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IDENTIFICATION OF ELECTRO - PHYSIOLOGICALLY ACTIVE COMPOUNDS IN FOUR SPECIES OF STINK BUG (HETEROPTERA).




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MASTER'S THESIS

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Table of contents

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Abstract

The current study aimed to identify compounds that could elicit cross EAD-responses in males and females of four species of stink bug: *Piezodorus hybneri*, *Dolycoris baccarum*, *Halyomorpha halys* and *Riptortus clavatus*. Using GC-EAD and GC-MS techniques, various compounds that evoked cross-antennal responses in mated males and females of the above insects were identified from hexane extracts obtained from the same four species. The insects were raised on soybean seeds throughout the study. The hexane extracts had many EAD-active compounds in common. Of all the identified compounds, 4-oxo-(E)-2-hexenal, (E)-2-hexenal and tridecane occurred in nearly every extract and evoked antennal responses in each of the four insects. 4-oxo-(E)-2-hexenal had three dimers, all of which elicited strong antennal responses. Tridecane was the most abundant compound in all of the extracts. Hexane extracts from *P. hybneri* had very few identifiable EAD-active compounds; not even one of three pheromone compounds of *P. hybneri* previously reported were identified in our study. All three already known aggregation pheromone compounds of *R. clavatus* (i.e. trans-2-hexenyl-cis-3-hexenoate, trans-2-hexenyl-trans-2-hexenoate and tetradecyl isobutanoate) were identified from male *R. clavatus* extracts. It is reported for the first time here that antennae of male *D.baccarum* and *P.hybneri* both responded to trans-2-hexenyl-cis-3-hexenoate. Save for cyclohex-2-enedione, cyclopentasiloxane- decamethyl, and 2-hexen-1-ol, all of the electrophysiologically- active compounds identified in our study have been reportedly identified in various stink bugs including the species used in the current investigation. With a few exceptions, the EAD-active compounds identified broadly fall into one of four chemical groups: aldehydes, alkanes, acetates or oxo-(E)-2-alkenal and they generally serve defensive functions for the stink bugs. Many EAD-active compounds remained unidentified due to their low concentrations in the hexane extracts.

Key words: *Piezodorus hybneri*, *Dolycoris baccarum*, *Halyomorpha halys*, *Riptortus clavatus*, GC-EAD, GC-MS, electrophysiologically-active compounds.

CHAPTER ONE

1.0 General introduction

1.1 Communication in insects: a general perspective.

By definition, communication is a process by which information is exchanged between organisms (Jackson et. al., 1993). Communication can occur between members of the same or different species (Dicke and Sabelis, 1992). Within the world of insects, communication is vital for a number of reasons. The insects need to recognize their kins or nestmates. For the sexually reproducing insects, communication facilitates mating by mediating mate finding and courtship of the opposite sex. Upon locating a food or other suitable resource, the insects need to inform their conspecifics of availability of the same. When danger looms in a certain habitat, for example presence of a natural enemy and such danger is sensed by some individuals, then the perceivers of the danger will warn members of the same species to flee. Certain species of insects effectively defend themselves against natural enemies by mimicking the appearance of a well self-defended heterospecific insect. Depending on availability and distribution of resources including food and nesting sites, insects need to regulate, through communication, spatial distribution of members of their own colony (Nordlund and Lewis, 1976; Dicke and Sabelis, 1992).

1.2 Choice of communication mode.

Intra- and inter-specific communications among insects and between them and their environment are mediated by a range of cues including tactile, visual, acoustic, and chemical signals (Lewis, 1984). Nature of the habitat, prevailing ecological conditions, and the significance to the species of spacing and social grouping all influence the mode of communication that insects adopt. Of all the possible modes of communication among insects, chemical mode is the preferred choice, particularly in communication over long range (Law and Regnier, 1971; Sandler et al 2000; Tegoni et al., 2004). In chemical communication, the insects use volatile organic molecules to communicate messages with remarkable sensitivity and specificity (Sandler et al., 2000; Tegoni et al., 2004). Chemical communication system involves the production and release into the

environment of organic compounds that are detected and used to modify the behavior of the receiver (Jurenka, 1996; Tegoni et al., 2004).

Signals in chemical information conveyance are referred to as info- or semiochemicals (Dicke and Sabelis, 1992; Wertheim et.al. 2005). Semiochemicals are of two types: pheromones and allelochemicals (Dicke and Sabelis, 1992). Pheromones facilitate intraspecific interactions, for example, sexual communication between male and female insects of the same species. On the other hand, allelochemicals mediate interactions between organisms of different species. For instance, an insect herbivore identifies and locates its host plant by perceiving volatile chemicals emitted by the plant (Dicke and Sabelis, 1988, 1992).

