at 27^{0}C.

Table 8.10 Release rate data for sachets (50cm^2 surface area, 135 μ wall thickness) with different loadings of (-)-limonene and dioctyl phthalate

Percent (-)-Limonene	No. of Days Over Which Release Rate Remained Constant	Release Rate (mg/h)
100	0.28	4.30
90	0.28	6.28
60	1	3.35
30	2	1.22
15	2	0.62
5	3	0.17
2.5	3	0.05

To prevent rapid depletion and loss of uniform release in less than one day at least 70% dioctyl phthalate (30% limonene) had to be added to the formulation in the sachet. Even with 97.5% dioctyl phthalate (2.5% limonene) a constant release rate was only maintained for 3 days under the conditions in the wind-tunnel (Table 8.10).

Subsequently a double layered sachet formulation was used to further slow down the release rate of (-)-limonene. A 2.5 x 2.5cm sachet loaded with formulation (0.5 ml) was placed inside a 4 x 5cm outer sachet. With 85% dioctyl phthalate (15% limonene) the release rate of limonene from the double walled sachet was 0.19mg/h which was sustained for 6 days at 27^{0}C.

Experiment 4: To investigate release rates from sachets loaded with L. odouratus *compounds*

Aliquots (1 ml) of diacetone or benzyl alcohol were pipetted into polyethylene sachets of 50cm^2 surface area (5cm x 5cm) and 135 μ thickness. There were 2 replicate sachets for each compound. Sachets were hung in a wind-tunnel maintained at 27^{0}C, 2.2 m/sec windspeed.

As seen in Table 8.12, consistent release rates for more than a week were obtained for both benzyl alcohol and diacetone. Release rates from sachets for the other two sweet pea compounds, linalool and phenylacetaldehyde, had already been investigated (Expt. 1, Table 8.5).

Table 8.12 Release Rates from Sachets (50cm^2 surface area, 135 μ wall thickness, 27^{0}C) in Experiment 4

Compound	No. of Days Over Which Release Rate Remained Constant	Release Rate (mg/h)	Half life (days)
Benzyl alcohol	9+	0.23	90
Diacetone	9+	0.31	67

Sachet Formulations for use as Trap Baits in Field Trapping Experiments

Field experiments were designed to last one week each and new sachets were used at the start of each new experiment (Chapter 9). When using the medium (135μ) and thick (255μ) sachet material uniform release rates of benzaldehyde, benzyl alcohol, diacetone, (±)-linalool and phenylacetaldehyde were obtained for at least one week (Table 8.5) which meant that these were suitable for use in the field. These compounds were added to the same sachet because they had broadly similar release rates. The release rate of (-)-limonene was much higher than the other bio-active compounds (Table 8.9) and this was formulated in a separate sachet with dioctyl phthalate for field use. The antioxidant 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) (10% of the formulate) was added to lures containing benzaldehyde and phenylacetaldehyde used in the field experiments.

T. erecta

Three of the compounds identified from flora volatiles of *T. erecta* (benzaldehyde, (±)-linalool and phenylacetaldehyde) were all added into the same sachet when used as trap baits in the field. Since the emission rate of benzaldehyde and phenylacetaldehyde from *T. erecta* flowers was a 4 : 1 ratio of phenylacetaldehyde : benzaldehyde (Table 5.10) but benzaldehyde was released more rapidly from polyethylene sachets a higher loading of phenylacetaldehyde was needed when compounds were mixed together and put in the same sachet in the field. By air entrainment of sachets and GC analysis it was found that a 40 : 1 : 1 mixture of phenylacetaldehyde, benzaldehyde and linalool gave an emission ratio of compounds similar to that of the flowers. Due to the high volatility of (-)-limonene it was decided to use a separate, double layered, sachet for it in which the plasticiser dioctyl phthalate (85%) was included. Release rates from sachets under field conditions are reported in Table 9.18.

L. odouratus

When the four *L. odouratus* compounds, benzyl alcohol, diacetone, phenylacetaldehyde and linalool, were put into a sachet in a 1 : 1 : 1 : 1 ratio it was found, by air entrainment and GC analysis, that the emission ratio was approximately the same as found with a *L. odouratus* flower.

8.4 Discussion

Emission Rate from Filter Papers

The emission rate of the 5-component synthetic blend of *T. erecta* floral volatiles from the filter paper which elicited significant increases in upwind flight response of female *H. armigera* in wind-tunnel bioassay Series 5 (Table 8.3) was calculated to be 120μg/h (2μg/min). In bioassays with a component of the sex pheromone *H. armigera*, Z11-16:Ald, Kehat & Dunkelblum (1990) were able to demonstrate attraction of male moths with an emission rate of 8.08 ng/min, 250 times less than found with the floral attractant.

Effect of Temperature and Surface Area on Release Rate from Sachets

Increasing the temperature at which sachets were exposed from 22°C to 27°C increased the emission rate of benzaldehyde, (±)-linalool and phenylacetaldehyde approximately twofold (Tables. 8.6, 8.7 & 8.8). The effect of increasing surface area was less consistent and was possibly influenced by temperature since the increase in emission rate of benzaldehyde, (±)-linalool and phenylacetaldehyde obtained by doubling surface area appeared to be larger at 22°C than at 27°C. At 22°C doubling surface area usually lead to an approximate doubling of emission rate for benzaldehyde and linalool (Figs. 8.6 & 8.7) whereas at 27°C the increase was closer to 1.5-fold. For phenylacetaldehyde sachets (Fig. 8.8) doubling the surface area lead to smaller increases in emission rate than for benzaldehyde and linalool and similarly increases were lower at 27 °C.

The effect of humidity on release rate was not determined. Within norml limits it is anticipated that the hydrophobic polyethylene would be unaffected but very high humidity or rainfall when the surface of the sachet is moist might be anticipated to affect release rate (D.R. Hall, *pers. comm.*)

Choice of Formulation for Use in Field Experiments

Release rate of attractant formulation can be critical to the biological effectiveness of a trap (Leonhardt, 1990). Sustained release rates of *T. erecta* and *L. odouratus* floral volatiles from polyethylene sachets were obtained but the very

volatile compound (-)-limonene required addition of a dioctyl phthalate to retard its release rate. Limonene was the only simple hydrocarbon tested, the other compounds all had oxygemated functionality. Limonene would be expected to be relatively more soluble in the hydrocarbon polyethylene sachet and hence have a higher release rate. The main reasons for choosing polyethylene sachets for use in field trapping experiments were that they give higher release rates than alternative formulations such as vials or septa due to their thin, permeable walls and that the polyethylene sachet formulation has the advantage of having zero order release rate kinetics (constant release rate) until the lure becomes depleted. Polyethylene vials and septa which are typically used for sex pheromone lures give first order release kinetics, the amount released declining as the lure depletes (Campion *et al.* 1978).

Chapter 9

FIELD TRIALS WITH FLORAL ODOUR-BAITED TRAPS IN ISRAEL AND PAKISTAN

9.1 Introduction

After identification of synthetic blends of volatiles that were significantly attractive in the wind-tunnel it was necessary to investigate whether this attraction could be demonstrated under field conditions using wild populations of *Helicoverpa armigera*. It was known from previous reports in the literature that female *H. armigera* are attracted to the flowering stage of host plants (Roome, 1975; Fitt, 1989) and that nectar foraging is a high a priority activity for recently emerged moths (Beerwinkle *et al.*, 1993). To test the hypothesis that olfaction plays a major role in this attraction, the field trapping experiments tested the efficacy of floral volatiles identified from host plants of *H. armigera* as trap baits. Increases in trap catches when a bait is present provides evidence of attraction (Cardé & Elkinton, 1984; Hsiao, 1985). Field experiments were thus designed to test whether the native field insects were sufficiently attracted to floral odour-baited traps to be caught in significantly higher numbers than in unbaited control traps. A controlled release formulation of floral volatile compounds using polyethylene sachets was used to make lures that were suitable for baiting funnel traps (see Chapter 8).

The field trapping experiments described here were designed not only to test whether attraction of *H. armigera* observed in the laboratory could be demonstrated under field conditions but also to test which other insects were attracted to the floral odour baited traps. Another objective of the field testing was to assess whether the traps baited with blends of floral volatiles identified in the laboratory had any potential for use in pest management.

9.2 Methods and Materials

9.2.1 Israel Field Trial

A series of field experiments was conducted at the Volcani Center, Bet Dagen, Israel, to screen different floral lures in an attempt to maximise their attractiveness to *H. armigera* and minimise attraction of non-target insects.

Experimental Sites

Traps were situated in a chickpea field (Plate 9.1) for the first month (10/5/99-31/5/99) and in a cotton field (Plate 9.2) for the second month (1/6/99-29/6/99). Both fields had a uniform crop. The chickpea, which was sown at the beginning of January, was in the podding growth stage. The cotton, which was sown at the end of April, was in the vegetative growth stage until 16/6/99 when bud development was noted. The cotton was variety "Acala" and started to flower on 23/6/99. Insecticides were applied to the crops when pest risk was perceived because the fields were being managed commercially.

Plate 9.1 Grid of Floral Odour-baited Traps in the Chickpea Field, Volcani Center

Plate 9.2 Grid of Floral Odour-baited Traps in the Cotton Field, Volcani Center

Layout of Traps in the Field

'Unitrap' cone traps (International Pheromone Systems, S. Wirral, UK; Plate 9.3) baited with floral lures were set up 50 cm above crop height. A Latin square experimental layout was used to minimise the variation in trap catch caused by trap interactions, differences in population density in different areas or by directional effects. Distance between traps was 12m in the chickpea field and 20m in the cotton field. Trap catches were recorded daily and the position of traps was re-randomised with a new Latin square layout each morning.

Plate 9.3 'Unitrap' Funnel Trap used in Field Trapping Experiments. The opening under the lid where moths enter was 3cm height.

Independent Assessment of H. armigera *Population Levels*

To obtain an independent measure of the *H. armigera* population density during the field experiments, four pheromone traps (Unitraps; Plates 9.3 & 9.4) and a Robinson portable light trap (15W ultraviolet flourescent bulb, Plate 9.5) which emitted bluish-white light were also set up. They were located in the same field as the traps baited with floral lures but approx. 60 m away from the nearest floral trap to avoid interference with the main experiment. Light and pheromone traps were 100m apart from each other.

Plate 9.4 Male *H. armigera* Caught in a Pheromone Trap (Chickpea Field, Week 1)

Plate 9.5 Light Trap by Cotton Field, Volcani Center

Controlled Release Formulations of Floral Volatiles

Compounds used in the lures, benzaldehyde, (±)-linalool, phenylacetaldehyde, (*S*)-(-)-limonene, benzyl alcohol and diacetone (4-hydroxy-4-methyl-2-pentanone), were purchased from Sigma Israel Chemicals Ltd. 2,6-Di-*tert*-butyl-4-methyl-phenol (BHT) was added (10% of the a.i.) as an antioxidant in lures that contained benzaldehyde and phenylacetaldehyde. Dioctyl phthalate (85%) was used to slow the release of (-)-limonene (15%) which had to be formulated in separate double-thickness sachets because of its high volatility (see Chapter 8). Lures were made by pipetting a known amount (usually 0.5ml) of various mixtures of compounds into polyethylene sachets cut from a roll of polyethylene tubing (500 gauge, 125µm thickness, Transatlantic Plastics, Southampton, UK) and sealed using a 'Futura' electrical heat sealing device (Audion Elektro, Holland). Representative lures from all treatments were hung in the shade and weighed daily to measure field release rates.

Treatments Used in Floral Odour Baited Traps

Five trapping experiments with floral odour-baited traps were carried out. All experiments had an unbaited control trap treatment. In Experiment 1 (10/5/99-15/5/99) lures based on floral volatiles of marigold, *T. erecta*, and sweet pea, *L. odouratus*, were tested along with a treatment combining odours from both plant sources. The *T. erecta* lures contained benzaldehyde, (±)-linalool, phenylacetaldehyde and (*S*)-(-)-limonene. The *L. odouratus* lures contained (±)-linalool, phenylacetaldehyde, benzyl alcohol and diacetone (4-hydroxy-4-methyl-2-pentanone). Lures were formulated to emit these compounds in the natural ratio but at a higher release rate (approx. 250 flower equivalents, see Chapter 8). Experiment 2 (18/5/99-24/5/99) compared three different types of lure formulation. Experiments 3 (25/5/99-31/5/99) and 4 (6/6/99-9/6/99; 13/6/99-16/6/99) evaluated reduced component blends. Experiment 5 (17/6/99-27/6/99) tested the effect of altering the ratio of compounds released. Full details of the loadings of compounds in different lure treatments are given in the results section.

Dissection of Female H. armigera

Female moths caught in the floral traps were dissected and examined for the presence of spermatophores to determine their mated status.

Statistical Analysis of Trap Catch Data

When the experiments were planned it was hoped that there would be large enough trap catches and differences between treatments in the weekly experiments to subject *H. armigera* trap catch data to an analysis of variance (ANOVA) and make comparisons between treatments in individual experiments. However, this was not possible due to low trap catches. Catches for the control and standard 4-component marigold blend were summed for each experiment which had both these treatments and compared using a Mann Whitney 'U' test with the different experiments being treated as different replicates (Table 9.9). An ANOVA using contrasts (Mead *et al.*, 1993) was carried out for honeybee, *A. mellifera*, catches which were several times higher than *H. armigera* catches.

9.2.2 Pakistan Field Trials

Field experiments were also carried out at Ali Khan Tareen farm, Lodhran, Punjab, Pakistan, October to November 1999. Testing the floral odour-baited traps in a different country allowed further investigation into the range of 'non-target' insects that were caught with the floral-odour baited trap. Methods were similar to those used in Israel. All grids of traps were set up in uniform fields of cotton which were in the flowering stage. A 20m spacing between traps was used. Benzaldehyde, (-)-linalool, (±)-linalool, phenylacetaldehyde, (*R*)-(+)-limonene and (*S*)-(-)-limonene were purchased from Sigma Aldrich (Gillingham, UK), (-)-piperitone was obtained from Daniels (UK) and these were air freighted into Pakistan. A larger area of land and more traps were available than in Israel and so it was possible to set up different grids of traps at the same time. Thirteen pheromone traps were used to monitor male *H. armigera* populations on the farm. Four experiments with *T. erecta* based floral odour-baited traps were carried out. Effects of using different *T. erecta* synthetic blend ratios, addition of piperitone to the 4-component blend, use of different enantiomers of linalool and altering trap colour and trap height were investigated.

9.3 Results

9.3.1 Israel Field Trial

Experiment 1 To Evaluate Lures based on Different Plant Sources

(10/5/99-15/5/99)

The first field experiment compared standard synthetic *T. erecta* and *L. odouratus* synthetic blends, as well as a mixed blend containing volatiles from both plants as shown in Table 9.1. This was to compare the attractiveness of the two different blends to *H. armigera* and to investigate if any synergism was obtained by using odours from both plant sources. Two Latin squares with a 4 x 4 array of traps were used.

Table 9.1 Treatments in Field Experiment 1, Israel (40 trap-nights per treatment)

Treatment	Treatment Details
Control	Unbaited control trap
M1	*Full Marigold Blend*,Two sachets: • 1.5 x 5cm sachet containing 0.5ml of a 40:1:1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool +10%BHT. • 2.5 x 2.5cm sachet containing 0.5ml of (-)-limonene in dioctyl phthalate (15% (-)-limonene) inside a 4 x 5cm outer sachet.
S1	*Full Sweet Pea Blend*, *1.5* x 5cm sachet containing 0.5ml of a 1:1:1:1 mixture of benzyl alcohol, diacetone, (±)-linalool and phenylacetaldehyde +10%BHT.
Combined Blend	Two sachets: • 1.5 x 5cm sachet containing 0.5ml of a 1:1:1:1:1 mixture of benzaldehyde, benzyl alcohol, diacetone, (±)-linalool and phenylacetaldehyde +10%BHT. • 2.5 x 2.5cm sachet containing 0.5ml of (-)-limonene in dioctyl phthalate (15% (-)-limonene) inside a 4 x 5cm outer sachet.

Table 9.2 Total catches of insects during Experiment 1 in Israel, 1999, with lures based on different plant sources (8 replicates for 5 nights). Treatments as in Table 9.1.

Treatment	*H. armigera*			*A. gamma*			Other Moths	*Apis sp.*	*Halictus* sp.	Diptera	Lacewings
	F	M	Total	F	M	Total					
Control	0	1	1	0	0	0	3	3	5	38	2
M1	7	0	7	12	8	20	15	222	81	267	38
S1	6	1	7	5	3	8	23	122	62	114	29
Combined	6	4	8	2	2	4	17	140	63	108	23

All insects in Table 9.2 were caught in higher numbers in the baited than in the control traps. It was observed that the traps caught other insects as well as *H. armigera.* Other insects caught comprised not only other Lepidopterous species such as Silver 'Y' moth, *Autographa gamma*, but also substantial numbers of Hymenoptera (*Apis mellifera* and *Halictus* spp.) and Diptera (small detritus feeding flies and fruit flies). Lacewings (mostly *Chrysopa carnea*) were also caught by the floral odour-baited traps.