1.3 Allelochemicals

Allelochemicals are sub-classified into allomones, kairomones, synomones and apneumones (Nordlund and Lewis, 1976; Dicke and Sabelis, 1992). Transmission of allomones favors the source insect. A case in point is when a prey emits volatile compounds that drive away its potential predator (Nordlund and Lewis, 1976). Kairomone compounds benefit the receiving insect only (Dicke and Sabelis, 1988; Ruther et al., 2002). An instance of this is when a predator/parasitoid locates its prey/host by eavesdropping on volatile organic substances emitted by the latter. Kairomones are also useful for communication between phytophagous insects and their host plants (Hansson and Anton, 2000). This can be exemplified when green leaf volatiles such as (Z)-3-hexenyl acetate and (E)-2-hexenal attract insect herbivores to the emitting brassicaceous host plants (Sarfraz et al., 2006). A review by Ruther et al., (2002) groups kairomones into two categories using two criteria: function of the kairomone to the benefiting organism (in which case we have foraging, enemy-avoidance, sexual, and aggregation kairomones) and effect of the kairomone on the receiving organism (i.e. primer and releaser kairomones). Synomones, on the other hand benefit both sender and receiver (Nordlund and Lewis, 1976). This is well illustrated when plants upon herbivory, emit volatile organic compounds (VOCs) that attract natural enemies (i.e. predators or parasitoids) of the attacking insect (Dicke and Sabelis, 1992; Vet and Dicke, 1992). Such VOCs are also known as herbivore-induced plant volatiles (HIPVs). Apneumones are substances discharged by nonliving materials that induce a behavioral or physiological

reaction adaptively favorable to a receiving organism, but detrimental to a heterospecific organism, which may be found in or on the nonliving material (Nordlund and Lewis, 1976).

1.4 Pheromones

Like the allelochemicals, pheromones are organic molecules (Jurenka, 1996). They comprise either a single chemical or more often, multi-component blends of chemicals (Read and Haines, 1975; Hodges et al., 1984; Tillman et al., 1999; Blomquist and Vogt, 2003; Altstein, 2004; Tegoni et al., 2004). It is important, however, to note that the various components of a pheromone blend when tested individually do not have equal efficacy in inducing behavioral response in insects (Read and Haines, 1975; Hodges et al., 1984). As such, mixtures of pheromone components are more active in stimulating behavioral responses than individual components (Raman, 1988).

1.5 Pheromone classification

Pheromones may be classified based on their effect on receiver and also their functions. On the basis of effect on receiver, insect pheromones fall into two broad categories: primer and releaser pheromones (Nordlund and Lewis, 1976; Jackson et. al. 1993). A primer pheromone elicits physiological changes in the recipient, which then equip the organism with a new variety of options. These pheromones are difficult to study, as their effect is generally long-term, without necessarily having an immediate effect. On the other hand, a releaser pheromone elicits immediate and reversible change in behavior mediated directly by the central nervous system (Jackson et. al. 1993). Releaser pheromones are classified into various categories according to the kind of behavior they evoke (i.e. their functions) (Nordlund and Lewis, 1976). These categories are: sex pheromone, dispersal or spacing pheromone, alarm pheromone, trail pheromone, surface pheromone, and aggregation pheromones. Chemically, pheromones can fall into one of two structural groups: Type I compounds comprising straight-chain C10-C18 alcohols, aldehydes, and acetates, with 0-3 double bonds; and Type II compounds consisting of C17-C23 polyunsaturated hydrocarbons, or the corresponding mono- or diepoxides (Millar, 2005b).

A sex pheromone mediates coming together of opposite sexes for mating purpose. The sex pheromone induces responses such as orientation, precopulatory behavior, and mating in conspecifics (Roelefs and Cardé, 1977). A sexually active male or female insect identifies and locates its mating partner upon perception of sex pheromone emitted by the opposite sex.

Aggregation pheromones elicit aggregation of members of the same species in a given area. Insects of the same species come together for mating, or they come to a food source, or a habitat that is conducive.

Dispersal (or spacing) pheromones cause the spacing between conspecifics to increase thus minimizing intraspecific competition (Dicke and Sabelis, 1992). These may be useful in preventing overcrowding of resources such as mates, food, egg-laying sites, and refugia.

Insects employ alarm pheromones to warn members of the same species that danger is looming. The alarm pheromones stimulate such behavioral responses as agitated walking, dropping from the host plant/feeding substrate and flight attempt (Blatt et al., 1998).

Trail pheromones aid orientation of insects to sources of food or new nesting sites. An insect marks a route with scent or odour traces so that other conspecifics can follow it.