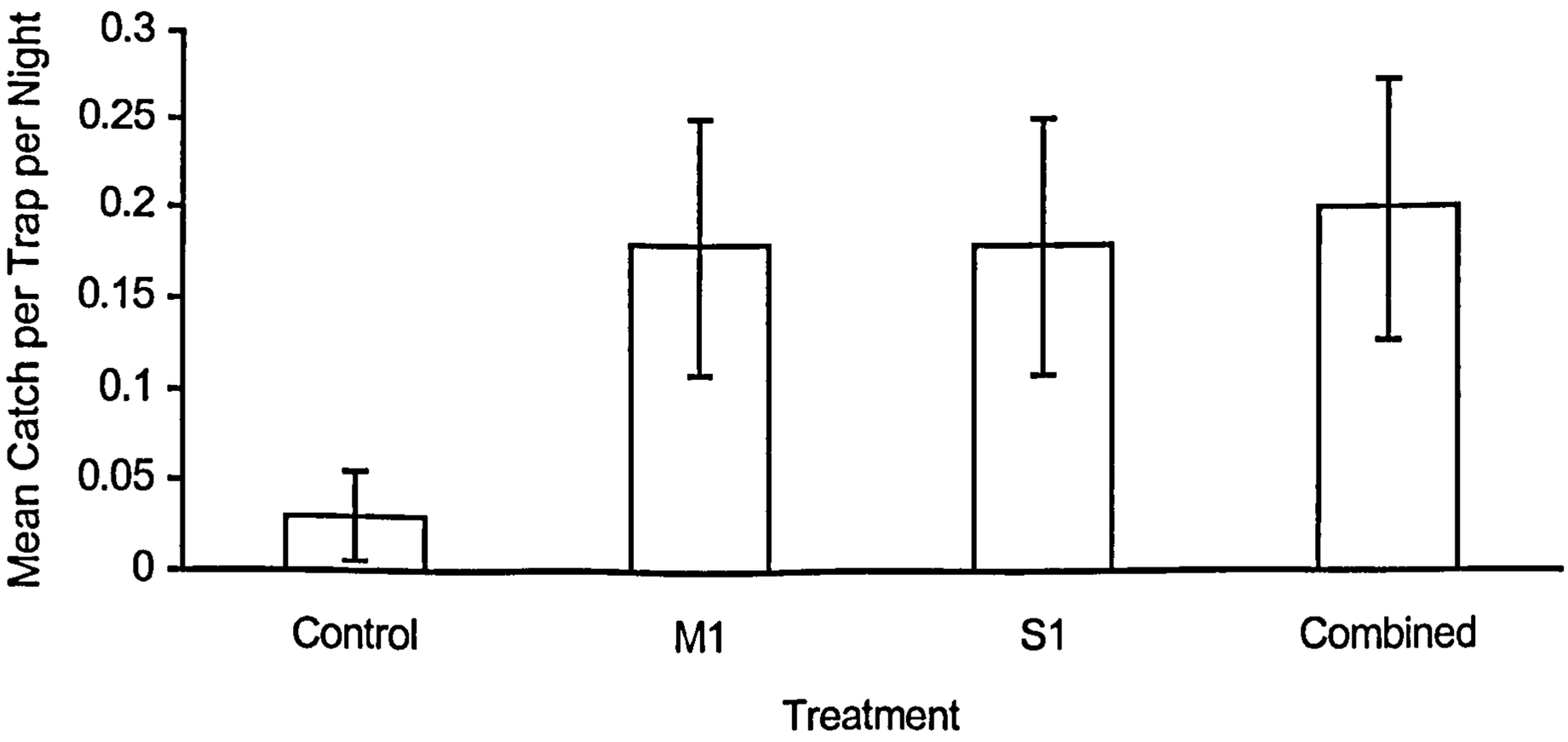

Fig. 9.1 Catches of *H. armigera* (males and females combined) in Experiment 1 (See Table 9.1 for details of treatments).

As shown in Fig 9.1, more *H. armigera* were caught in the baited than in the unbaited control traps ($P = 0.03$, Mann Whitney 'U' test, baited treatments compared with unbaited control treatments). However, there was no significant difference between the catches of *H. armigera* in the three floral odour-baited traps ($P = 0.20$). In addition to the *H. armigera* two *Helicoverpa peltigera* were caught by the combined blend in this experiment.

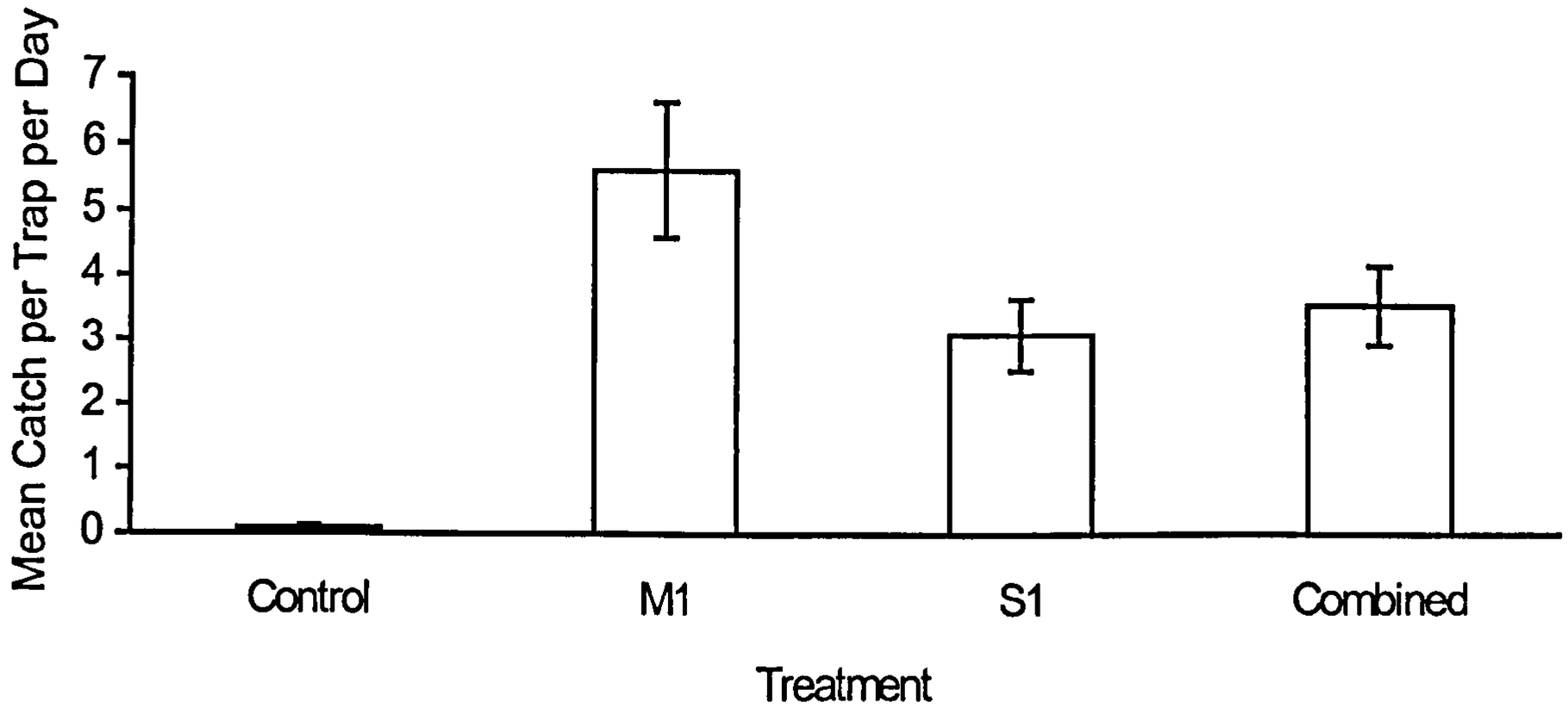

Fig 9.2 Catches of *Apis mellifera* Catch in Experiment 1.

As shown in Fig 9.2 there was a highly significant difference between numbers of *A. mellifera* caught in different treatments (ANOVA, $P < 0.001$) in Expt. 1. Contrasts (Mead *et al.*, 1993) were used to compare treatments. The floral odour-baited traps ('M1', 'S1' and 'Combined' treatments) caught significantly more *A. mellifera* than the unbaited control traps ($P < 0.001$). The marigold (M1) blend caught significantly more than the sweet pea (S1) blend ($P = 0.007$). However, catches with the combined treatment were not significantly different from those with blends derived from one source, 'M1' or 'S1' treatments ($P = 0.31$).

Experiment 2, To Evaluate Different Formulations of Synthetic Floral Odours *(18/5/99-24/5/99)*

Experiment 2 investigated the effect of using different formulations of the synthetic floral compounds. A rubber septum formulation was compared with single and double layered polyethylene sachet formulations. Two trials were set out at the same time using Latin squares (4 x 4) arrays of traps Experiment 2a investigated different *T. erecta* formulations while experiment 2b investigated different *L. odouratus* formulations. Treatments used in Experiments 2a and 2b are shown in Tables 9.3 and 9.5 respectively.

Experiment 2a, To evaluate different synthetic T. erecta *formulations*

Table 9.3 Treatments in Experiment 2a (24 trap-nights per treatment).

Treatment	Treatment Details
C	Unbaited control trap
M1	*Marigold Blend, single layered sachet* Two sachets: • 2.5 x 5cm sachet containing 0.5ml of a 40:1:1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool +10%BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M2	*Marigold Blend, double layered sachet* Two sachets contained inside an additional 4 x 5 cm sachet. • 2.5 x 2.5cm sachet containing 0.5ml of the same 3-component mixture as "M1". • 1.5 x 2.5cm sachet containing 0.2ml of (-)-limonene in dioctyl phthalate (15% (-)-limonene) inside a 2.5 x 3.5cm outer sachet.
M3	*Marigold Blend, Rubber Septum* Rubber septum (Maavit, Tel Aviv, Israel) impregnated using 0.5ml of a 10% solution of synthetic marigold compounds (as above) in hexane.

Table 9.4 Total catches of insects during Experiment 2a in Israel, 1999, with different formulations of the marigold blend (4 replicates for 6 nights). Treatments as in Table 9.3.

Treatment	*H. armigera*			*A. gamma*			Other Moths	*Apis sp.*	*Halictus* sp.	Diptera	Lacewings
	F	M	Total	F	M	Total					
Control	0	0	0	0	0	0	1	3	6	23	1
M1	4	1	5	3	5	8	29	102	41	571	44
M2	2	3	5	1	2	3	20	75	37	671	37
M3	1	0	1	1	0	1	4	17	44	62	10

Catches of insects with different formulations of the marigold, *T. erecta*, blend are shown in Table 9.4. All categories of insects were caught in higher numbers in the baited than control traps. The traps baited with the higher release rate sachets of *T. erecta* volatiles (M1) caught more *A. gamma* but in general there were larger differences between the sachet baited traps (M1 plus M2) and the traps baited with septa (M3) than between traps baited with the two types of sachet (M1 and M2). All insects apart from *Halictus* were captured in significantly higher numbers in treatments M1 and M2 than in M3.

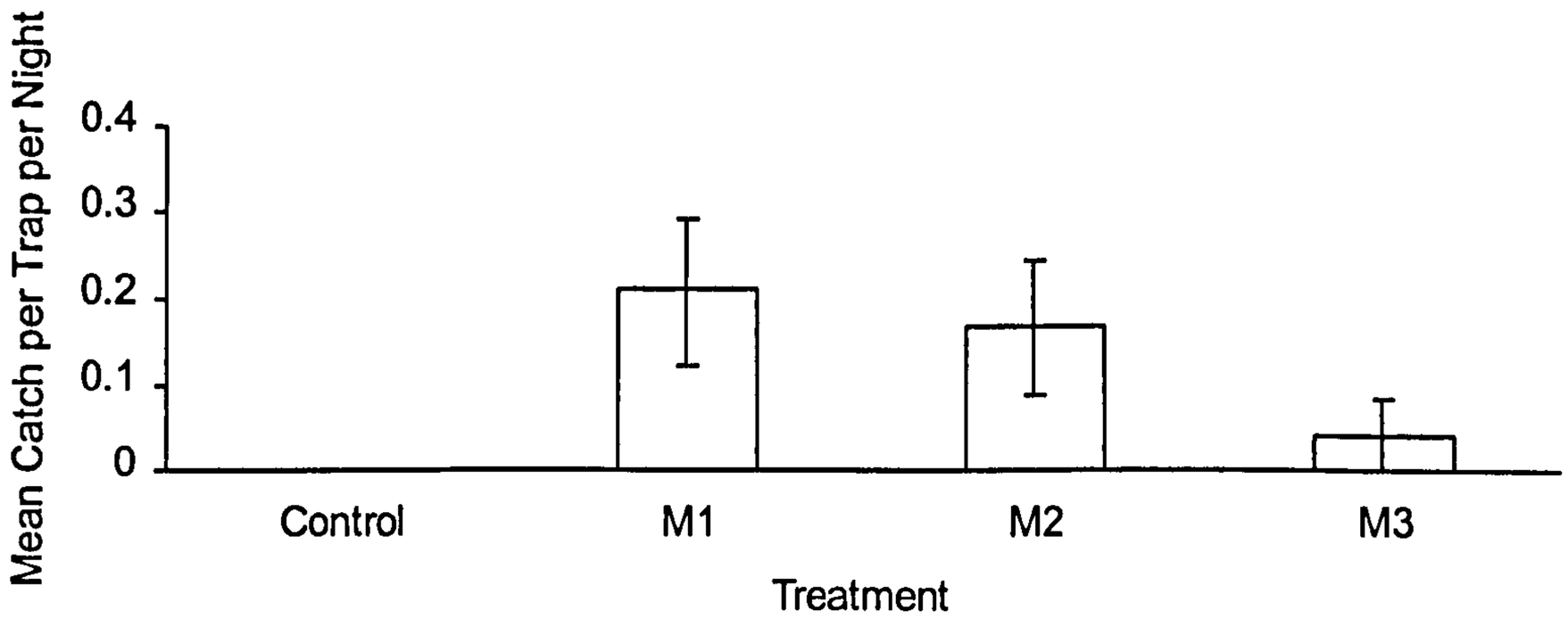

Fig 9.3 Catches of *H. armigera* (males and females combined) in Experiment 2a (See Table 9.3 for details of treatments).

More *H. armigera* were caught in baited than in unbaited control traps ($P = 0.05$, Mann Whitney 'U' test, baited treatments compared with unbaited control treatments). There was no significant difference between *H. armigera* catches in traps with different formulations of *T. erecta* odours although fewer moths were caught with the rubber septum formulation (Fig 9.3).

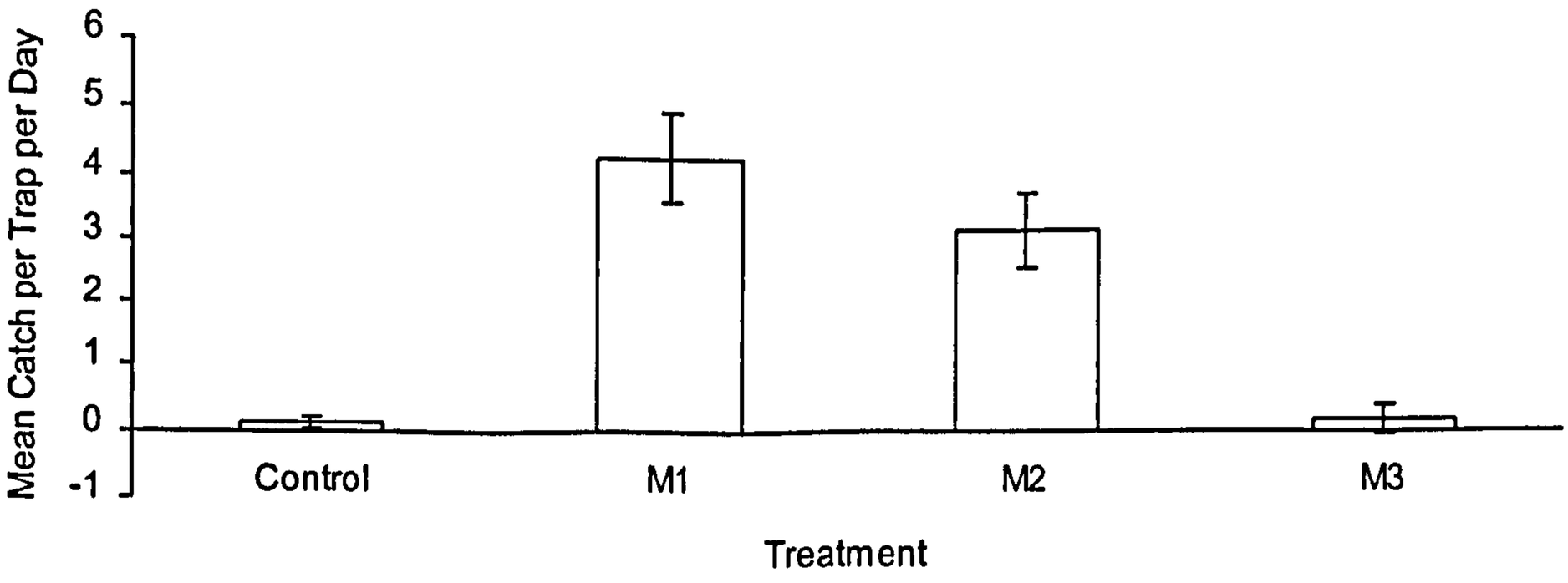

Fig 9.4 Catches *Apis mellifera* in Experiment 2a

There were highly significant differences between treatments (ANOVA, $P < 0.001$ overall) for catches of *A. mellifera* (Fig 9.4). Contrasts were calculated: Floral odour-baited traps caught more than the unbaited control traps ($P < 0.001$).

There was no significant difference between catches with the two sachet baited traps (M1 and M2) ($P = 0.085$). Sachet baited traps (M1 and M2) caught significantly more *A. mellifera* than traps baited with septa (M3) ($P < 0.001$).

Experiment 2b, To evaluate different synthetic L. odouratus *formulations.*

Table 9.5 Treatments in Experiment 2b (24 trap-nights per treatment)

Treatment	Treatment Details
C	Unbaited control trap
S1	*Sweet Pea Blend, Single Layered Sachet* 2.5 x 5cm sachet containing 0.5ml of a 1:1:1:1 mixture of benzyl alcohol, diacetone, (±)-linalool and phenylacetaldehyde +10%BHT.
S2	*Sweet Pea Blend, Double Layered Sachet* 2.5 x 2.5cm sachet containing 0.5ml of a 1:1:1:1 mixture of benzyl alcohol, diacetone, (±)-linalool and phenylacetaldehyde +10%BHT inside a 4 x 5 cm sachet.
S3	*Sweet Pea Blend, Rubber Septum* Rubber septum (Maavit, Tel Aviv, Israel) impregnated using 0.5ml of a 10% solution of synthetic sweet pea compounds (as above) in hexane.

Table 9.6 Total catches of insects during Experiment 2b in Israel, 1999, with different formulations of the sweet pea blend (4 replicates for 6 nights). Treatments as in Table 9.5.

Treatment	*H. armigera*			*A. gamma*			Other Moths	*Apis sp.*	*Halictus* sp.	Diptera	Lacewings
	F	M	Total	F	M	Total					
Control	0	0	0	0	0	0	0	0	0	35	0
S1	4	0	4	3	2	5	26	60	53	171	33
S2	0	1	1	2	1	3	30	41	56	190	18
S3	1	1	2	3	2	5	8	33	45	70	19

There were higher catches with floral odour-baited than unbaited control traps for Lepidoptera, Hymenoptera, Diptera and lacewings and there was a general trend of reduced catches with the rubber septum formulation compared with the polyethylene sachet formulations.