Surface pheromones are primarily produced and absorbed on the body surface and other insects of the same species perceive them by direct contact or over a short distance. These aid social insects to recognize conspecifics, nest mates, kin, or even members of different castes.

1.6 When is a pheromone treated as an allelochemical?

Thus far we have discussed communication between insects and their environment including fellow insects. We have seen that such communication is largely mediated by infochemicals. Furthermore, we have looked at the various classifications of the infochemicals according to their source of biosynthesis, emissions and also their functions. At this point, it is perhaps tempting to think that a compound or blend of compounds can only be a pheromone or an allelochemical and not both. However, such an assumption would not be absolutely right. Sometimes a compound or mixture of

compounds that serve as a pheromone for a certain species of insects can also be regarded as an allelochemical, mediating communication between insects of different species. An example of this has already been mentioned in one of the preceding paragraphs where it was said that a natural enemy (predator/parasitoid) may intercept volatile chemical-borne messages between conspecific insects and use them to track their prey/host. Another scenario is where an insect, upon perception of a natural enemy, emits defensive compounds aimed at driving away the enemy. However, such compound(s) may not only deter the enemy but also alert conspecifics to the emitter that danger is lurking. In that case, (a) compound(s) may both be a pheromone and an allelochemical. As such, sometimes there is no clear-cut boundary between a pheromone and an allelochemical in terms of the function. This phenomenon of (a) compound(s) serving both as a pheromone and an allelochemical will be become more authentic as more examples will be given elsewhere in the current document.

1.7 Infochemicals and crop protection

Having seen that volatile organic chemicals play a significant role in mediating communication in the world of insects, advantages have and continue to be taken of such a phenomenon. This is with respect to control of insects that have assumed a pest status. This use of infochemicals to control pest insects is currently applicable in various spheres of life including agriculture and forestry (Groot, 2000). Success of this is largely attributable to an ever growing collaboration between entomologists and chemists (Review by Ayasse et al., 2001). Use of infochemicals in pest control can take various dimensions. The chemicals can be used in monitoring the population level of pests before a judicious use of synthetic pesticides can be employed. This helps cut economic as well as ecological costs of pesticide use. Infochemicals can also be used in mating disruption, whereby synthetic versions of chemicals naturally mediating mating in insects are placed in a crop field. This way a sexually mature insect would not locate its mating partner. The mechanism driving this is that the target pest will get habituated hence they will stop responding to their genuine mating partners. However, this technique has a weakness in that it does not completely inhibit mating. Hence with an occurrence of even a few mating instances, it is still possible for insect populations to reach a level where they can cause economic damage (Groot, 2000). Lure and kill is yet another pest control strategy

in which infochemicals are potentially applicable. In this, a lure containing the attractant infochemical is placed in a trap that contains substances that can cause death to an insect. Following olfactory and flight response to the lure, the insect will enter a trap and then come into contact with the killer substance, usually formulated as a liquid. Mass trapping of pest insects is another window of opportunity for employing infochemicals. This technique is similar to lure and kill except that the trapped insects do not get into contact with a lethal substance. Rather, the insects are held up in a trap keeping them out of the mating and/or damaging population. Of all these four possible methods of pest control using infochemicals, population monitoring is the most commonly applied.

1.8 Infochemicals for control of heteroptera pests

Heteroptera (also known as true bugs) rank fourth among the most important insect orders in terms of the economic damage they cause to their host crops (Groot, 2000). Their host range is diverse, varying from vegetables to fibers to trees (Funayama, 2006). For along time, control of heteropteran pests has been achieved by use of broad range pesticides synthesized for use against insects from other taxonomic orders (Groot, 2000). However, intensification of campaigns against use of broad range insecticides is taking such chemicals off the market. Furthermore, there is a general push across the board for use of alternatives to purely chemical-based pest control. As a result of these concerns/actions, various techniques including use of target-specific insecticides and Bt-crops that can resist insect damage are being put into practice (Armer et al., 2000; Siebert et al., 2005). However, currently available Bt toxins target insects in three taxonomic orders only: Lepidoptera, coleoptera and diptera (Schuler et al., 1999; Armer et al., 2000 Groot and Dicke, 2002), thus leaving out heteroptera. The heteroptera may be insensitive to Bt toxins due to various possibilities including the likelihood of the insects lacking receptor cells sensitive to the toxins, or the enzymes contained in the saliva of the insect breaking down the toxic proteins during ingestion (Armer et al., 2000). Furthermore, to the best of our knowledge, no insecticides specific to heteroptera are presently available in market. Consequently, heteroptera continue to pose a problem to growers (Siebert et al., 2005). To address this problem, other options including infochemical-based control are being pursued. Therefore, research activities have been directed towards identifying, synthesizing and using infochemicals to control members of heteroptera group.