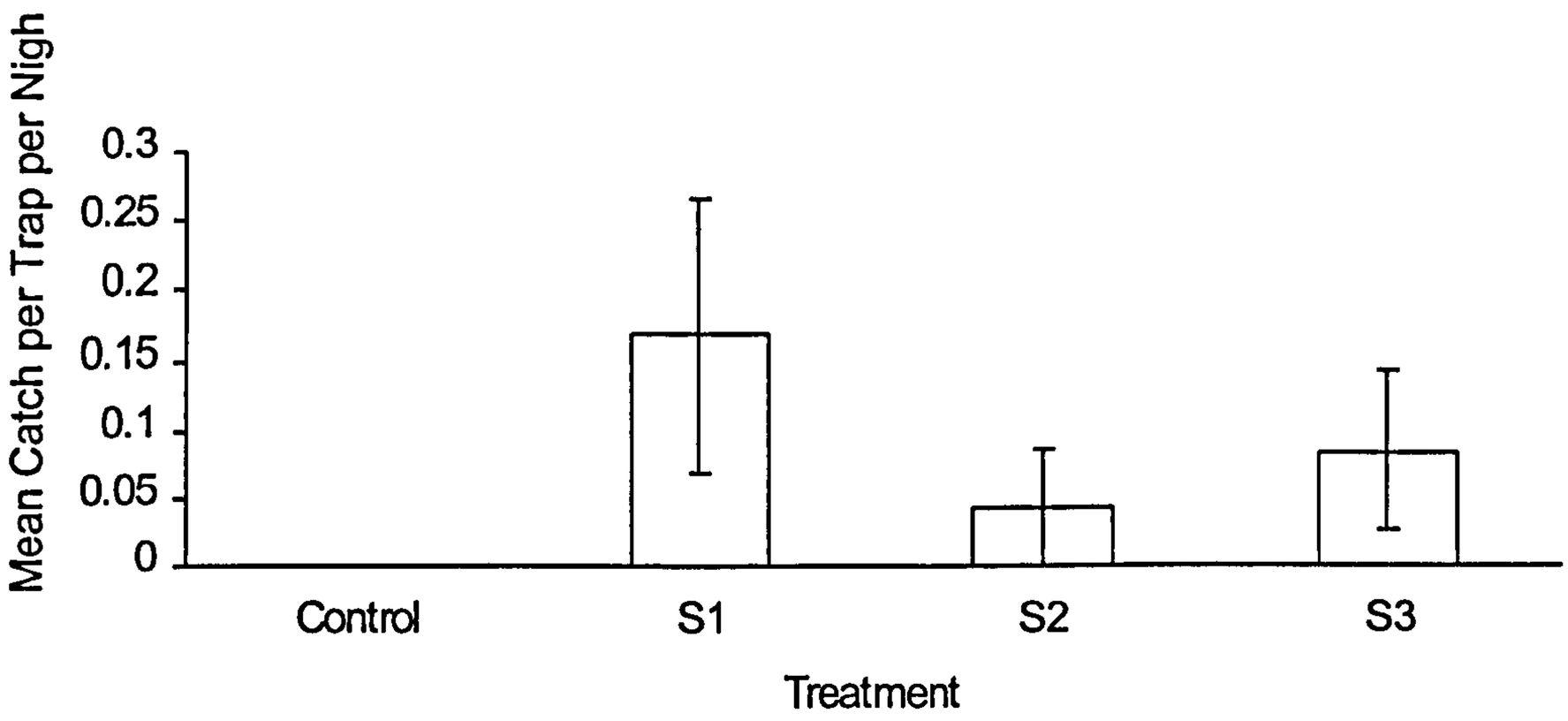

Fig 9.5 Catches of *H. armigera* (males and females combined) in Experiment 2b (See Table 9.5 for details of treatments).

H. armigera catches with different formulations of the sweet pea blend in Experiment 2b are shown in Fig 9.5. Due to the low catches per trap per night, there were no statistically significant differences between treatments (Mann Whitney 'U' test comparing baited and unbaited traps, $P = 0.15$; ANOVA for whole experiment, $P = 0.25$). However, reducing the release rate from the polyethylene sachets reduced the mean trap catch of *H. armigera* but the difference was not significant. There appeared to be some attraction to traps baited with rubber septa (S3) but catches with these baits only occurred in the first two days of the trial.

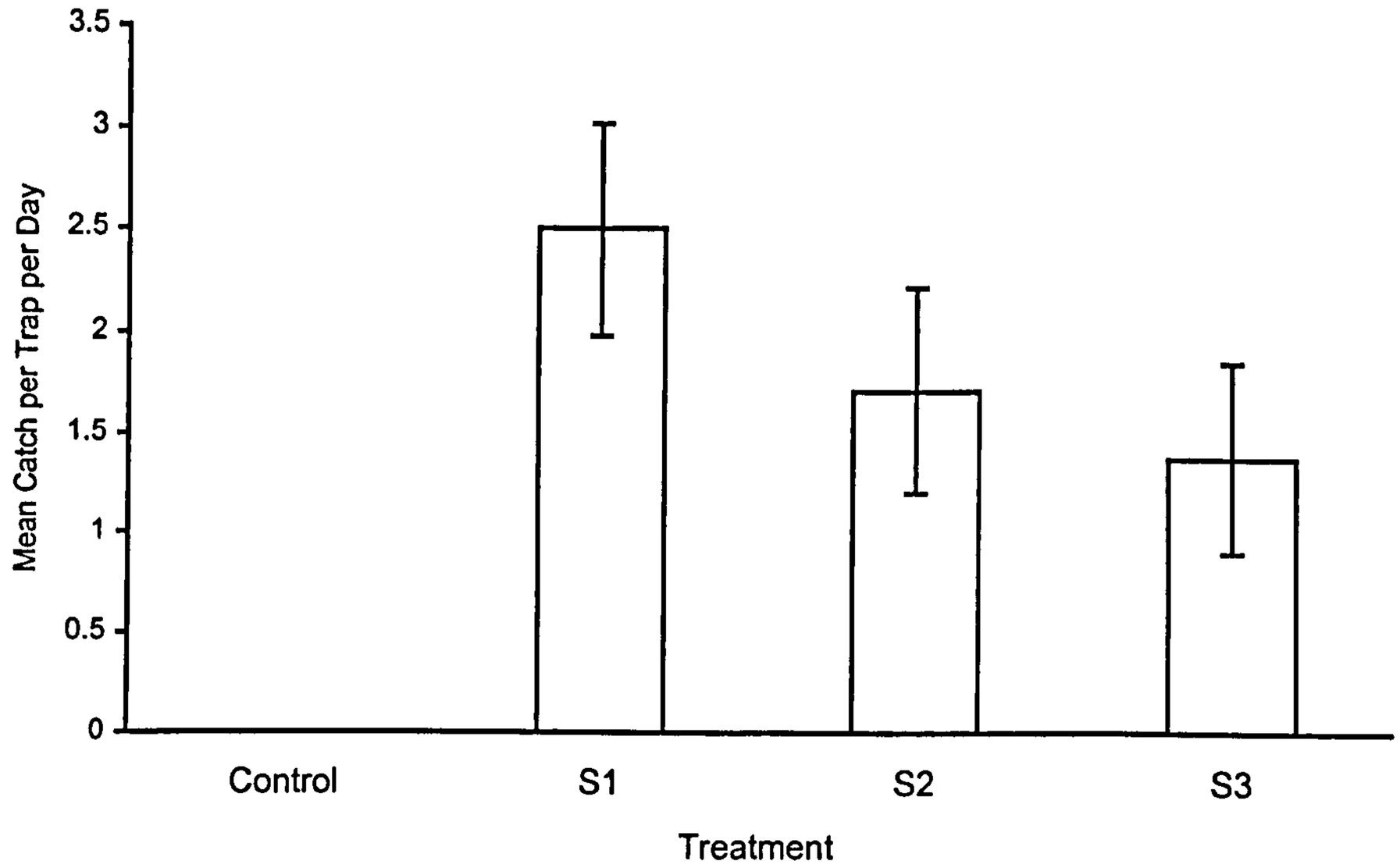

Fig 9.6 Catches of *Apis mellifera* in Experiment 2b

A. mellifera catches with different formulations of the sweet pea blend (Fig 9.6) were significantly different between treatments (ANOVA, $P < 0.001$) largely due to the difference between floral baited and control treatments ($P < 0.001$). However, there was no difference *A. mellifera* catch between different formulations of the sweet pea bait ($P = 0.163$).

Experiment 3, To Evaluate Reduced Component Synthetic Floral blends

Experiment 3 investigated reduced component blends in which one of each of the four components of the synthetic *T. erecta* blend (Experiment 3a) and synthetic *L. odouratus* blend (Experiment 3b) were omitted. These were tested along with the full 4-component blend and unbaited control traps in each case. There was one Latin square with a 6 x 6 array of traps in each experiment. Treatments used in Experiment 3 are shown in Tables 9.7 and 9.9.

Experiment 3a: Reduced component synthetic T. erecta *blends (25/5/99-31/5/99)*

Table 9.7 Treatments in Experiment 3a (36 trap-nights per treatment).

Treatment	Treatment Details
C	Unbaited control trap
M1	*Marigold, Full Blend* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 40:1:1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool +10%BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M-B	*Marigold minus benzaldehyde* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 40:1 mixture of phenylacetaldehyde and (±)-linalool +10%BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M-Lin	*Marigold minus (±)-linalool* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 40:1 mixture of phenylacetaldehyde and benzaldehyde +10%BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M-Lim	*Marigold minus (-)-limonene*: • 4 x 5cm sachet containing 0.5ml of a 40:1:1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool +10%BHT
M-P	*Marigold minus phenylacetaldehyde* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 1:1 mixture of benzaldehyde and (±)-linalool +10%BHT. • (-)-limonene sachet (as in Expt.1, "M1").

Table 9.8 Total catches of insects during Experiment 3a in Israel, 1999, with reduced component *T. erecta* synthetic blends (6 replicates for 6 nights). Treatments as in Table 9.7.

Treatment	*H. armigera*			*A.gamma*			Other Moths	*Apis sp.*	*Halictus* sp.	Diptera	Lacewings
	F	M	Total	F	M	Total					
Control	0	0	0	0	0	0	3	1	1	16	2
M - B	0	0	0	1	1	2	28	85	39	186	30
M - Lim	1	0	1	2	3	5	25	121	61	206	45
M - Lin	1	1	2	1	3	4	18	107	41	177	30
M - P	3	2	5	2	1	3	17	27	6	66	7
M1	0	1	1	0	1	1	16	73	44	167	37

As shown in Table 9.8 removing benzaldehyde (M - B), (±)-linalool (M - Lin) or (-)-limonene (M - Lim) from the marigold blend had little effect on trap catches of most insects. Omission of phenylacetaldehyde (M - P) reduced catches of Hymenoptera, Diptera and Lacewings although catches of Lepidoptera did not appear to be reduced (Table 9.8).

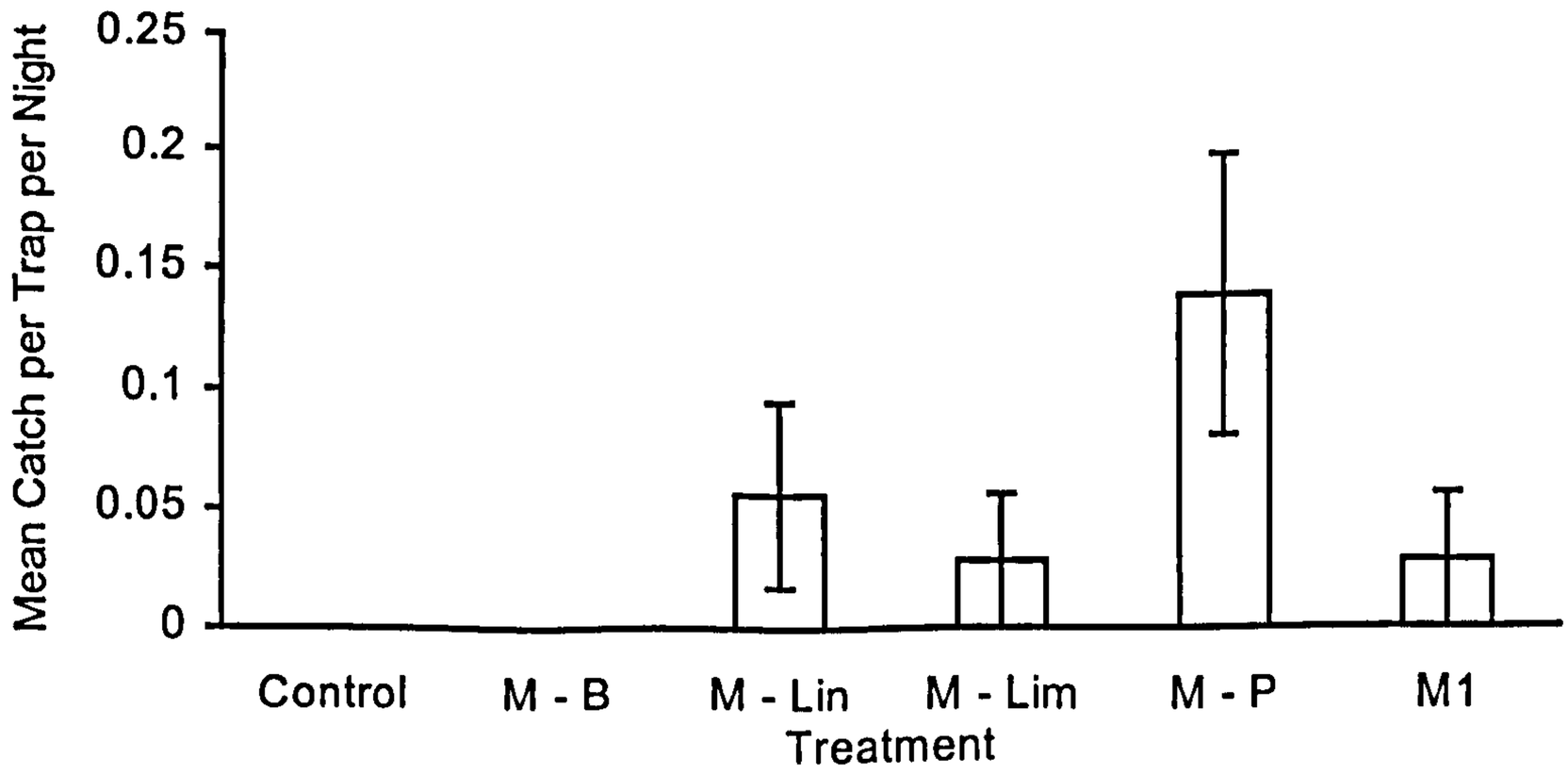

Fig 9.7 Catches of *H. armigera* (males and females combined) in Experiment 3a (See Table 9.7 for details of treatments).

As shown in Fig 9.7, no *H. armigera* were caught in traps with the blend in which benzaldehyde was omitted (M-B). However, *H. armigera* were still caught with blends omitting (±)-linalool, (-)-limonene and phenylacetaldehyde. In this experiment more *H. armigera* were caught with the blend missing phenylacetaldehyde than with the full marigold blend (M1). However, differences between treatment means for *H. armigera* were not statistically significant due to the low catches per trap per night and the consequent high standard errors.

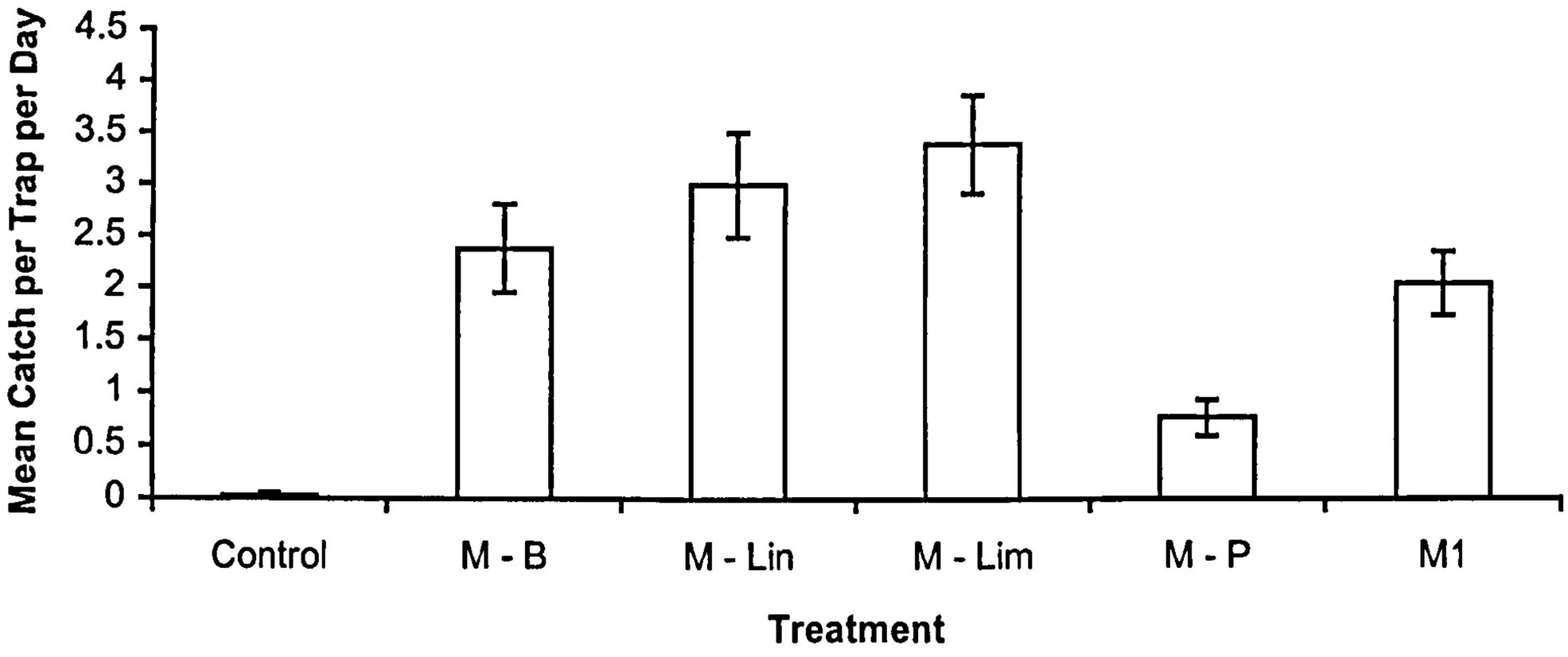

Fig 9.8 Catches of *Apis mellifera* in Experiment 3a.

Significant differences were found between treatments for *A. mellifera* in Expt. 3a (ANOVA, $P < 0.001$). All floral baited treatments combined caught significantly more than the control treatment (Fig 9.8). The blend omitting (-)-limonene (M-Lim) caught significantly more than the full marigold blend (M1) ($P = 0.010$). The blend omitting phenylacetaldehyde (M-P) caught significantly fewer *A. mellifera* than the full marigold blend ($P = 0.013$).

Experiment 3b, Reduced component synthetic L. odouratus *blends (1/6/99-6/6/99)*

Table 9.9 Treatments in Experiment 3b (30 trap-nights per treatment).

Treatment	Treatment Details
C	Unbaited control trap
S1	*Full synthetic sweet pea blend.* One sachet. 4 x 5cm sachet containing 0.5ml of a 1:1:1:1 mixture of benzyl alcohol, diacetone, (±)-linalool and phenylacetaldehyde +10%BHT.
S-BA	*Sweet Pea minus benzyl alcohol.* [One sachet. 4 x 5cm sachet containing 0.5ml of a 1:1:1 mixture of diacetone, (±)-linalool and phenylacetaldehyde +10%BHT.]
S - D	*Sweet Pea minus diacetone.* [One sachet. 4 x 5cm sachet containing 0.5ml of a 1:1:1 mixture of benzyl alcohol, (±)-linalool and phenylacetaldehyde +10%BHT.]
S - Lin	*Sweet Pea minus (±)-linalool.* [One sachet. 4 x 5cm sachet containing 0.5ml of a 1:1:1 mixture of benzyl alcohol, diacetone and phenylacetaldehyde +10%BHT.]
S - P	*Sweet Pea minus phenylacetaldehyde.* [One sachet. 4 x 5cm sachet containing 0.5ml of a 1:1:1 mixture of benzyl alcohol, diacetone and (±)-linalool +10%BHT.]

Table 9.10 Total catches of insects during Experiment 3b in Israel, 1999, with reduced component *L. odouratus* synthetic blends (6 replicates for 5 nights). Treatments as in Table 9.9.

Treatment	*H. armigera*			*A. gamma*			*S. littoralis*			Other Moths	*Apis* sp.	*Halictus* sp.	Diptera	Lacewing
	F	M	Total	F	M	Total	F	M	Total					
Control	0	0	0	0	0	0	0	0	0	68	1	3	8	0
S - BA	3	1	4	2	0	2	1	1	0	36	25	61	49	26
S - D	3	1	4	0	0	0	2	1	1	17	9	59	36	14
S - Lin	2	1	3	0	0	0	1	0	1	19	16	74	25	23
S - P	0	0	0	0	0	0	0	0	0	6	1	10	7	7
S1	2	2	4	0	0	0	1	1	0	25	22	57	51	27

Catches of insects with reduced component *Lathyrus odoratus* blends are shown in Table 9.10. For all species, except lacewings, trap catches with the *L. odouratus* blend in which phenylacetaldehyde had been omitted (S - P) were similar to the unbaited control traps. Omission of the any one of the other three compounds did not have a noticeable effect on trap catches.

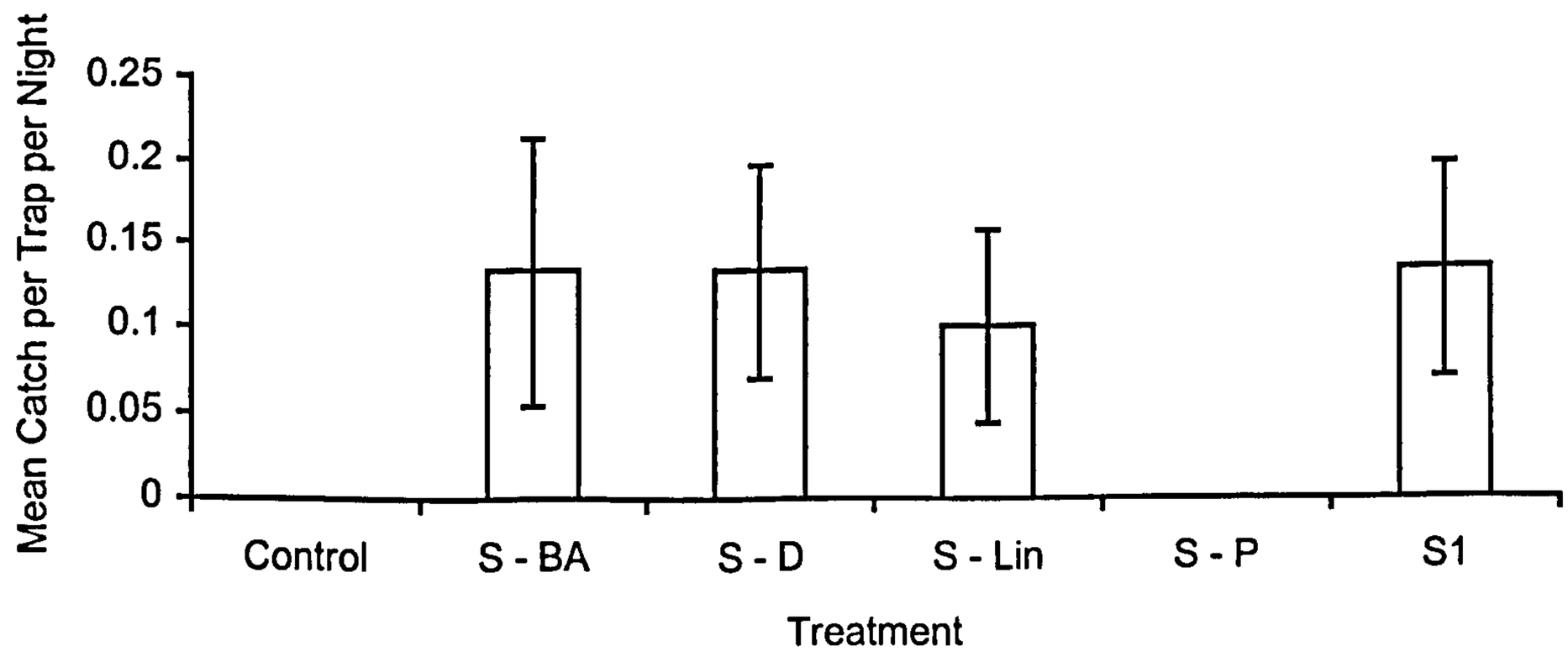

Fig 9.9 Catches of *H. armigera* (males and females combined) in Experiment 3b (See Table 9.9 for details of treatments).

There was no significant difference in *H. armigera* catches between treatments (ANOVA $P = 0.19$) (Fig 9.9). No *H. armigera* were caught by unbaited control traps or with traps baited with *L. odouratus* lures not including phenylacetaldehyde (S – P). Omission of any one of the other three components of the *L. odouratus* blend did not significantly affect *H. armigera* trap catches.

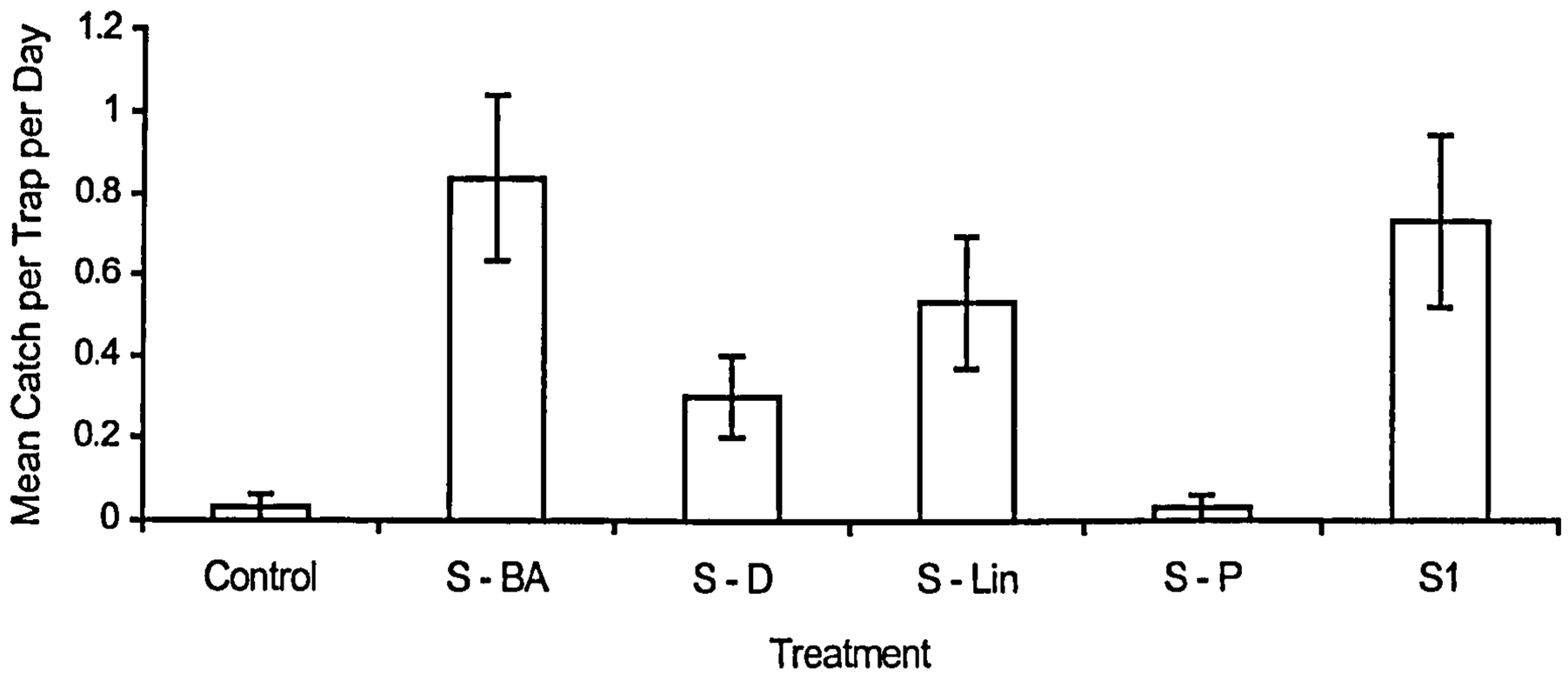

Fig. 9.10 Catches of *Apis mellifera* in Experiment 3b.

There were significant differences between treatments in *A. mellifera* catches (ANOVA, $P < 0.001$). Floral baited traps caught significantly more *A. mellifera* than unbaited control traps (P=0.004). Catches with the blend omitting phenylacetaldehyde (S - P) were not significantly different from catches with the unbaited control treatment ($P = 1.00$). However, removal of any one of the other three components did not significantly affect attractiveness of the lure to *A. mellifera* (see Fig 9.10).

Experiment 4, Further evaluation of* T. erecta *based reduced component blends (6/6/99-9/6/99; 13/6/99-16/6/99).

Experiment 4 continued testing reduced component synthetic *T. erecta* blends. The 3-component blend omitting benzaldehyde which had not caught any *H. armigera* in Experiment 3a was tested again to see whether this had been due to low adult populations present at the time of the trial. The 3-component blend in which phenylacetaldehyde was omitted was tested further because it had appeared to give lower *A. mellifera* catches in Experiment 3a while catches of Lepidoptera were similar to the 4-component blend (Table 9.10). A 2-component blend containing benzaldehyde and (-)-limonene and benzaldehyde alone was tested to see if it was attractive to *H. armigera.* Treatments used in Experiment 4 are shown in Table 9.11 and traps were laid out in a 6 x 6 Latin square.

Table 9.11 Treatments in Experiment 4 (36 trap-nights per treatment).

Treatment	Treatment Details
C	Unbaited control trap
M1	*Marigold, Full Blend* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 40 : 1 : 1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool + 10% BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M - B	*Marigold minus benzaldehyde* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 40 : 1 mixture of phenylacetaldehyde and (±)-linalool + 10% BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M - P	*Marigold minus phenylacetaldehyde* (2 sachets): • 4 x 5cm sachet containing 0.5ml of a 1:1 mixture of benzaldehyde and (±)-linalool + 10% BHT. • (-)-limonene sachet (as in Expt.1, "M1").
M – PL	*Marigold minus phenylacetaldehyde and (±)-linalool.* (2 sachets): • 4 x 5cm sachet containing 0.5ml of benzaldehyde + 10% BHT. • (-)-limonene sachet (as in Expt.1, "M1").
B	*Benzaldehyde alone.* • 4 x 5cm sachet containing 0.5ml of benzaldehyde + 10% BHT

Table 9.12 Total catches of insects during Experiment 4 in Israel, 1999, with reduced component *T. erecta* synthetic blends (6 replicates for 6 nights).
Treatments as in Table 9.11.

Treatment	*H. armigera*			*A. gamma*			*S. littoralis*			Other Moths	*Apis* spp.	*Halictus* spp..	Diptera spp.	Lacewing spp.
	F	M	Total	F	M	Total	F	M	Total					
Control	0	0	0	0	0	0	0	1	1	17	0	0	13	0
M - B	3	0	3	1	2	3	0	1	1	35	18	43	60	33
M - P	2	2	4	0	0	0	0	0	0	20	6	15	65	10
M - PL	1	1	2	0	0	0	0	1	1	9	8	15	45	30
B	0	0	0	0	0	0	0	0	0	16	18	28	74	32
M1	2	0	2	0	1	1	1	5	6	33	27	50	84	26

As shown in Table 9.12 catches with the marigold blend omitting benzaldehyde (M - B) were not very different from those with the full blend (M1). *H. armigera* were caught with the "M - B" treatment in this field trial unlike Experiment 3a. *H. armigera* were still caught by the marigold blend without phenylacetaldehyde (M - B) whereas catches of Hymenoptera and lacewings were reduced compared with the full marigold blend (M1), consistent with findings in Experiment 3a. Traps baited with a binary blend of benzaldehyde and (-)-limonene (M – PL), and benzaldehyde alone (B) caught low numbers of Lepidoptera but still caught Hymenoptera and lacewings.

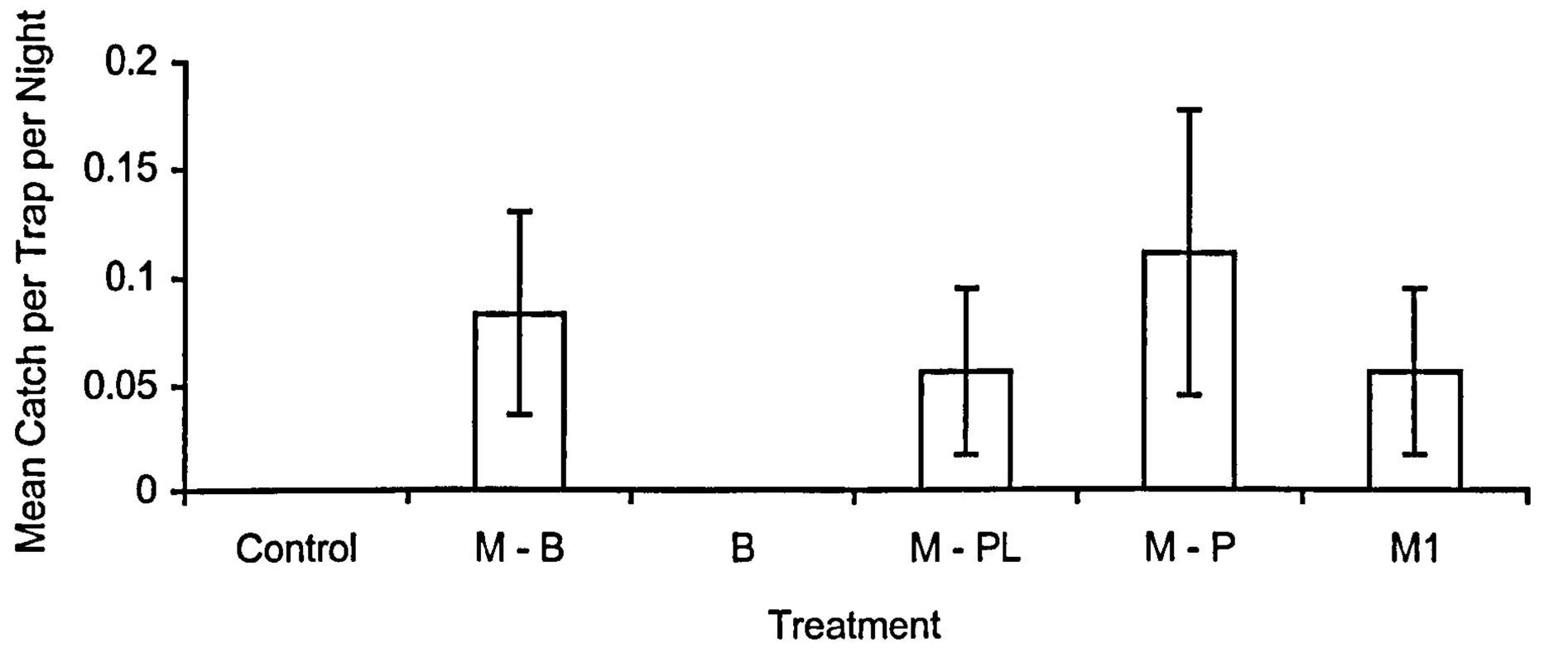

Fig 9.11 Catches of *H. armigera* (males and females combined) in Experiment 4 (See Table 9.11 for details of treatments).

There were no significant differences in *H. armigera* catches between treatments (ANOVA, $P = 0.27$). The largest number of *H. armigera* was caught using treatment 'M - B' despite the omission of phenylacetaldehyde (Fig 9.11). This result was very different from that when the same blend was tested in Experiment 3a and no *H. armigera* were caught, which emphasises that care has to be taken when interpreting trap catch data when numbers are low.

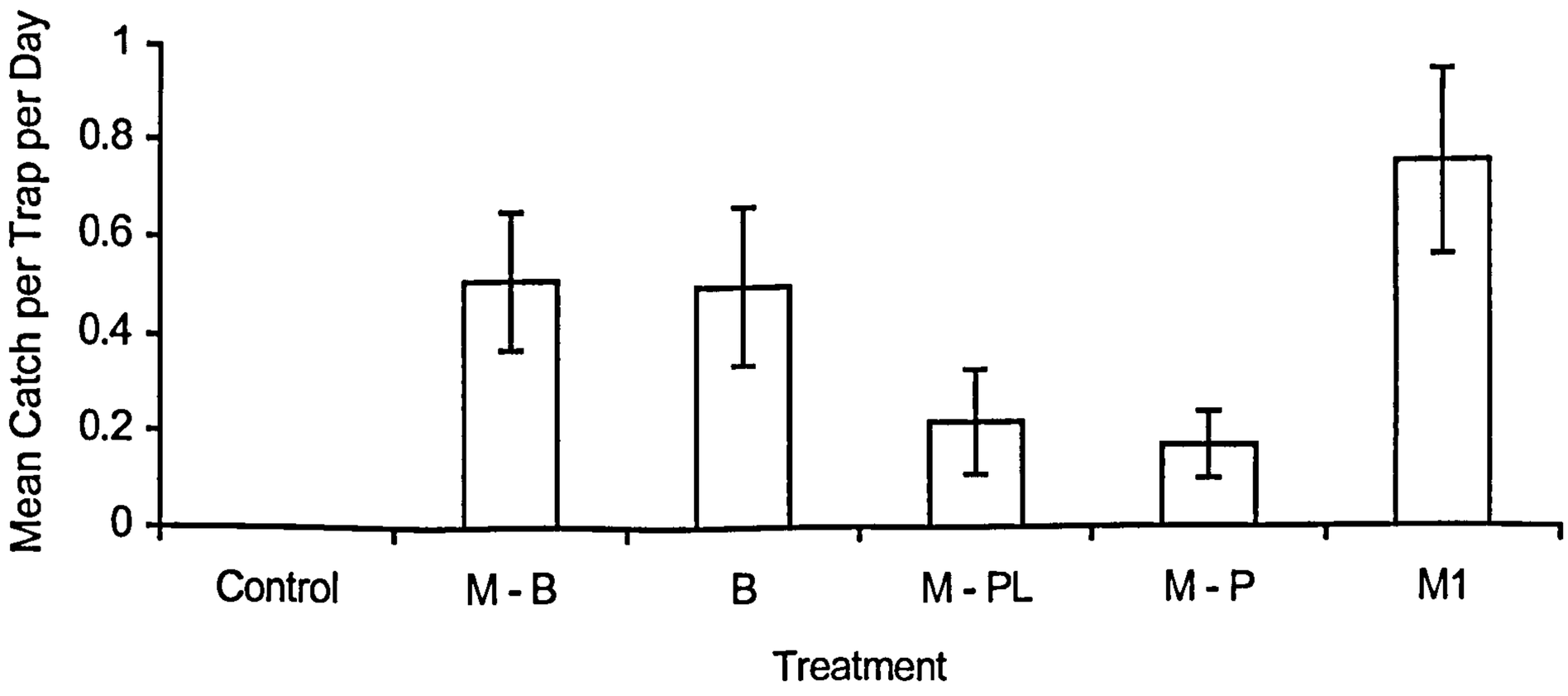

Fig 9.12 Catches of *Apis mellifera* in Experiment 4.