1.9 Stink bugs

Subject of the current MSc thesis was on infochemicals of four species of insects that all belong to the order Heteroptera. The thesis report is part of a sequence of activities aimed at developing infochemicals for pest control. Aim of the thesis was to identify infochemicals that would elicit antennal response in each of four species of stink bugs. These bugs were: *Halyomorpha halys, Piezodorus hybneri, Riptortus clavatus* and *Dolycoris baccarum*.

CHAPTER TWO

2.0 Literature review

2.1 Heteroptera in general

The heteroptera are also known as true bugs (Millar, 2005a). This group comprises 79 families with approximately 38,000 described species (Millar, 2005a). Some of the popular families include stink bugs, bed bugs, plant bugs, assassin bugs, and water striders (Millar, 2005a). True bugs are ubiquitous in distribution, inhabiting various habitats including aquatic environments (Aldrich, 1988; Millar, 2005a). They undergo incomplete metamorphosis, by-passing the pupal life stage. Their eggs hatch into nymphs that directly mature into adults. They have sharp-pointed feeding mouthparts that they use to inject enzyme-containing saliva into the feeding substrate (McBrien and Millar, 1999). This enzyme liquefies and predigests the food, which is then sucked by the proboscis (McBrien and Millar, 1999). Most true bugs use plants as their food sources, with some species attaining the status of economically serious pests. In all, the true bugs place fourth in the ranking of insect groups according to the seriousness of economic damage they cause (Groot, 2000). A handful species are predators, facultatively or obligatorily preying on other arthropods, snails, and even small fish. Some species have been applied as biocontrol agents of pests, for example, *Anthocoris nemoralis*, *Dicyphus hesperus* and *Orius spp*. have been used against pear psyllids (Scutareanu et al., 1997).

Like other insects, true bugs also communicate by use of chemical signals. However, the true bugs stand out from the rest of other insects in that the former are typified by synthesis and emission of massive amounts of defensive chemical secretions, particularly when under stress (Aldrich, 1988; Blatt et al., 1998). Such secretions give off strong pungent smell, driving away the offending living organism. The secretions may also contain alarm pheromones (Blatt et al., 1998). The glands from which such defensive compounds are synthesized vary in their location depending on the stage of development of the insect (Borges and Aldrich, 1992). In nymphs, the scent glands are located in the abdomen while in adults, they occur in the metathorax (Aldrich 1988; Borges and Aldrich, 1992; Millar, 2005a). There is variable level of plasticity with regard to site of biosynthesis and the functions of such defense compounds. The plasticity also varies with the family, genera or species but also with the stage of growth and development of insects

in question (Borges and Aldrich, 1992; Millar, 2005a). In members of the plant bug (Miridae) and stink bug families (Pentatomidae), it has been shown that the same defense compounds can also serve as sex pheromones. This is determined by the dosage at which they are emitted. At high dosage, they serve a defensive function while at lower dosage they mediate intra-specific sexual and aggregation communication purposes (James et al., 1996; Lockwood and Story (1985), cited by Blatt et al., 1998; Millar, 2005a). Again, a heteropteran at a different growth stage may use a different compound as an alarm pheromone. For example, nymphal *Leptoglossus occidentalis* (Hemiptera: Coreidae) use (E)-2-hexenal as an alarm pheromone while for adults the alarm pheromone is quite a different compound (Blatt et al., 1998). Sometimes there is a specialization when it comes to the site of synthesis of infochemicals. Biosynthesis site of defense compounds may be remarkably different from the location of sex pheromone manufacture (Millar, 2005a).

2.2 Pentatomidae (stink bugs)

The stink bugs are polyphagous insects, feeding on a wide range of plants including members of such families as Leguminosae, Gramineae, Solanaceae, Compositae, and Rosacea (Kobayashi, 1972; Higuchi and Suzuki, 1996; Nakamura, 2002; Funayama, 2006; Nakamura and Numata, 2006). The stink bugs overwinter as adults (Kobayashi, 1972). The number of generations they have per year is determined by the temperature and photoperiod regimes (Kobayashi, 1972; Conradilarsen and Somme, 1978; Nakamura, 2002). Both nymphal and adult stink bugs cause damage by feeding on various plant parts like leaves, pods, seeds and fruits (McBrien and Millar, 1999). As with other heteroptera, they feed by piercing and sucking from their substrates, leaving behind necrotic lesions. The damage caused by stink bugs is characterized by premature abortion and deformation of fruits and wilting of leaves (McBrien and Millar, 1999; Siebert et al., 2005). The bugs have wings and are highly mobile. Sometimes they transfer phytopathogens from infected to uninfected plants (McBrien and Millar, 1999; Correa-Ferreira and Azevedo, 2002).