There were significant differences between treatments in terms of numbers of *A. mellifera* caught (ANOVA, P < 0.001). As shown in Fig 9.12 significantly more *A. mellifera* were caught with floral baited traps than with the unbaited control traps (P = 0.002). There were no significant differences in captures between the full marigold blend (M1), benzaldehyde alone (B) and the 3-component blend omitting benzaldehyde (M - B) (P = 0.120). Captures with the 2-component blend containing benzaldehyde and (-)-limonene (M – PL) and the 3-component blend with benzaldehyde, (±)-linalool and (-)-limonene (M – P) were not significantly different from unbaited control trap captures (P = 0.215).

Experiment 5, To investigate the effect of altering the ratio of compounds released (17/6/99-29/6/99)

Different ratios of a 3-component *T. erecta* blend containing benzaldehyde, (±)-linalool and (-)-limonene were investigated. Phenylacetaldehyde was omitted because it had been observed in previous experiments (Tables 9.10 & 9.12) that captures of insects other than *H. armigera* such as *A. mellifera* were lower without phenylacetaldehyde. A full *T. erecta* 4-component blend treatment (M1) was included for comparison. One Latin square with a 6 x 6 array of traps was used. Treatments used in Experiment 5 are shown in Table 9.13.

Table 9.13 Treatments in Experiment 5 (72 trap-mights per treatment).

Treatment	Treatment Details
1	Unbaited control trap
2	*Benzaldehyde, (±)-linalool, (-)-limonene; standard ratio* [Two sachets. 4 x 5cm sachet containing 0.25ml benzaldehyde and 0.25ml (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]
3	*Benzaldehyde (increased), (±)-linalool, (-)-limonene* [Two sachets. 4 x 5cm sachet containing 0.4ml benzaldehyde and 0.1ml (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]
4	*Benzaldehyde, (±)-linalool (increased), (-)-limonene* [Two sachets. 4 x 5cm sachet containing 0.1ml benzaldehyde and 0.4ml (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]
5	*Benzaldehyde, (±)-linalool, (-)-limonene (increased)* [Two sachets. 4 x 5cm sachet containing 0.25ml benzaldehyde and 0.25ml (±)-linalool + 10% BHT. Modified (-)-limonene sachet: single thickness, 4 x 5cm dimension containing 0.5ml (-)-limonene in dioctyl phthalate (15% (-)-limonene), replaced daily]
6	*Marigold, Full Blend* [Two sachets. 4 x 5cm sachet containing 0.5ml of a 40:1:1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]

Table 9.14 Total catches of insects during Experiment 5 in Israel, 1999, with *T. erecta* synthetic blends with altered ratios of components (6 replicates for 12 nights). Treatments as in Table 9.13.

Treatment	*H. armigera*			*A. gamma*			*S. littoralis*			Other Moths	*Apis* spp.	*Halictus* spp	Diptera spp.	Lacewing spp.
	F	M	Total	F	M	Total	F	M	Total					
1	0	0	0	0	0	0	0	2	2	15	2	1	5	2
2	1	1	2	2	0	1	0	3	3	13	60	13	47	7
3	7	3	10	1	0	1	1	1	2	31	57	11	75	9
4	3	1	4	1	0	1	0	1	1	10	35	9	39	4
5	4	0	4	0	1	1	0	0	0	26	47	12	38	10
6	5	1	6	0	1	1	4	5	9	59	186	113	174	43

Table 9.13 Treatments in Experiment 5 (72 trap-mights per treatment).

Treatment	Treatment Details
1	Unbaited control trap
2	*Benzaldehyde, (±)-linalool, (-)-limonene; standard ratio* [Two sachets. 4 x 5cm sachet containing 0.25ml benzaldehyde and 0.25ml (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]
3	*Benzaldehyde (increased), (±)-linalool, (-)-limonene* [Two sachets. 4 x 5cm sachet containing 0.4ml benzaldehyde and 0.1ml (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]
4	*Benzaldehyde, (±)-linalool (increased), (-)-limonene* [Two sachets. 4 x 5cm sachet containing 0.1ml benzaldehyde and 0.4ml (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]
5	*Benzaldehyde, (±)-linalool, (-)-limonene (increased)* [Two sachets. 4 x 5cm sachet containing 0.25ml benzaldehyde and 0.25ml (±)-linalool + 10% BHT. Modified (-)-limonene sachet: single thickness, 4 x 5cm dimension containing 0.5ml (-)-limonene in dioctyl phthalate (15% (-)-limonene), replaced daily]
6	*Marigold, Full Blend* [Two sachets. 4 x 5cm sachet containing 0.5ml of a 40:1:1 mixture of phenylacetaldehyde, benzaldehyde and (±)-linalool + 10% BHT. (-)-limonene sachet (as in Expt.1, "M1").]

Table 9.14 Total catches of insects during Experiment 5 in Israel, 1999, with *T. erecta* synthetic blends with altered ratios of components (6 replicates for 12 nights). Treatments as in Table 9.13.

	H. armigera			*A. gamma*			*S. littoralis*							
Treatment	F	M	Total	F	M	Total	F	M	Total	Other Moths	*Apis* spp.	*Halictus* spp	Diptera spp.	Lacewing spp.
1	0	0	0	0	0	0	0	2	2	15	2	1	5	2
2	1	1	2	2	0	1	0	3	3	13	60	13	47	7
3	7	3	10	1	0	1	1	1	2	31	57	11	75	9
4	3	1	4	1	0	1	0	1	1	10	35	9	39	4
5	4	0	4	0	1	1	0	0	0	26	47	12	38	10
6	5	1	6	0	1	1	4	5	9	59	186	113	174	43

As shown in Table 9.14, catches of *A. mellifera*, *Halictus*, Diptera and lacewings appeared to be reduced in traps baited with the marigold blend omitting phenylacetaldehyde (Treatments 2, 3, 4 and 5) compared with the full marigold blend (Treatment 6). This was consistent with observations in Experiment 3a and 4 that improved selectivity was obtained by omitting phenylacetaldehyde. Sequentially altering the proportion of individual components of the 3-component blend did not result in a significant change in trap catches although Treatment 3 (increased proportion of benzaldehyde) caught more Lepidoptera than other treatments, although the difference was not statistically significant.

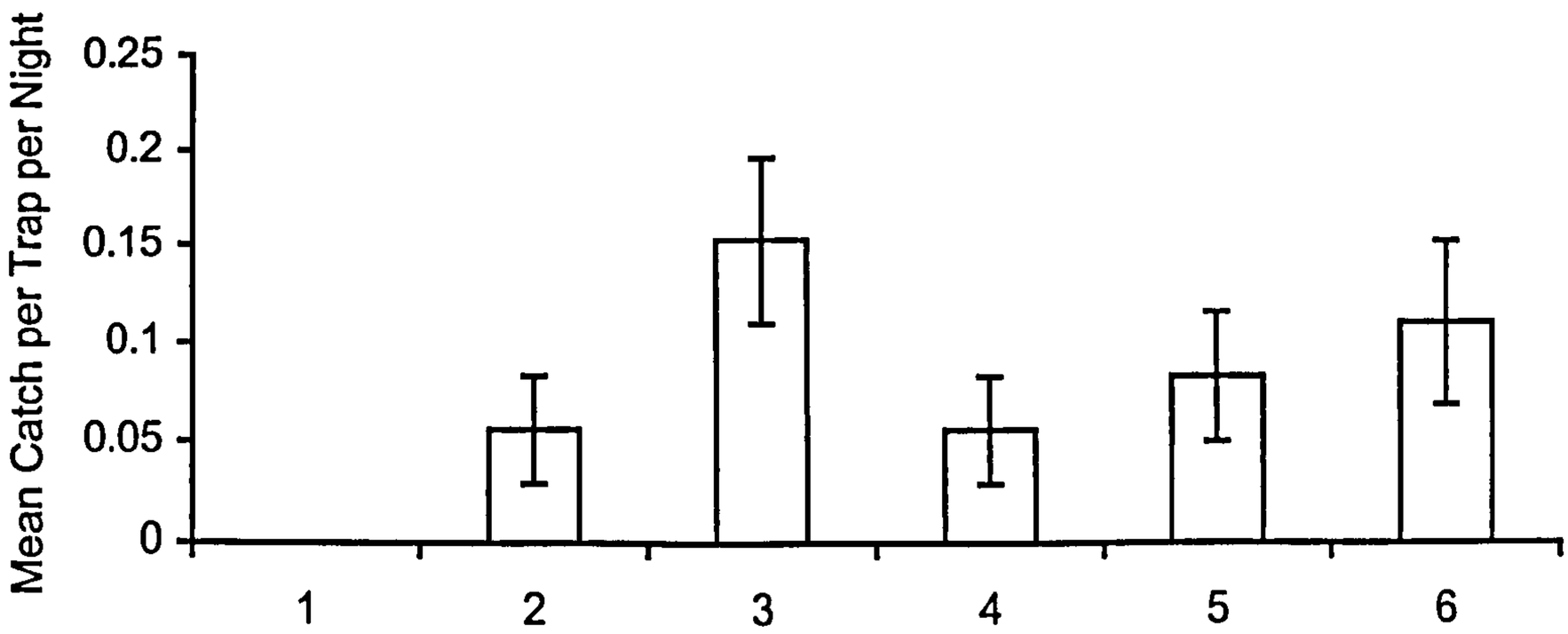

Fig 9.13 Catches of *H. armigera* (males and females combined) in Experiment 5 (See Table 9.13 for details of treatments).

H. armigera catches in Experiment 5 with altered ratios of the synthetic *T. erecta* synthetic blend are shown in Fig 9.13. A 3-component blend blend omitting phenylacetaldehyde but with an increased benzaldehyde emission (blend 3) actually caught more *H. armigera* than the full 4-component blend (blend 6) although this difference was not statistically significant.

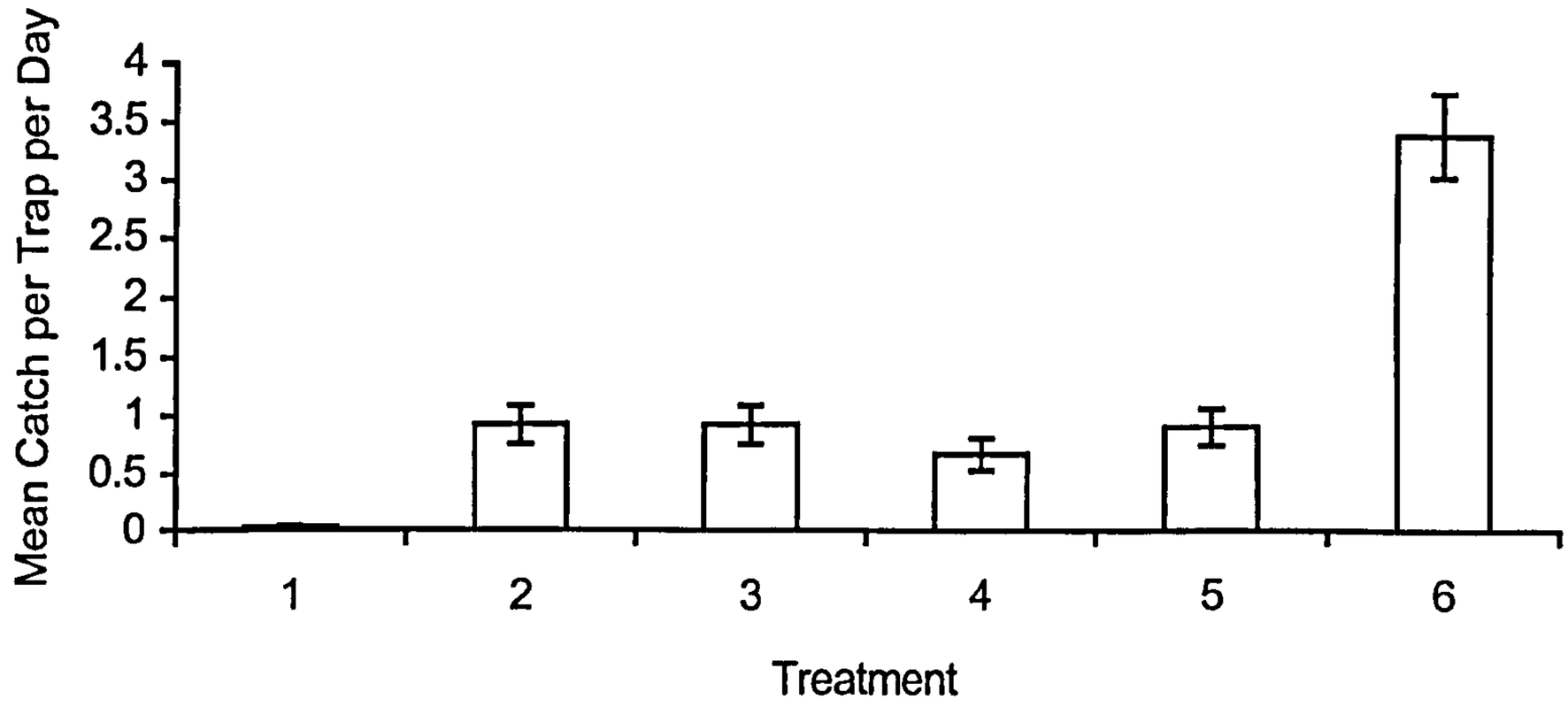

Fig 9.14 Catches *Apis mellifera* in Experiment 5.

A. mellifera catches in Experiment 5 are shown in Fig 9.14. Significantly fewer *A. mellifera* were caught with the 3-component blends omitting phenylacetaldehyde (blends 2, 3, 4 & 5) than with the full marigold blend (blend 6) ($P < 0.001$). Significantly more *A. mellifera* were captured with the floral baited traps than the unbaited control traps ($P < 0.001$).

Statistical Analysis of H. armigera *Trap Catches over the Whole Season in Israel*

ANOVA with contrasts was used to analyse trap catch data for *A. mellifera* catches. However, due to the low catches in floral odour-baited traps and high standard errors this form of statistical analysis could not be undertaken for *H. armigera*. Nevertheless, when different experiments including the standard 4-component *T. erecta* trap bait (M1, see Table 9.1) and the unbaited control treatment were treated as different replicates instead of separate experiments it was possible to perform a Mann Whitney 'U' test statistical analysis.

A comparison was made between catches with the standard 4-component *T. erecta* floral bait (M1) and the control treatment over the whole season. Experiment 5 was treated as two replicates because it lasted two weeks unlike the other experiments which each lasted one week. *H. armigera* catches were summed for these two treatments for each replicate experiment and the Mann Whitney 'U' test was used to compare medians. There was a significant difference in *H. armigera* catches in

traps with the standard 4-component marigold lure compared with the unbaited control trap catches over the whole season (Table 9.15) (P = 0.0023, Mann Whitney 'U' test).

Table 9.15 Mean *H. armigera* Catch in Standard Marigold Baited and Unbaited Control Traps in Israel Trial (both sexes, per trap, per night)

Experiment	Control	4-Component Marigold Blend (M1)
1	0.025	0.175
2	0.000	0.208
3	0.000	0.028
4	0.000	0.056
5 (week 1)	0.000	0.111
5 (week 2)	0.000	0.111
Mean	0.004	0.115

Female Dissection to Determine Mated Status

Of the 48 female *H. armigera* from the floral traps that were examined, 36 contained spermatophores i.e. 25% were unmated. Ten out of twelve (83%) of the female silver Y moth, *A. gamma*, (Lepidoptera; Noctuidae) examined were unmated. Insects in Experiment 1 were not dissected and in Experiments 4 and 5 only *H. armigera* were dissected. These data are shown in Table 9.16.

Table 9.16 Female Dissection Data for *H. armigera* and *A. gamma*

Insect	Fertility	Expt. 2	Expt. 3	Expt.3a	Expt. 4 & 5	Total
H. armigera	Mated	6	3	5	22	36
	Virgin	4	3	3	2	12
A. gamma	Mated	2	0	0	na[a]	2
	Virgin	7	3	0	na	10

[a] Not available

Independent Assessment of H. armigera *Population Density*

Results of the independent assessment of *H. armigera* population density carried out using sex pheromone and light baited traps are reported in Table 9.17.

Table 9.17 Mean *H. armigera* Catch in Pheromone and Light Traps in Israel Trial

Experiment	Dates	Pheromone Trap (no. per trap, per night)	Light Trap (no. per trap, per night)
1	10/05-15/05/99	19.9	na[a]
2	18/05-24/05/99	12.3	3.0
3	25/05-31/05/99	11.0	0.0
3a	01/06-06/06/99	6.2	1.0
4	06/06-16/06/99	2.5	1.75
5	17/06-27/06/99	1.0	1.0
Mean		8.8	1.35

[a] Not available

Release Rates of Floral Volatiles

Representative sachets from each treatment were hung in the shade and weighed daily to record weight loss from which release rates were calculated. These data are shown in Table 9.18.

Release rates ranged from 0.12 mg / h for the double thickness sweet pea sachet (S2) used in Experiment 2b to 4.40 mg / h for the sachet containing benzaldehyde alone in Experiment 4. The sachets containing limonene which were formulated with dioctyl phthalate (Experiments 1, 2a, 3a & 4) released from 0.20 to 0.37 mg / h. The Marigold (M1) sachets (Experiments 1, 2a, 3a & 4) released from 0.30 to 0.75 mg / h. These changes in emission rate are of a similar size to the changes observed when temperature was changed by 5°C in the laboratory (Chapter 8) and thus could have been caused by differences in temperature between experiments.

Table 9.18 Release Rates from Sachet Formulations used in Israel (See above in results of individual experiments for details of sachets used).

Experiment	Sachet Treatments & Release Rates (mg/h)				
1	Marigold 0.30	Sweet Pea 0.17	Mixture 0.51		Limonene 0.20
2a	M1 0.75	M2 0.33			Limonene 0.37
2b	S1 0.47	S2 0.12			
3a	Marigold 0.47	M - B 0.54	M - P 1.38	M - Lin 0.55	Limonene 0.24
3b	Sweet Pea 0.40	S - BA 0.50	S - D 0.44	S - Lin 0.24	S - P 0.40
4	Marigold 0.60	Benzaldehyde 4.40	M - B 0.63	M - P 2.02	Limonene 0.32

B = benzaldehyde; P = phenylacetaldehyde; Lin = linalool; BA = benzyl alcohol; D = diacetone; M = 4-component *T. erecta* blend

9.3.2 Pakistani Field Trials

There was an exceptionally low population of *H. armigera* during the time of the field trial in Lodhran, Pakistan, Oct-Nov 1999 (D. Chamberlain, pers. com.). In pheromone monitoring traps on average less than one *H. armigera* per trap per night was caught over the season (Table 9.25). Because floral odour-baited traps caught other moths than *H. armigera*, comparisons between treatments were made in terms of total Lepidoptera caught and numbers of spiny bollworm, *Earias insulana* and spotted bollworm, *E. vittella*, caught. Far fewer Hymenoptera were caught in Pakistan than in Israel.

Pakistan Experiment 1, Comparison of Different Ratios of Components in the Synthetic* T. erecta *Blend

Experiment 1 was based on the 4-component *T. erecta* blend (Israel Experiment 1, "M1" Treatment) used in field trials in Israel. Benzaldehyde, linalool and phenylacetaldehyde were held in a 4 x 5 cm polyethylene sachet, and (-)-limonene was held in a vial with 100µl cotton seed oil added to slow its release rate. The plasticiser dioctyl phthalate used in Israel was not available in Pakistan and air freight safety regulations prevented its importation. Treatments varied by changing the ratios of benzaldehyde, linalool and phenylacetaldehyde (Table 9.19). The trial was laid out as two 7 x 7 Latin squares and run for 24 nights (336 trap-nights).

Table 9.19 Treatments in Pakistan Field Experiment 1

Treatment	Volume Used (µl)			
	(-)-Limonene	Benzaldehyde	(-)-Linalool	Phenylacetaldehyde
1	100	125	125	125
2	100	375	125	125
3	100	1250	125	125
4	100	170	17	340
5	100	50	17	340
6	100	17	17	340
Control	0	0	0	0

Table 9.20 Total catches of insects during Experiment 1 in Pakistan, 1999, testing effect of changes in composition of 4-component floral attractant on trap catch (7 replicates for 24 nights). Treatments as in Table 9. 19.

Total insect catch	Treatment						
	1	2	3	4	5	6	Control
Male	2	1	2	0	2	2	0
Female *H. armigera*	0	1	0	1	0	0	0
E. vittella	64	59	73	72	64	122	33
E. insulana	48	85	87	95	87	133	23
P. gossypiella	0	0	0	0	0	0	0
Spodoptera spp.	3	0	1	1	2	4	0
D. indica	14	16	8	11	5	20	0
Other Moth spp.	70	79	84	88	63	105	53
Total Lepidoptera	201	241	255	268	223	386	109
Jassid spp.	27	20	30	18	20	36	26
Lacewing spp.	38	35	52	50	45	69	7
Coleoptera spp.	2100	1797	1635	2198	1917	3363	1124
Diptera spp.	103	124	159	141	121	169	204
Halictus	15	2	19	9	13	20	2
Apis spp.	0	1	0	0	1	1	0
Other Hymenoptera	3	1	1	2	2	2	0
Spider spp.	3	11	3	6	7	12	4

Total trap catches in Experiment 1 are shown in Table 9.20. An ANOVA was carried out and significant differences were found between treatments for numbers of *Earias* spp. caught ($P = 0.001$) and total Lepidoptera caught ($P < 0.001$). Significantly more *Earias* spp. and total Lepidoptera were caught in floral, odour-baited traps than in unbaited control traps ($P < 0.001$). However, there were no significant differences in trap catches between treatments.

Pakistan Experiment 2, Investigation of Effect of Addition of Piperitone to* T. erecta *Synthetic Blend and Using Racemic Linalool

In Experiment 2 the effects of including (-)-piperitone in the *T. erecta* synthetic blend and of using racemic linalool unstead of (-)-linalool were tested. Treatments are shown in Table 9.21. The *T. erecta* synthetic compounds were pipetted into 4 x 5 cm sachets except (-)-limonene which was contained in a vial (100µl in vial + 100µl cotton seed oil). There were 370 trap-nights per treatment.

Table 9.21 Treatments in Pakistan Field Experiment 2

Treatment	Volume Used (µl)					
	(-)-Limonene	Benz-aldehyde	(-)-Linalool	(+)-Linalool	Phenylacet aldehyde	(-)-Piperitone
1	100	17	8.5	8.5	333	0
2	100	17	17	0	333	0
3	100	17	8.5	8.5	333	133
4	100	17	17	0	333	133
Control	0	0	0	0	0	0

Table 9.22 Total catches of insects during Experiment 2 in Pakistan, 1999, testing changes in composition of 4-component floral attractant (10 replicates for 37 nights). Treatments as in Table 9. 21.

Total insect catch	Treatment				
	1	2	3	4	Control
Male *H. armigera*	0	0	2	0	0
Female *H. armigera*	0	1	0	0	0
E. vittella	28	20	22	15	1
E. insulana	49	53	26	39	7
P. gossypiella	0	0	0	0	1
Spodoptera spp.	3	3	5	1	1
D. indica	18	11	13	11	0
Other Lepidoptera	151	172	190	185	59
Total Lepidoptera	249	260	258	251	69
Jassid spp.	21	15	11	12	20
Lacewing spp.	91	88	103	101	3
Coleoptera spp.	1139	1211	919	1021	405
Diptera spp.	298	287	252	292	333
Halictus	0	0	2	1	2
Apis spp.	0	0	0	1	0
Other Hymenoptera	1	0	1	0	1
Spider spp.	0	0	1	0	0

Total trap catches in Experiment 2 are shown in Table 9.22. Significantly more Lepidoptera and *Earias* spp. were caught in floral odour baited traps than in unbaited traps (ANOVA, $P < 0.001$). There were no significant differences between treatments with (±)-linalool (1 and 3) or (-)-linalool (2 and 4) or between treatments including piperitone (3 and 4) and those omitting piperitone (1 and 2).

Pakistan Experiment 3, Effect of Trap Colour on Catch

In Experiment 3 different coloured traps were compared in an 8 x 8 grid in which blue-yellow-white traps (blue lid, yellow funnel, white base) were alternated with all green traps. The standard 4-component *T. erecta* blend (Israel Experiment 1, "Marigold" Treatment) was used as the lure. There were 288 trap-nights per treatment.

Table 9.23 Total catches of insects during Experiment 3 in Pakistan, 1999, testing different trap colours (32 replicates for 9 nights).

	Funnel trap colour	
Total insects caught	Blue-Yellow-White	Green
Male *H. armigera*	1	3
Female *H. armigera*	0	0
E. vittella	29	27
E. insulana	16	22
P. gossypiella	0	0
Spodoptera spp.	6	1
D. indica	13	9
Other Moth spp.	30	49
Total Lepidoptera	95	111
Jassid spp.	60	52
Lacewing spp.	30	34
Coleoptera spp.	1193	1331
Diptera spp.	210	182
Halictus spp.	68	3
Apis spp.	4	0
Other Hymenoptera spp.	8	1
Spiders	7	13

Total trap catches in Experiment 3 are shown in Table 9.23. There was no significant effect of trap colour on insect catches. It is possible that more Hymenoptera were caught with blue-yellow-white coloured than the green coloured traps but the numbers caught were too low for statistical analysis.

Pakistan Experiment 4, Comparison of Different Trap Heights

In Experiment 4 the effect of placing floral odour-baited traps at 1.0 and 3.0 m on trap catch was compared (1.0m traps were level with the top of the crop canopy, 3.0m traps were 2m above it). The standard 4-component *T. erecta* blend (Israel Experiment 1, "Marigold" Treatment) was used as the lure. Traps were laid out in a 4 x 4 matrix with traps of different heights systematically alternated. The trial was conducted for a total of 416 trap-nights per treatment.

Table 9.24 Total catches of insects during Experiment 4 in Pakistan, 1999, testing different trap heights (16 replicates for 26 nights). Treatments as in Table 9. 21.

Insects caught	Trap height	
	Short (1.0m)	Tall (3.0m)
Male *H. armigera*	0	0
Female *H. armigera*	1	2
E. vittella	66	22
E. insulana	37	7
P. gossypiella	0	0
Spodoptera spp.	21	5
D. indica	121	26
Other Moth spp.	138	69
Total Lepidoptera	384	131
Jassid spp.	28	7
Lacewing spp.	48	55
Coleoptera spp.	655	578
Diptera spp.	254	307
Halictus spp.	0	2
Apis spp.	0	0
Other Hymenoptera	1	0
Spider spp.	10	2

Total trap catches in Experiment 4 are shown in Table 9.24. Most insects, except Diptera, were caught in lower numbers in the 3.0m traps than in the 1.0m traps. Thus for example, traps at 1 m caught significantly more Lepidoptera than those located at 3 m height (Mann Whitney 'U' test, $P = 0.001$).

Independent Assessment of H. armigera *Population Density*

Thirteen *H. armigera* pheromone-baited traps were situated around the experimental farm to provide an independent measure of adult population density over time. Mean male moth catch (per trap per night) for each week was calculated as shown in Table 9.25.

Table 9.25 Mean *H. armigera* Pheromone Trap Catches on experimental farm

Dates	Mean Trap Catch (per trap per night)
24/07/99 - 30/07/1999	0.01
31/07/99 - 06/08/1999	0.02
07/08/99 - 13/08/1999	0.02
14/08/99 - 20/08/1999	0.00
21/08/99 - 27/08/1999	0.05
28/08/99 - 03/09/1999	0.11
04/09/99 - 10/09/1999	0.06
11/09/99 - 17/09/1999	0.33
18/09/99 - 24/09/1999	0.75
25/09/99 - 01/10/1999	1.67
02/10/99 - 08/10/1999	1.15
09/10/99 - 15/10/1999	0.87
Mean	0.42

9.4 Discussion

Experiments in Pakistan are discussed separately from those in Israel due to the much lower *H. armigera* population during the time of the field trapping experiments in Pakistan. The mean pheromone trap catch per trap per night in Pakistan was 0.42 (Table 9.25) compared with 8.8 in Israel (Table 9.17).

9.4.1 Trapping Experiments in Israel

Main Conclusions of Field Experiments

The following three observations concerning floral odour baited traps and *H. armigera* were apparent from the results of the field trapping experiments in Israel:

1. Synthetic floral odour-baited traps caught significantly more *H. armigera* than unbaited control traps. This difference was highly significant ($P = 0.0023$) when catches in the standard 4-component *T. erecta* based floral odour baited traps were compared with those in the control traps over the whole season (Table 9.15).
2. Despite this the mean *H. armigera* trap catch in floral odour baited traps of 0.11 moths/trap/night for the standard *T. erecta* blend was much less than the mean catch in pheromone traps (9.8 moths/trap/night) and in light traps (1.35 moths/trap/night) (Table 9.17).
3. The floral odour-baited traps were non-selective catching large numbers of Hymenoptera and Diptera in addition to other moth species.

Findings in Relation to the Research Hypothesis

The significant increase in *H. armigera* catch with floral odour-baited traps in the field ($P = 0.0023$, Mann Whitney 'U' test) supported the hypothesis that olfactory cues play a role in host location by *H. armigera* (Section 1.2). This finding validates the wind-tunnel results in which insects were observed to increase upwind flight significantly when synthetic blends of *T. erecta* and *L. odouratus* floral volatiles were present.

Insects Other than H. armigera *Attracted to the Floral Lure*

The traps had little selectivity since other flower visiting insects were also attracted and trapped. Significantly more *H. armigera*, *Autographa gamma*, the honeybee *A. mellifera*, a wasp *Halictus* spp. and lacewings (Chrysopidae, mostly *Chrysopa carnea*) were caught in traps baited with synthetic marigold, *T. erecta*, and sweet pea, *L. odouratus*, floral volatiles than in unbaited control traps. A large proportion of the insects caught were beneficial, mostly because they were pollinators (*A. mellifera*, *Halictus* spp.) but lacewings, which are predators of aphids and whitefly, were also caught. The lack of selectivity was an advantage, to a limited extent, in that two other pest noctuids were caught in the floral baited traps. These were silver Y moth, *A. gamma*, which was caught more with the marigold than the sweet pea blend, and the armyworm *Spodoptera littoralis*. Due to the large numbers of beneficials captured the lack of selectivity was on the whole undesirable as far as potential use of the traps for pest management purposes is concerned. Laboratory studies with one species do not give an indication of effects such as these and the mixture of species attracted to plant odours tends not to be mentioned in the literature when single species are being reported on, although Meagher & Mitchell (1999) noted the large numbers of Hymenoptera caught by traps baited with phenylacetaldehyde.

Release Rate of Floral Volatiles from Sachets

It was decided to start with a formulation that gave at least the same release rate as the filter paper used in the wind-tunnel bioassay because traps in the field have to attract moths from a much larger volume of air. In the order of 0.5 mg/h of benzaldehyde, (-)-limonene, (±)-linalool and phenylacetaldehyde, depending on field conditions, was emitted from the sachets described here (Table 9.18). The release rates of these compounds from filter papers in the wind-tunnel was 0.05mg/h (Table 8.4) and their release rate from a live *T. erecta* flower was approximately 0.002 mg/h (Table 5.11). Thus, the release rate of *T. erecta* volatiles from the formulation used in the field was about 250 flower equivalents.

Comparison of Different Floral Blends and Emission Rates

The synthetic blends tested were based on two different plant sources which were remarkably similar in their ability to attract *H. armigera.* In Experiment 1 there was no significant difference between trap catches of *H. armigera* in traps baited with a synthetic marigold, *T. erecta*, blend and traps baited with a synthetic *L. odouratus* blend. Two alternative formulations were tested against the standard single-walled sachet in Expt. 2. These were a double-walled sachet and a baited rubber septum. The double-walled sachets gave a release rate approximately 3 times lower than the single-walled ones (Table 9.18). Even when using polyethylene sachets, which already give a high emission rate compared with other formulations such as vials and septa, the higher release rate provided by a single rather than double sachet layer increased the *H. armigera* trap catch with the *L. odouratus* blend. Due to the low captures per trap per night this difference was not statistically significant. There was no difference in trap catch of female and male *H. armigera* with single- and double-walled synthetic *T. erecta* sachets, although both types of sachets caught more than rubber septa which would have given a high release rate but only for a short period (P. Innocenzi, *pers. comm.*).

Reduced Component Synthetic Floral Blends

Tests with incomplete synthetic blends (Experiments 3 and 4) implied that the presence of phenylacetaldehyde was important for the attractiveness of the synthetic sweet pea blend (Table 9.10) to female and male *H. armigera.* However, when phenylacetaldehyde was removed from the *T. erecta* 4-component blend and a larger amount of benzaldehyde was used, the numbers of *H. armigera* and other Lepidoptera caught were similar to those caught with the complete blend whereas numbers of other insects decreased (Tables 9.8 & 9.12). Significantly fewer *A. mellifera*, were caught when phenylacetaldehyde was omitted from the synthetic *T. erecta* blend (ANOVA contrast, $P = 0.013$, Expt. 3; Fig 9.8). The marigold blend still caught *H. armigera* when reduced to a 2-component blend comprising benzaldehyde and (-)-limonene although less so than the 3-component blend ((±)-linalool, benzaldehyde and (-)-limonene).

Independent Assessment of H. armigera *Population Density*

To obtain an independent assessment of *H. armigera* pest population density in the field trial, two adult monitoring methods other than the floral odour-baited traps in the main experiments were employed in Israel. A light trap caught on average 1.35 *H. armigera* per night during the experiments and pheromone traps caught on average 8.8 *H. armigera* per trap per night (See Table 9.17). They indicated that *H. armigera* population densities were moderate but not at epidemic levels. Up to 762 *H. armigera* have been caught in one pheromone trap over one night at ICRISAT, India (Pawar *et al.*, 1988) which would be considered an epidemic. Nevertheless, the economic threshold of seven moths per pheromone trap per night (Deva Prasad *et al.*, 1993) was exceeded.

9.4.1 Trapping Experiments in Pakistan

In Pakistan there was a very low *H. armigera* population over the whole season as judged by pheromone trap catches (mean 0.42 moths/trap/night). Consequently, the low catches in floral odour-baited traps meant that no comparisons between different treatments in terms of numbers of *H. armigera* caught could be made. However, significantly more *E. insulana* and *E. vittella*, were caught in floral odour-baited traps than in unbaited traps ($P < 0.001$). Using different ratios of behaviourally-active compounds identified in flowers of *T. erecta*, addition of (-)-piperitone to the 4-component blend or using different trap colours had no significant effect on catches of Lepidoptera in general and *Earias* spp. in particular (Tables 9.20, 9.22 & 9.23) but increasing trap height from 1 to 3 m significantly reduced catches (Table 9.24). Sinha & Mehrotra (1993) found that the optimal trap height for pheromone trap catches of male *H. armigera* was when the trap was just above the vegetation (1.9m). The findings of Firempong & Zaluki (1990a) also imply that a trap prominent above the vegetation would be more effective because sunflower was preferred by *H. armigera* at its natural height than when its flowers were presented at a lower height. However, raising the traps 2m above crop height reduced trap catches of Lepidoptera in the current study. It would appear that the trap should be just above

the crop but not raised excessively high to maximise catches with the floral bait. It was noted that catches of Hymenoptera in Pakistan were approximately two orders of magnitude lower than in Israel. This was possibly due to use of broad spectrum pesticides in Pakistan that killed Hymenoptera.

9.4.3 Potential Practical Applications of Floral Odour Baited Traps in Pest Management

Trap performance can be evaluated using the relatively simple criterion of number of moths caught per trap per night in relation to the size of the local *H. armigera* population as measured by more conventional monitoring methods such as light and pheromone traps. In Israel mean *H. armigera* catch per trap per night over the whole season in unbaited traps, floral odour traps, pheromone traps and light traps were 0.004, 0.11, 8.8 and 1.35 respectively (Tables 9.15 & 9.17).