The stink bugs have highly developed dorsal and metathoracic abdominal glands, (DAGs) and (MTGs) respectively (Aldrich, 1988). DAGs are mostly found in nymphs while MTGs in adults (Aldrich, 1988). Some adults have fully functional DAGs, though

the behavioural functions of their secretions are largely unknown (Aldrich et al., 1995). The posterior DAGs in larvae that feed on plants have been shown to secrete such compounds as C6, C8 and C10 alk-2-enals, C6 and C8 4-oxo-alkenals, and various alkanes including tridecane (Aldrich 1988). Alkanes, C8 and C10 alk-2-enals, C6 and C8 4-oxo-alkenals abound in the MTG secretion of pentatomidae (Aldrich, 1988). Acetates of alk-2-enols are also found in those glands but in low quantities.

Generally, in the phytophagous pentatomid bugs, it is the males that produce sex pheromones (Moraes et al., 2005).

2.2.1 *Riptortus clavatus*

R. clavatus is also known as bean bug. It belongs to the family Alydidae (Aldrich, 1988). Unlike other pentatomids, female *R. clavatus* lay single scattered eggs both on host and non-host plants (Leal et al., 1995). This could be a strategy evolved by the adult females to spread the risk of parasitism by an egg parasitoid, *Ooencyrtus nezarae* (Leal et al., 1995). However, the adults and nymphs have an aggregated distribution and both cause damage to host plants including their favorite, soybean (Kono, 1989, 1990). Late-instar nymphs are more destructive than the early-instars (Kono, 1989). *R. clavatus* overwinter as adults in diapause (Numata and Nakamura, 2002).

Figure 1 Adult *R. clavatus* feeding on soybean leaves.

Adult males emit an aggregation pheromone that attracts conspecific nymphal (2^{nd} instars) and adult males and females under field conditions (Numata et al., 1990; Leal et al., 1995). The aggregation pheromone is a three-part mixture comprising (E)-2-hexenyl (E)-2-hexenoate (E2-6:E2Hx), (E)-2-hexenyl (Z)-3-hexenoate (E2-6:Z3Hx) and

tetradecyl (= myristyl) isobutyrate (14: iBu) (Leal et al., 1995). Yasuda et al., (2007) discovered that males additionally produce octadecyl isobutyrate (18: iBu), which when added to the three compounds significantly increase the number of adult and nymphal *R. clavatus* caught in a trap.

2.2.2 *Halyomorpha halys*

Not much information is available on the life-cycle of *H. halys* (Funayama, 2006).The insect has a wide range of host plants and the adults keep moving between the hosts seasonally (Funayama, 2006). It can feed on more than 51 plant species belonging to 27 families (Funayama, 2006).

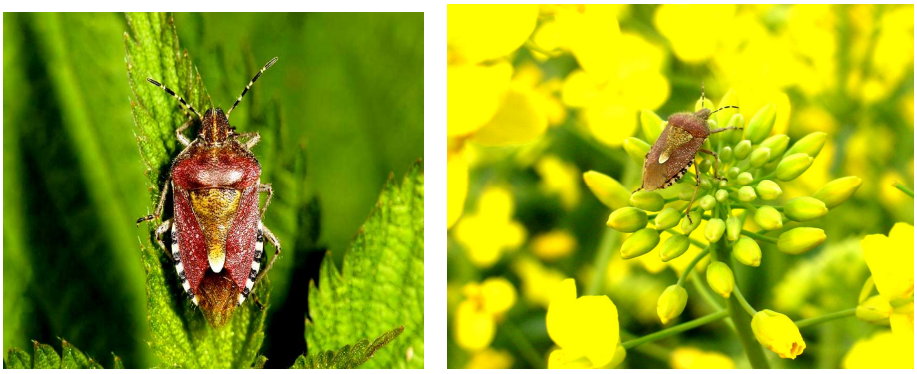
(a) **(b)**

Figure 2 Nymphal **(a)** and adult **(b)** *H. halys* feeding on pod of common beans and peach fruit hosts, respectively.

Its life style is as follows: adults migrate into houses and sheds during autumn, overwinter there, and emerge again in early spring (Funayama, 2004). Both male and female *H. halys* are attracted to methyl (E, E, Z)-2, 4, 6-decatrienoate, an aggregation pheromone of brown-winged green bug, *Plautia stali* (KyuChul et al, 2002). Female *H. halys* can attract male conspecifics. Toyama et al., (2006) found that *H. halys* when placed close together in space under cold overwintering conditions tended to aggregate and such aggregations did not occur when antennae of the bugs were clipped off. This strongly suggests that even at close range, conspecific aggregations within *H. halys* are mediated by pheromones. However, the pheromone compound(s) emitted by this insect has/have not been reportedly identified.