Monitoring

Monitoring requires the trap catch to give a good indication of the potential threat to the crop. Numbers caught per trap per night need not be high so long as they correlate well with the likelihood of crop damage by the larvae developing from the eggs of the female moths. Since a floral trap can catch female insects, on which oviposition depends, it is possible that they could be useful for monitoring purposes. Maini & Burgio (1999) found a good correlation between female European corn borer, *Ostrinia nubilalis*, captures in phenylacetaldehyde baited traps and ensuing crop damage. Because *H. armigera* is so mobile any improvements in monitoring its populations by keeping track of populations of adult female moths in an area could lead to better informed pest management decisions about pesticide applications.

Apart from correlation of trap catch with ensuing crop damage the other, perhaps even more important, consideration for monitoring is the sensitivity of the trap to early infestation. If the trap does not catch moths until there has already been considerable oviposition in the crop the warning given could be too late for applying a pesticide spray or other crop protection measure. The economic threshold can be exceeded by fewer than 5 females per acre (Lingren *et al.*, 1982). It should be noted

that large larvae are a more difficult spray target than small larvae because they require a higher dose of active ingredient to be killed (Lingren *et al.*, 1982). This means that monitoring should give as much warning as possible so that arrangements for an early insecticide application can be made.

A problem for use of the traps by farmers is their lack of selectivity. Whoever checked the trap would have to distinguish adult *H. armigera* from the other insect species that were caught by the trap. There was the advantage that other moth pests were caught with the floral traps, *e.g. Autographa gamma*, *Spodoptera littoralis*, *Earias vittella* and *Earias insulana* but again this would require more taxonomic training to evaluate than many farmers might have. Perhaps traps would be best used by extension personnel in their monitoring programmes. Modifications to the trap and bait could be made to improve selectivity, possibly by adding a bee repellent. Possible bee repellents include methyl salicylate and 3-octanone (Henning *et al.*, 1992). If the number of traps used was small then traps could simply be removed during the daytime to prevent bee captures.

For any pest population prediction from monitoring to be useful for management purposes (Maelzer & Zalucki, 1999) it would need to:

- provide information of value to the manager
- be cost effective
- be made well in advance of when it would be used

Information on the arrival of egg-laying female *H. armigera* into a crop is valuable information and monitoring the adult stage before larvae develop would give a timely warning. However, with only a low level of attraction into the floral trap the number of traps needed might lead to unacceptable costs and might compromise capabilities for early detection. Cardé & Elkinton (1984) considered the key features sought in a trapping system used for monitoring to be:

- low cost
- sensitivity & specificity for the target species
- user convenience

The floral traps developed in this study, unfortunately, do not meet any of these criteria of Cardé & Elkinton particularly well.

Traps were not used for predicting the threat to the crop or for timing of pesticide applications in the current study and so there is insufficient evidence to decide whether the floral baited traps used here would be reliable for monitoring. The low catches per trap per night in the Israel trial (average 0.11) mean that caution would be needed when considering basing spray timing decisions on floral trap captures. Pesticide applications were made because of concern about potential damage by *H. armigera* in the fields in Israel while the trapping experiments were in progress. If the attractancy of traps designed to catch female moths meant that they were sensitive enough then the next step would be to calibrate the traps in terms of the trap catch at which it becomes worthwhile to control the pest.

Mass Trapping

For mass trapping purposes the floral lure used in the field trials reported here caught too few moths per night. Although the field population level was only moderate in Israel and low in Pakistan (as assessed by sex pheromone traps), the numbers of female moths caught in floral traps would have had to have been higher for the idea of mass trapping to be viable as a crop protection method.

Mass trapping using pheromone lures is ineffective for control of *H. armigera* populations because only male moths are caught. With an average male mating with five females, up to 80% of the males could be removed without affecting female fecundity (Lanier, 1990). Despite the capture of egg-laying females being more effective in reducing populations than catching males, catches of males in *H. armigera* pheromone traps themselves have been considered too low for mass trapping (Cork & Hall, 1998) and catches with a floral trap ideally should exceed those with the pheromone trap, if mass trapping is to work. However, the floral traps were observed to catch far fewer *H. armigera* than pheromone traps. With a low level of attraction into the trap there is too much of a risk that a mated female moth could lay eggs in the crop before being attracted into the trap. One female can lay in excess of 1000 eggs (Fitt, 1989). Also with low attraction the number of traps required per hectare would need to be higher and this might be uneconomic. A large number of traps could interfere with other cultivation practices where a tractor requires access to the crop.

Advantages of Floral Odour-Baited Traps

Having discussed some limitations of floral baited traps, some advantages should also be mentioned. Being able to trap female insects may mean that the floral odour-baited traps described here might provide different information on pest population densities compared with pheromone traps for pest monitoring. The high mobility of adult moths (Gaines, 1933) could mean that numbers of male moths caught in pheromone traps were not related to the number of eggs in an area. Maini & Burgio (1999) found a better correlation between female captures of European corn borer, *O. nubilalis*, in traps baited with phenylacetaldehyde and subsequent crop damage by larvae than with male captures in pheromone traps. Being able to catch female moths also means that their mated status can be determined which can provide useful information about the threat of oviposition in a crop. Unlike a light trap the floral odour-baited trap does not require a power source avoiding the need for a long cable or continual recharging and replacement of batteries. Pest monitoring and forecasting has taken an increasingly important role in insect management and insecticide resistance management because some pest problems are induced by over-application of insecticides and there is a need to rationalise the insect pesticide applications (Wightman & Ranga Rao, 1993).

Research on Floral Lures for H. armigera

When making conclusions it should be remembered that the floral trap used in this study might not be the best possible floral trap. However the multicomponent lure used is probably an improvement on previous single component lures which only contained phenylacetaldehyde (Pawar *et al.*, 1983). Because other research groups have been working on floral attractants for *Helicoverpa* species for a number of years with limited success in the USA (Shaver *et al.*, 1998, USDA) and Australia (Gregg *et al.*, 1998) some more fundamental considerations regarding use of host-plant volatiles as trap baits might need to be addressed. If host-plant odours elicit an increase in searching flight which is not well directed towards the odour source and might require additional visual cues once the insect is closer to the source even the best synthetic kairomonal blend might be too weak an attractant for use as a trap bait. Moths landing

on the crop in the vicinity of traps rather than directly in them would be not be prevented from ovipositing even though the floral volatiles identified in the current study were of importance in host-plant location. Night observations indicated that moths were flying about near the traps rather than directly into them but there was no quantitative statistical analysis of these observational data.

Other Possible Applications of the Floral Odours

Knowledge of the chemical identity of olfactory cues emitted from flowers that make them attractive to insect pests could have practical implications other than their potential for use as trap baits. Once the attractive volatiles are known they could be a trait to select against when breeding resistant varieties (Jackson, 1990). Since insect behaviour drives insect-plant interactions it is an important aspect to consider when attempting to identify characters that make crops resistant to insect attack (Benedict *et al.*, 1988). Attractive chemicals could be sprayed onto unfavourable host plants, which would not support larval development, to distract insects from oviposition on more favourable crops (Unnithan & Saxena, 1990) or be used as a component of a "push-pull" pest management strategy (Pyke *et al.*, 1987).

The efficacy of the traps in catching *A. mellifera* could be exploited as a positive feature. Bees captured in the floral baited traps could be used to spread *H. armigera* nuclear polyhedrosis virus which is specific to *H. armigera*. An attractant baited trap in which insects are innoculated with pathogen and subsequently disperse it in a crop has been described by Pell *et al.* (1993). The idea of using bees to deliver biocontrol agents against any insect that feeds on flower heads that bees visit has been suggested by Corliss & Adams (1992). However, this might be too complex for field implementation (D. Grzywaczs, pers. com.) where new technologies have a better chance of implementation if they are kept simple.

Use of Floral Odours to Monitor Apis mellifera *Populations*

Instead of being used to monitor *H. armigera* the floral baited traps could be used to monitor honey bee, *A. mellifera*, populations in agricultural situations where pollination by bees is important and it is suspected that conventional insecticides with broad spectrum unselective action are reducing bee populations. A trap effective at

sampling the local bee population could provide a useful indicator of population levels. Information provided by bee monitoring traps could be used to support grower decisions about whether to artificially supplement the bee population by introducing bees in hives or whether to change the type or quantity of insecticides applied to the crop.

Admittedly the traps were developed with a different application in mind, namely to monitor moth populations, but it is not surprising that bees are attracted to a floral odour based lure. It has been documented that odours provoke much stronger discrimination between flowers than do visual stimuli for bees and that some moth-pollinated plants keep their flowers open during the day, suggesting that other, diurnally active insects may participate in their pollination (Dobson, 1994). There is no reason why the floral-baited traps could not be used for a different application if is it proven to be useful. Further tests would be necessary to discover the relationship between bee catches in floral traps and local bee population levels.

Chapter 10

GENERAL DISCUSSION

10.1 Introduction

The null hypothesis being tested by the current research, as stated in Chapter 1, was:

"Volatile floral compounds play no role in the attraction of the polyphagous moth species *Helicoverpa armigera* to the flowers of its host plants"

The alternative hypothesis was that floral volatiles do play a role in attraction to host-plant flowers.

The potential role of volatile floral compounds emitted from host plants of *H. armigera* in the host-plant finding process by adult moths of this species was evaluated. Electrophysiological analyses were used to screen for compounds in the natural samples which could be detected by the antennae, wind-tunnel bioassays were carried out to investigate whether collections of floral volatiles and synthetic blends altered the behaviour of flying moths, and field trapping experiments were conducted to verify laboratory responses in a field context. The main host-plant from which volatile collections were made was the African marigold, *T. erecta*, although some work was also done on another host-plant, the sweet pea, *L. odouratus*, as Leguminosae are favoured hosts (Fitt, 1989).

10.2 Evidence obtained supporting a role for olfaction in the process of host-plant finding by *H. armigera*

Wind-Tunnel Responses to Natural Samples

Behavioural responses to the floral headspace samples, collected on Porapak Q, were demonstrated in wind-tunnel bioassays. Significant increases in upwind flight of *H. armigera* were obtained when air entrained *T. erecta* and *L. odouratus* headspace samples were present (Chapter 7, Series 3 & 12, Fig. 7.2 & 7.14). Air

entrained extracts of volatiles from *T. erecta* flowers on filter paper were found to be significantly more attractive than the solvent control by comparing the median number of approaches (P = 0.014, Fig 7.2), furthest distance flown upwind (P = 0.034, Fig 7.3) and number of source contacts (P = 0.005, Table 7.5). A headspace sample of sweet pea, *L. odouratus*, floral volatiles also appeared to be attractive giving a significantly higher mean number of upwind approaches (P = 0.008, Fig 7.14) when it was present and elicited source contacts which were not obtained with the control treatment. *Buddleia davidii*, which is not a host plant of *H. armigera* floral volatiles did not increase upwind flight activity when collected and tested in the same way as the *T. erecta* and *L. odouratus* samples.

GC-EAG Responses to Specific Floral Compounds

It is likely that volatile attractants are perceived by receptors on the antennae. The behaviourally active collections of floral volatiles were shown to elicit electroantennogram responses from female *H. armigera*. Linked GC-EAG analyses were used to determine which particular components of the mixture caused EAG responses and prioritise compounds for testing in subsequent wind-tunnel bioassays. With *T. erecta* floral headspace samples five EAG responses above the background level were identified using a polar column and again when using a non-polar column (Table 4.3). Four EAG-active compounds were identified in sweet pea, *Lathyrus odouratus*, air entrained samples (Table 4.4). Retention indices of the compounds were calculated. Typically an increase in depolarisation of around 175% of the background response was observed at the retention times where there was an olfactory response.

Identification of Compounds

Compounds in the *T. erecta* and *L. odouratus* extracts were identified using gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS) analytical techniques (see Chapter 5). The best fits for the EAG-active *T. erecta* compounds from EI-MS were (*E*)-myroxide, benzaldehyde, linalool, phenylacetaldehyde and piperitone (Table 5.1). These tentative structural assignments were confirmed by obtaining synthetic compounds of known chemical

purity and comparing Retention Indices and mass spectra on both polar and non-polar columns. Other components of *T. erecta* headspace were identified (Table 5.1) as (*Z*)-ocimene, limonene, β-caryophyllene and myrcene. Configurations of chiral compounds limonene, linalool and piperitone were determined by comparison of GC retention times with authentic synthetics as (+)-linalool, (-)-linalool, (-)-limonene and (-)-piperitone using the cyclodextrin column (Table 5.4). EAG-active compounds in *L. odouratus* floral headspace samples were identified as diacetone (2-hydroxy-2-methyl-4-pentanone), (-)-linalool, phenylacetaldehyde and benzyl alcohol (Table 5.6).

EAG with Synthetic Compounds

EAG tests with identified synthetic compounds were carried out to confirm their electrophysiological activity. The five *T. erecta* compounds myroxide, benzaldehyde, (±)-linalool, phenylacetaldehyde and (-)-piperitone which were suspected to have EAG activity in linked GC-EAG analyses were confirmed as being EAG-active at a dose of 1μg on filter paper (Table 6.1 & 6.2). EAG responses ranged from -0.60 (± 0.11) mV for (±)-linalool to -0.85 (± 0.15mV) for (-)-piperitone compared with a mean control response of –0.43 (± 0.06 mV). Significantly higher EAG responses than with the solvent control were obtained ($P < 0.05$, Tables 6.1 & 6.2). Four other compounds present in the *T. erecta* air entrained samples were also tested. (+)-Limonene, β-caryophyllene and myrcene were not EAG-active at a dose of 1μg on filter paper but (*Z*)-ocimene and (-)-limonene were. The *L. odouratus* compounds diacetone, linalool, phenylacetaldehyde and benzyl alcohol also gave significant EAG responses (Table 6.1 & 6.4). Thus, it was shown that *H. armigera* was capable of detecting specific volatile floral compounds.

Wind-tunnel Responses to Synthetic Blends of Floral Volatiles

Synthetic kairomonal blends based on floral odours collected from *T. erecta* and *L. odouratus* were tested for their attractiveness using the wind-tunnel bioassay. Significant increases in number of upwind flights were obtained with synthetic *T. erecta* and *L. odouratus* blends ($P = 0.0008$ and 0.014 respectively, Fig 7.4 & 7.16). If insects flew upwind beyond the solvent control treatment they tended not to

fly downwind again or make any further upwind forays. However, insects presented with natural floral odour sources and the synthetic mimic blends derived from them made numerous attempts to re-orient towards the source, exhibiting a 'seeking type' behaviour (Brantjes, 1978). Mean furthest distance flown upwind was also significantly higher with synthetic blends than the control treatment (Fig 7.5 & 7.17).

Field Trapping Experiments

Wiseman *et al.* (1988) pointed out that laboratory tests with plant volatiles can be unrealistic when there is no choice of odours. They mention that *Helicoverpa* moths will accept even non-host stimuli when there is no choice. Thus, field trapping experiments were carried out to confirm laboratory findings. Significantly more *H. armigera* were caught in floral baited than in unbaited traps: there was a significant difference in *H. armigera* catches in traps with the standard 4-component *T. erecta* lure compared with the unbaited control trap catches over the whole season ($P = 0.0023$, Table 9.15). This provided further evidence for a role of floral volatiles in attraction to the host-plant.

In a field situation host odours released from floral odour-baited traps are not presented in isolation: volatiles released from nearby plant sources were likely to exert considerable influence on trap catches (Creighton *et al.*, 1973; Foster & Harris, 1997). Three of the four compounds used in the synthetic blend in the Israel field trials, (-)-limonene, benzaldehyde and (±)-linalool are emitted by cotton plants (Hedin, 1976). Nevertheless, in the current study significant increases in trap captures were obtained when floral volatiles were used as trap baits.

Results in Relation to the Hypothesis

Thus, significant electrophysiological and behavioural responses were observed with *H. armigera* in EAG, wind-tunnel and field trapping tests with floral volatiles. This disproved the null hypothesis that volatile floral compounds play no role in host-plant finding behaviour of *H. armigera* and confirmed the alternative hypothesis that they do play a role.

However, olfactory cues are only one of a number of cues probably used in host plant location and these are discussed below. It is likely that olfactory cues form part of a sequence of cues associated with a favourable host and that host plant choice is made after alightment on the plant and contact chemoreception has occurred. Due to time constraints in the present study, the focus was simply on establishing whether or not floral odours were involved at all.

10.3 Identification of behaviourally relevant volatiles from headspace samples

Combination of EAG and Bioassay Approaches

The floral headspace samples of *T. erecta* and *L. odouratus* each contained at least 45 volatile compounds and it was necessary to determine which of them were responsible for stimulating the increases in upwind flight observed in the wind-tunnel (Bioassay Series 3 & 12: Figs 7.2 & 7.14). A combination of electroantennographic techniques to screen for EAG-active compounds (Chapters 4 & 6) followed by wind-tunnel bioassay with putative synthetic kairomonal blends (Chapter 7) was used. This enabled the formulation and testing of simplified synthetic blends containing fewer compounds than the natural samples they were based on. The blends of compounds tested in the wind-tunnel had greater behavioural activity than single compounds tested individually. In bioassay Series 9 a 3-component blend signifcantly increased upwind flight compared to the solvent control whereas the same dose of phenylacetaldehyde alone had no effect (Fig. 7.10). To understand the sensory basis of insect host-plant finding both electrophysiological and behavioural bioassays were needed, as recommended by Hsiao (1985) and Agelopoulos *et al.* (1999).

Wind-Tunnel Responses to Blends of EAG-Active Compounds

Synthetic blends which stimulated similar levels of upwind flight from *H. armigera* to those obtained with natural samples were found (Chapter 7). However, this was achieved more quickly for *L. odouratus* than for *T. erecta*. In

bioassay Series 13 a synthetic blend of the four EAG-active compounds identified in linked GC-EAG analyses of *L. odouratus* samples elicited significantly more upwind approaches than the solvent control ($P = 0.014$, Fig 7.16) and the furthest distance flown upwind was significantly higher ($P = 0.046$, Fig 7.17). For *T. erecta* a synthetic blend containing five EAG-active compounds identified in linked GC-EAG analyses did not elicit more upwind flight in the wind-tunnel than the solvent control in bioassay Series 4 (Tables 7.7 & 7.8). There were two reasons for this complication. Firstly, the synthetic blend initially tested contained a mixture of (*E*)- and (*Z*)-myroxide whereas the natural sample contained only the (*E*)-isomer. Secondly, a compound in *T. erecta* headspace which had low EAG activity, limonene, was found to increase behavioural activity when added to the synthetic kairomonal blend.

Significant increases in upwind flight activity were obtained with a synthetic *T. erecta* blend containing the EAG-active compounds used in series 4 (benzaldehyde, (±)-linalool and phenylacetaldehyde) but omitting the mixture of myroxide isomers and including (+)-limonene[1] ($P = 0.0008$ for number of upwind approaches, bioassay Series 5, Fig 7.4). Subsequently, (*E*)-myroxide was separated from a mixture of isomers using silica column chromatography and a synthetic *T. erecta* blend containing only (*E*)-myroxide was tested in bioassay Series 7. Significant increases in upwind flight approaches were obtained with this blend ($P = 0.015$, Fig 7.8) and there was no significant difference in the response to this blend and the natural floral extract.

In bioassay Series 11 there was no significant difference in upwind flight response to odours ducted from a live *T. erecta* flower and a 6-component synthetic floral blend on a filter paper releasing approximately the same dose of volatiles (Fig 7.12). This implied that the synthetic blend was as attractive as a live flower and that no essential components had been missed.

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[1] (+)-limonene was used before the enantiomeric composition of the natural sample had been determined.

Relationship Between EAG-Activity of Plant Volatiles and their Behavioural Effect

When (-)-limonene was omitted from the synthetic *T. erecta* blend in bioassay Series 10 the level of upwind flight was reduced to that of the solvent control indicating that (-)-limonene was an important component of the host odour (Fig 7.9).

Unlike the other floral compounds used in the synthetic *T. erecta* and *L. odouratus* blends an EAG response to limonene was not detected under the conditions of linked GC-EAG analyses. A significant EAG response was only obtained in EAG studies with a 1μg dose of (-)-limonene ($P = 0.012$, Table 6.4) and the level of significance was lower than for other compounds tested. However, limonene was found to be behaviourally important in wind-tunnel bioassay Series 4 (Figs 7.4 & 7.5). The size of female EAG responses elicited by host-plant odours in Lepidoptera are typically lower than male EAG responses elicited by sex pheromone components indicating a lower sensitivity to plant volatiles (Topazzini *et al.*, 1990). The finding that EAG-activity is not directly correlated with the behavioural activity of plant volatiles agrees with the comments made by Suckling *et al.* (1996) after comparing the EAG-activity of compounds and oviposition responses of light brown apple moth, *E. postvittana*.

Advantages of Using Linked GC-EAG Analyses for Identification of Host-Odours

Assuming that volatile olfactory stimulants are percieved by the insect through the antennae, EAG provides a relatively rapid screening process for prioritising compounds that may be behaviourally active. Four EAG-active compounds identified in *T. erecta* headspace, myroxide, benzaldehyde, linalool and phenylacetaldehyde, were only minor components making up less than 4% of the total area under the GC peaks observed. If only the larger components of the natural extract had been tested in the wind-tunnel then these minor components would have been missed. Linked GC-EAG analyses were very effective for identification of the components of the synthetic *L. odouratus* blend. Given the large number of components in the natural extracts it would have been very time consuming to carry out bioassays with 4- and 5-component blends until a behaviourally active blend was

found had linked GC-EAG analyses not been used to screen the compounds beforehand.

Furthermore EAG provides a means of locating individual compounds that are perceived by receptors on the antennae. Behavioural tests may require synergistic activity of several compounds to show an effect. Thus in the present work, phenylacetaldehyde elicited an EAG response from *H. armigera* but alone caused no behavioural response in the wind-tunnel when presented at the same dose as the total of a 3-component blend (Bioassay Series 9, Fig 7.10). However it was an important component of the blend with other EAG active compounds which did cause a behavioural response.

As indicated above, there is no clear relationship between the magnitude of the EAG response and behavioural activity. In this work limonene was not detected in GC-EAG analyses (although it was subsequently shown to have EAG activity at a higher dose than in GC linked EAG analyses, Table 6.4) but was an essential component of the attractive blend (Fig. 7.9). It is possible that another behaviourally-active component could have been missed, but it is considered that no major attractive components went undetected as a 6-component synthetic blend with doses of compounds equivalent to those in the Porapak Q collection (2.6 μg phenylacetaldehyde, 0.6 μg benzaldehyde, 0.4 μg (±)-linalool, 1.0 μg (-)-limonene, 0.2 μg (*Z*)-ocimene and 1.6 μg (-)-piperitone) was shown to have the same behavioural activity as the complete sample of volatiles collected from *T. erecta* flowers (Fig 7.12).

The GC-EAG technique has been used to identify semiochemicals other than sex pheromones. For example, Hall *et al.* (1984) used a combination of GC-EAG analyses followed by wind-tunnel bioassay and field trapping experiments to identify 1-octen-3-ol as an attractant for tsetse flies from cattle odours. Cork *et al.* (1991) used GC-EAG in the identification of a major component of the male produced aggregation pheromone of the lesser grain borer, *Prostephanus truncatus*.

10.4 Host-plant Finding Behaviour of *H. armigera*

According to Dobson (1994) there are only a few examples of insect-flower interactions that have been examined in terms of olfactory interactions and reports showing links between floral odours and insects have been largely founded on circumstantial rather than experimental evidence. Natural selection would be expected to favour mechanisms which maximise benefits and minimise costs associated with searching behaviour (Bell, 1990) but the nature of the mechanisms and cues involved largely remain to be discovered.

A particular interaction has been focused on here and studied in detail in relation to olfactory cues and the findings contribute to the theory of how phytophagous insects find their host-plants. However, it must be remembered that what occurs in one insect-plant interaction could be different in another, especially if the physical attributes, life history or foraging strategy of the insect are different. The cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), was chosen due to its importance as a pest species. It is an example of a highly polyphagous *r*-selected (short generation time, high fecundity) Noctuid moth.

Biological Characteristics of H. armigera *which Influence Host-Finding Behaviour*

H. armigera is nocturnal and so visual cues would be expected to play a lesser role than with diurnal Lepidoptera. It is a relatively strong flyer and so it has more control over its direction of movement than a weaker flying insect such as an aphid and thus would be more able to react to cues perceived at a distance.

As a polyphagous insect it would not be expected to have very specialised cues for host-plant recognition. Monophagous and oligophagous insects have more rigid chemosensory requirements for their host-plants than polyphagous species (Hsiao, 1985; Ramaswamy, 1988). Species that lay few eggs over their lifetime or that lay all of their eggs on a few plants would be expected to evaluate plant quality more carefully (Damman & Feeny, 1988). However *H. armigera* is highly fecund and lays eggs singly (Fitt, 1989).

Cues Potentially Used in Host-Plant Location

There are at least four different sensory modalities that could be used by an insect in host-plant location. These are vision, olfaction, mechanoreception and gustation (Dethier, 1982). The first two stimuli occur before the insect contacts the plant. Mechanoreception and gustation, which include contact chemoreception, occur once the insect has contacted a plant. An insect's perception of its host-plant involves the integration of different stimuli and the importance of olfactory, visual, gustatory, and tactile cues varies over space (distance from the flower) and time, with some stimuli being more dominant than others (Hsiao, 1985). The interplay of stimuli from the flowers makes it a challenge to identify the effect that any single stimulus has on an insect.

The Role of Olfactory Cues

The results of the current study have demonstrated that olfactory cues play a role in host-plant finding by *H. armigera* (Section 10.2). Previously there has been some controversy about the role of olfactory cues in host-plant finding by polyphagous moth species, such as *H. armigera*. Ramaswamy (1988) suggested that for flight, orientation and landing no specific olfactory cues are involved and that host finding is mostly a random process with phototaxis and anemotaxis being important, whereas Rembold *et al.* (1991) hypothesised that polyphagous insects do utilise volatile olfactory cues for host recognition and location. Wiseman *et al.* (1988) reported that there was little actual hard evidence to support the contention that females of another Heliothine moth, *Helicoverpa zea*, orient over distances in response to host-plant volatile chemicals although there had been speculation about this (Buttery, 1978; Parsons, 1940). However, the current study has demonstrated that floral volatiles play at least some role in host-plant finding behaviour of *H. armigera* because significantly more moths were caught in traps baited with floral odours than in unbaited traps ($P = 0.002$). The compounds identified were ubiquitous and occur the floral headspace of a large number of plant species (Table 5.11) which is not surprising because *H. armigera* is a polyphagous species.

Evidence of Role of Olfactory Cues: Timing and Composition of Odours Emitted by Moth-Pollinated Plants

Periodicity in fragrance emission in moth-pollinated plants supports the idea that floral volatiles play a role in attracting pollinators: these flowers are strongly scented at night, but during the day they tend to emit weaker and qualitatively different aromas or close (Dobson, 1994; Kevan & Baker, 1983). Existence of a general moth-pollinated flower fragrance is suggested by the lack of flower specificity in the nectar feeding of moths and by the general attraction of naïve moths to floral fragrances (Cantelo & Jacobson, 1979). Night-blooming, white flowers visited by moths often contain acyclic terpenes (e.g., linalool, nerolidol, farnesol, and their corresponding hydrocarbons) accompanied by benzoid (e.g., *cis*-3-hexenyl benzoate) and nitrogen containing (especially indole) compounds (Dobson, 1994). There is still a large amount of work to be done in identifying the floral scents associated with different pollinators (Knudsen *et al.*, 1993).

Pre-Alighting Versus Post-Alighting Host-Plant Choice

Visser (1988) contrasted two possibilities regarded as extremes: insects could make their host-plant choice prior to landing on the plant (host-plant "finding") or alternatively they could make their choice on contact after alighting on plants randomly (host-plant "recognition"). If floral fragrances (pre-alighting cues) could provide species-specific signals they would increase an insect's ability to learn particular food sources. They would also enhance flower constancy which would ensure successful pollen transfer and thus sexual reproduction for the plants (Dobson, 1994). Being able to discriminate between plants prior to alighting would improve foraging efficiency and the evolutionary fitness of phytophagous insects.

Close Range Discrimination of the Host-Plant by Contact Cues

In a study of host choice at close range Ramaswamy *et al.* (1987) found that anntenectomised female *H. virescens* (which is closely related to *H. armigera*) were able to discriminate between host plants but not when all contact chemosensilla on the tarsi were destroyed. Thus, discrimination between host-plants can occur, at

close range, in the absence of olfactory cues. It is known that *H. armigera* prefers to oviposit on hairy leaves (King, 1994). The physical characteristics of the surface texture could have importance when a 'decision' on whether to accept a plant or not is made by the insect. Thus, if the plant is found unfavourable after alighting the insect could fly off and move to a different patch of habitat until reactivated by fresh olfactory cues.

Interaction of Different Cues

Insects integrate directional cues from more than one modality and so if only one modality such as olfaction is considered, it is an underestimate of the real amount of information available to the insect (Bell, 1990). Using more than one sensory modality can improve the quality of information on the host-plant available to the insect (Miller & Strickler, 1984).

A sequence of behavioural cues required by an insect in host-plant finding has been described (Jackson, 1990; Miller & Strickler, 1984; see Fig. 2.3). If subsequent cues required after olfactory ones, for example correct surface texture, are missing the insect reverts to less localised searching behaviour. Vision is also likely to play some role in the pre-alighting stage of host finding. It is well known that Lepidoptera active during the daytime are well tuned in to the colour of their host flowers (Bernays & Chapman, 1994). Nocturnal moths are less likely to be able to make clear distinctions about suitability of potential host plants on the basis of colour. Plant silhouette and reflectance of moonlight could be important though (Firempong & Zalucki, 1990a; Kevan & Baker, 1983).

According to Bernays & Chapman (1994) the olfactory signal is often the indicator of an appropriate host, causing the insect to take off and move towards the source of the odour while visual properties of leaves seem often to determine whether or not a landing is made. They see vision as giving the final degree of precision to an approach to a plant, "enabling the insect to approach and land on the target rather than simply blundering into it". Lack of odour gradients in the air means that it is unlikely that odour would lead an insect directly to a plant (Hsiao, 1985). The analogy when using a floral baited trap might be that floral odour cues

alone are insufficient to make the insect enter the trap after having flown near it and this might partly explain the low nightly trap catches in floral odour baited traps.

Perception of volatiles associated with the host-plant leads to a generalised searching behaviour (Brantjes, 1978) in which the insect becomes more likely to alight on surrounding vegetation. In a process known as 'cross-channel potentiation' a non-directional cue such as the odour from a host-plant may change the sensory threshold for another modality, for example visual pattern recognition (Bell, 1990).

Non-Directed Aspects of Host-Plant Finding

Hurtrel and Thiéry (1999) comment that well-orientated flight behaviour is not necessarily needed in order to find suitable host plants and that increased searching activity may be sufficient. Indeed, Bernays & Chapman (1994, p.156) comment that there is a "random element" in the host plant finding behaviour of phytophagous insects and that random movement coupled with arrestant responses on host plants might sometimes be a more efficient mechanism than foraging dominated by oriented responses.

Comparison of the Role of Olfactory Cues in Host-Plant Finding with their Role in Mate Finding

Volatile olfactory stimuli are known to play an important role in insect mating behaviour where male insects usually use the female emitted sex pheromone to locate calling females. However behavioural responses to volatile host-plant odours generally appear to be less specific (Hsiao, 1985) although specific responses have been obtained at the receptor level (Pickett *et al.*, 1997).

According to Miller & Strickler (1984):

> "*most insects do not find host plants by responding to volatiles with the same speed and pinpoint efficiency seemingly exhibited by responders to sex pheromones.*"

Even bees are much more sensitive to pheromones than to floral scents (Kevan & Baker, 1983). One of the main reasons for this is that olfactory responses to food odours are less specific at the behavioural level (Hsiao, 1985). Raguso (2000) reported that adult hawkmoth, *Manduca Sexta*, behavioural response to floral

fragrance, which is used as a cue in nectar foraging, is far less predictable than odour driven sexual behaviours. Plant odour blends also tend to comprise more compounds than pheromone blends (Hsaio, 1985) which means that determination of which compounds are behaviourally relevant could take longer.

In the current study, trap captures in floral odour baited traps were much lower than in nearby pheromone traps (See Chapter 9) and so it appears that volatile host-plant derived chemicals play a lesser role or are less potent in foraging behaviour than in mate location behaviour for *H. armigera*. Alternatively this may just mean that mating is a higher priority activity than feeding for adult *H. armigera* (P. Gregg, *pers. com.*). However, it is possible that to have maximal effect in host-plant finding and eliciting landing on potential hosts olfactory cues require simultaneous input from other cues e.g. the contrast of a light flower on a dark background (Kevan & Baker, 1983) or plant shape. A possible explanation for why more directed responses to olfactory stimuli involved in mate location occur is that female moths represent a smaller target area and thus require more sensitive detection mechanisms. In the case of *H. armigera* potential host-plants are often almost ubiquitous being the main ground cover in an agricultural situation.

Neurophysiology

Sex pheromone receptors are stimulated with a high degree of specificity by the chemicals involved and their very narrow response spectra do not overlap with those of other receptor cells. Stimulation produces overt behavioural responses indicating that they have direct access to the CNS and this type of encoding is known as a "labelled line" (Hsiao, 1985). On the other hand plant odours appear usually to be coded in a more complicated way according to the "across fibre pattern" where input from several receptor cells or fibres is integrated by the insect to obtain an overall picture (Dethier, 1982).

Conclusion: Process of H. armigera *Host Plant Finding*

Experimental evidence for the role of olfaction in host-plant selection behaviour by *H. armigera* has been obtained in the current study thus disproving the null hypothesis that floral volatiles played no role in such behaviour. Since

H. armigera is a polyphagous moth, the current research also disproves the hypothesis of Ramaswamy (1988) that olfactory cues are not used in host-plant location by polyphagous moths. Natural selection would be expected to favour searching mechanisms in which a preliminary assessment of the host-plant could be made prior to alighting because this would mean that time and risk could be concentrated on plants more likely to provide an oviposition site (Damman & Feeny, 1988).

However, it is unlikely that host-plant choice is made solely on the basis of olfactory cues. The amount of information that can be obtained about a plant from volatile chemicals is much less than with the chemicals contacted after alighting on the plant (Lance, 1983) because volatile compounds have simple, lower molecular weight structures. It seems probable that there is a sequence of cues leading to acceptance of a host-plant and that olfaction is more important in the earlier stages prior to contact with the plant (Miller & Strickler, 1984, Hsiao; 1985). Host-plant odours stimulate searching behaviour and make foraging moths such as *H. armigera* more responsive to other cues associated with host-plants (Brantjes, 1978; Hurtel & Thiéry, 1988; Bell, 1990; Bernays & Chapman, 1994).

10.5 Future Work

As discussed in Chapter 9 the attractiveness of the floral odour traps was too low for mass trapping. A more realistic option could be using the traps for monitoring purposes, especially in situations where information on numbers of female moths present or their mated status is required, but this also has limitations (discussed in Chapter 9) the most important of which are low sensitivity and selectivity of the traps. Studies to establish whether there is a good correlation between numbers of female moths caught in floral traps and subsequent crop damage might be worthwhile. Floral odour traps might give a more local picture of the threat of *H. armigera* damage to a particular field than pheromone traps which tend to give a more area-wide indication. A formulation of a floral kairomonal blend could possibly be applied to trap crops to increase their attractiveness (Agelopoulos *et al.*, 1999). Floral odours could also be tested as synergists for pheromone traps as

it is possible that they might increase moth captures in them. A different approach, after identifying kairomones, is to reduce the level of them by breeding out traits responsible for them in crops to increase host-plant resistance in otherwise acceptable cultivars (Mitchell *et al.*, 1991). Semiochemicals emitted by crops could be manipulated using genetic engineering.

The lure could be used to attract flower visiting insects to a source of pathogen inoculum which could subsequently be spread at sites where *H. armigera* moths visit. Using a selective biocontrol agent could overcome any problems with selectivity. Pell *et al.* (1993) used a pheromone trap for dispersal of the fungal pathogen, *Zoophthora radicans*, in diamondback moth, *Plutella xylostella*, populations. Possibly one of the most fruitful lines of future research would be development of floral odour baited traps as a device for monitoring *A. mellifera* rather than *H. armigera* populations due the highly significant increases in bee captures in the floral baited traps compared with control traps in Israel.

Having established that olfactory cues do play a role in host-plant location further work could be done to assess their relative importance compared with visual cues and the post-alighting gustatory and tactile cues. It would further our understanding of *H. armigera* host-plant seeking behaviour if the conditions necessary for alightment on a plant could be better defined. It has been shown in the present work that olfactory cues increase searching flight (more upwind appraoches in the wind-tunnel) which suggests that a visual cue, perhaps the silhouette of a flower might be required before alightment occurs. Alternatively olfactory cues associated with the host-plant might increase activity leading to more alightments on plants in the vicinity of the odour-source. Behavioural studies to establish whether the rate of alightment on a range of host and non-host plants or the leaving rate after having alighted is more important in host selection would help to show the relative importance of pre- and post-alighting cues.

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