2.2.3 *Dolycoris baccarum*

D. baccarum can infest about 50 species of plants (Kobayashi, 1972). It is a seed-sucking bug (Nakamura, 2002; Nakamura and Numata, 2006). No reports exist in regard to the infochemicals that this species uses to communicate.



(a) **(b)**

Figure 3 Adult *D. baccarum* on host plants (**a** and **b**).

2.2.4 *Piezodorus hybneri*

Nymphs and adults of *P. hybneri* including mated and diapausing females are attracted to a component [i.e. (E)-2-hexenyl (E)-2-hexenoate] of the aggregation pheromone of *R. clavatus* (Endo et al., 2006; Hu et al., 2006). Males of *P. hybneri* produce a sex pheromone comprising a mixture of b-sesquiphellandrene, (R)-15-hexadecanolide, and methyl (Z)-8-hexadecenoate (Leal et al., 1998). The adults are very mobile (Higuchi and Suzuki, 1996). An egg parasitoid, *Telenomus triptus*, has been shown to parasitize eggs of *P. hybneri*, significantly contributing to a reduction in population density of the bug (Higuchi and Suzuki, 1996).

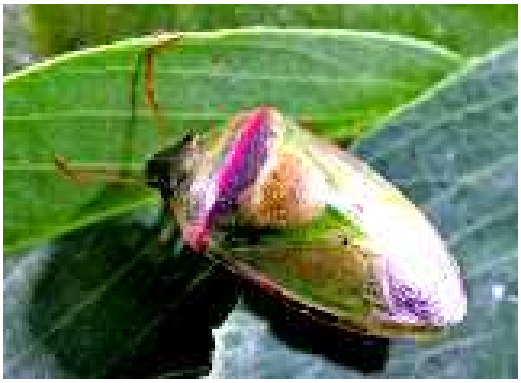


Figure 4 Adult *P. hybneri* occurring on a host plant.

Table 1: Summary of infochemicals known to influence behavior of the above four species of stink bug.

Responding species	Type of pheromone	Emitting species
H. halys	Aggregation pheromone	*Plautia stali* (Lee et al., 2002).
P. hybneri	Sex pheromone	Male *P. hybneri* (Leal et al., 1998).
R. clavatus & *P. hybneri*	Aggregation pheromone	Male *R. clavatus* (Leal et al., 1995).
D. baccarum	Unknown	Unknown

2.3 Difficulties in studying stink bug pheromones

Many factors impede identification of stink bug pheromones. The bugs synthesize large quantities of defense compounds that may fade out pheromone components (McBrien and Millar, 1999). Among the bugs, there is variability in synthesis of and response to pheromones between sexes across families and even genera; in some groups males emit pheromones while in others pheromone synthesis is the preserve of females (McBrien and Millar, 1999). As such, it is difficult to predict which of the two sexes will emit the pheromone compounds for species whose pheromones have not been identified yet. Furthermore, the bugs must be in an optimal physiological condition in order for them to synthesize or respond to pheromones. Species that have long life spans may take several days to attain sexual maturity and start producing and responding to pheromones. For species that overwinter as adults, the onset of winter season may trigger the bugs to enter the reproductive diapause and hence not respond to pheromones. Furthermore, stink bugs posses diverse glands that synthesize communication and defense compounds. Some species have discrete, multicellular glands; yet others have unicellular glands. In differing with several other insects, the pheromone glands are not essentially situated in typical locations where they can be readily found and dissected for examination. Again, some species cannot be reared in the laboratory with ease due to amongst other things, chronic release of defense compounds as a result of crowding (McBrien and Millar, 1999).

CHAPTER THREE

3.0 Problem statement and justification of the study

3.1 Background information

As dictated by the nature of its geography, only 17 % of total land area in the Republic of Korea (ROK) is cultivable (Anonymous, 2007). This fact coupled with a high population density exerts a great deal of pressure on the cultivable land. The land is under intense strain to optimize its productivity to feed the many people occupying it. Indeed the last decade has witnessed a significant increase in the production of such fruits as apples, oranges, grapes, persimmons, pears and peaches; thanks to chemical control of pests that would otherwise hinder production of the crops.

Of the fruits named here above, persimmon plays the greatest role in the economy of ROK through export earnings. At a global scale, ROK is second only to China in terms of acreage under production of persimmon. At present, the crop occupies the largest growing area of 29, 000 hectare, followed by apple and pear. Besides persimmon, the last two fruits are also important for export purposes. In the year 2003, ROK earned US $ 40.3 million from export of these three crops. Export of persimmon to countries in the South Eastern part of Asia and USA has grown fast over the last decade and multiplied by a factor of 5 between the years 1998 and 2002. However, persimmon production is facing a very serious challenge from a complex of stink bug species, which inflict serious economic damage to the crop (Hu et al, 2006). The stink bugs also infest the other fruits named above together with vegetables grown in ROK. Such stink bugs are namely: brown marmorated stink bug (*H. halys*), the bean bug (*R. clavatus*), and the green stink bug (*P. stali*) (Lee et al., 2002; Park et al., 2003; Toyama et al., 2006). Other economically important stink bugs are *P. hybneri* and *D. baccarum* (Kobayashi, 1972; Panizzi, 1997). Parts of damaged fruit turn blackish-green and become concave, and the fruits are not marketable. Synthetic chemicals are currently in application to control these pests.

3.2 The problem and justification of the study

Frequency of chemical use in fruit and vegetable production in ROK varies and can be as high as 40 times per growing season. Such a frequency and rate of application of synthetic chemicals in ROK is among the world's highest. However, this kind of pesticide application regime does not bode well for long term pest management and brings with it some unwanted problems. Such a high frequency of use points to unsustainability of chemical use in economic terms. The other economic problem that comes with use of synthetic chemicals in crop protection is loss of market, particularly the export market. This is due to unacceptably high level of pesticide residues in the produce. Then comes the ecological problem of emergence and/ or outbreak of secondary pests, natural enemies of which are eliminated by the generalistic synthetic pesticides in current use (Carde and Minks, 1995). Not even the farmer and agricultural workers are spared the wrath of adverse effects of such chemicals as the chemicals pose health hazards to them. Self regulatory roles and natural functions of the various ecosystems get their share too of the adverse effects of these synthetic chemicals.

Given the many problems associated with application of synthetic chemicals as cited above, it is increasingly becoming necessary, the use of novel economically and ecologically sustainable pest control strategies. Such novel strategies should be based on the challenges posed by specific crop-pest systems, as opposed to the current use of pesticides that have general efficacy. One such strategy could be found in a combined use of infochemical technology and biological control agents.

3.3 Infochemical technology as a potential solution to the problem

Some species of stink bug have been shown to use the same pheromone component. For instance, *Piezodorus guildinii* (*westwood*) and *Euschistus heros (F.)* are both attracted to field traps containing synthetic methyl 2, 6, 10-trimethyltridecanoate (Borges et al., 1998 cited by Borges et al., 1999). This sharing of pheromone component offers a great opportunity for using the same pheromone blend to attract heterospecific pest insects. Besides causing aggregation of stink bugs, it has been shown that aggregation pheromones can also attract such natural enemies of the bugs as parasitic flies and egg

parasitoids (Mizutani, 2006). One of three components of aggregation pheromone of *R. clavatus* i.e. (*E*)-2-hexenyl (*Z*)-3-hexenoate (E2HZ3H) was demonstrated to attract *Ooencyrtus nezarae*, an egg parasitoid of *R. clavatus* (Mizutani, 2006). The second pheromone component, (E)-2-hexenyl (E)-2-hexenoate (E2HE2H) can attract *P. hybneri*, a competitor of *R. clavatus* (Endo et al., 2003, cited by Endo et al., 2005; Huh et al., 2006). It is known that male *P. hybneri* emits pheromones that attract both conspecific sexes (Higuchi, 1999). Lee et al. (2002) also reported that *H. halys* can be attracted to aggregation pheromones of *P. stali*. However, to the best of our knowledge, no aggregation or sex pheromones have been reported for *D. baccarum*. Furthermore, no single pheromone compound/blend has been reported that can attract all four species of stink bugs, namely: *H. halys*, *P. hybneri*, *R. clavatus* and *D. baccarum*. Infochemical-based control of these pests would benefit immensely with the identification and synthesis of a single compound /blend that can attract all these four insects simultaneously.

CHAPTER FOUR

4.0 Research objective and question

4.1 Objective

To identify electrophysiologically-active compound(s) that can elicit antennal response in each of four species of stink bug (*H. halys, P. hybneri, R. clavatus* and *D. baccarum*) by electrophysiological and analytical chemical techniques.

4.2 Research question

We attempted to answer the following question:

What compound(s) contained in a stink bug extract from each of four stink bug species named above would evoke electroantennographic responses in conspecific and heterospecific males and females?

4.3 Rationale of the study

Identification of (a) stink bug-derived compound(s) that elicit(s) electroantennographic response on conspecifics and heterospecifics could help single out what compound(s) could be potentially useful infochemicals. Using infochemical compound(s) that elicit(s) behavioral responses in two or more species would be economically-viable as it would save growers the cost of having to use a species-specific compound or blends of compounds in protecting their crops. Such a strategy would be particularly useful when a stink bug complex attacks crops in a particular region. Furthermore, if a working product would have to be made commercially available, it would be cheaper to register only one as opposed to two or more.

4.4 Choice of study techniques

Several studies have demonstrated strengths of coupled gas chromatography - electroantennography (GC-EAD) (Review by Ayasse et al., 2001) and gas chromatography - mass spectrometry (GC-MS) techniques (Takken and Dicke, 2006). Respectively, these electrophysiological and analytical chemical techniques allow exceptionally sensitive and selective detection of potentially active infochemical components, even within a complex multicomponent sample (Review by Ayasse et al., 2001). Therefore, these two techniques were employed in our study.

4.5.0 Materials and methods

4.5.1 Insects for use in electrophysiological (GC-EAD) study

Four species of adult stink bugs were used in the coupled GC-EAD study. These were: *H. halys, R. clavatus, P. hybneri,* and *D. baccarum*. These insects were obtained from a colony of EntoCare C.V, Wageningen, The Netherlands. Prior to the experiments, the insects were maintained at 24 ± 2^{o}C, 50-70 ± 10 % R.H., under a 16L: 8D photoperiod. All the insects were mated adults fed on soybean seeds and regularly exposed to water-soaked filter paper.

4.5.2 Extraction of stink bug compounds.

Separate body extracts were prepared from one male and one female each of a whole *H. halys, R. clavatus, P. hybneri,* or *D. baccarum* by soaking it in 30 ml hexane for 30 minutes. Mated and fed adults were used. Stink bugs exhibit multiple mating and so can continue emitting sex pheromones even after they mate (Miklas et al., 2003). Other pheromones that they may give off following mating include aggregation and alarm pheromones. Since stink bugs synthesize and release defensive chemicals when disturbed, they were anaesthetized with CO_2 gas just prior to the extraction of compounds. This was aimed at preventing extraction of unwanted defense compounds. The extracts were filtered through a small wad of cotton, the residues were rinsed twice with the same volume of hexane, and the rinses were added to the extract. The extracts were stored below −20°C until use.

Hexane was used as a solvent because of its relatively low volatility, a property which ensures that the dissolved substances do not evaporate (Aldrich et al., 1993). Again, some bisabolene-derived compounds, which are found in the body of certain species of stink bugs, are unstable and therefore break down in acidic solvents and so use of hexane(a non-polar hydrocarbon) (Hong and Chao, 2004) solves this problem of instability (Aldrich et al., 1993). Furthermore, hexane is an inert solvent and therefore, antennae of most insects do not show electroantennographic response to it.

4.5.3 Coupled gas chromatography - electroantennographic detection (GC-EAD).

Antennal responses of males and females of *H.halys, D.baccarum, P. hybneri,* and *R.clavatus* to whole body extracts of each of four species of the same insects were studied

with the aid of a coupled gas chromatography - electroantennographic detection (GC-EAD) system. The GC-EAD system, Interscience Trace GC-2000 (Interscience, Breda, The Netherlands) with flame ionization detection, was used. A CP-Sil8- fused silica column with the following specifications was used: 30 m in length, internal diameter (ID) of 0.25 mm and a film thickness of 0.5 μm (Chrompack-Varian, Middelburg, The Netherlands). Conditions of operation were as follows: helium was the carrier gas at a constant flow of 2.5 ml/min; GC oven temperature programming was such that the initial level was 80ºC (0.8 min hold) then increased to 280 ºC (20 min hold) at 25 ºC/min. Detector temperature was 280 ºC. 1 μl of each extract was injected with the aid of an on-column injector of the GC-EAD system. The column effluent was split into two equal parts between a Flame Ionisation Detector (FID) and the EAG detector. The temperature of the transfer line between the GC oven (Syntech, Hiversum, The Netherlands) and the EAG detector was synchronized with the temperature programming of the GC oven. Over the antenna, a flow of purified, humidified air was maintained at a rate of 80 cm/sec. The antennae of males and females reared in a group were used. The antenna was mounted between two glass electrodes filled with a ringer solution (6.4mM KCl (0.048 g), 12mM $MgCl_2.6H_2O$ (0.24 g), 9.6mM KOH (0.054 g), 12mM NaCl (0.07 g), 20mM KH_2PO_4 (0.272 g), 1mM $CaCl_2$ (0.015 g) and 354mM glucose (6.379 g). All these compounds were dissolved in 100 ml of deionised water. The electroantenographic (EAG) responses were obtained simultaneously with FID recordings. To identify all electrophysiologically-active compounds and to distinguish electroantennographic responses from noise, 4-6 GC-EAD runs were performed with each sample of crude extract. GC-EAD-active compounds were identified by performing GC-MS analyses of the same crude extracts as used in the GC-EAD.

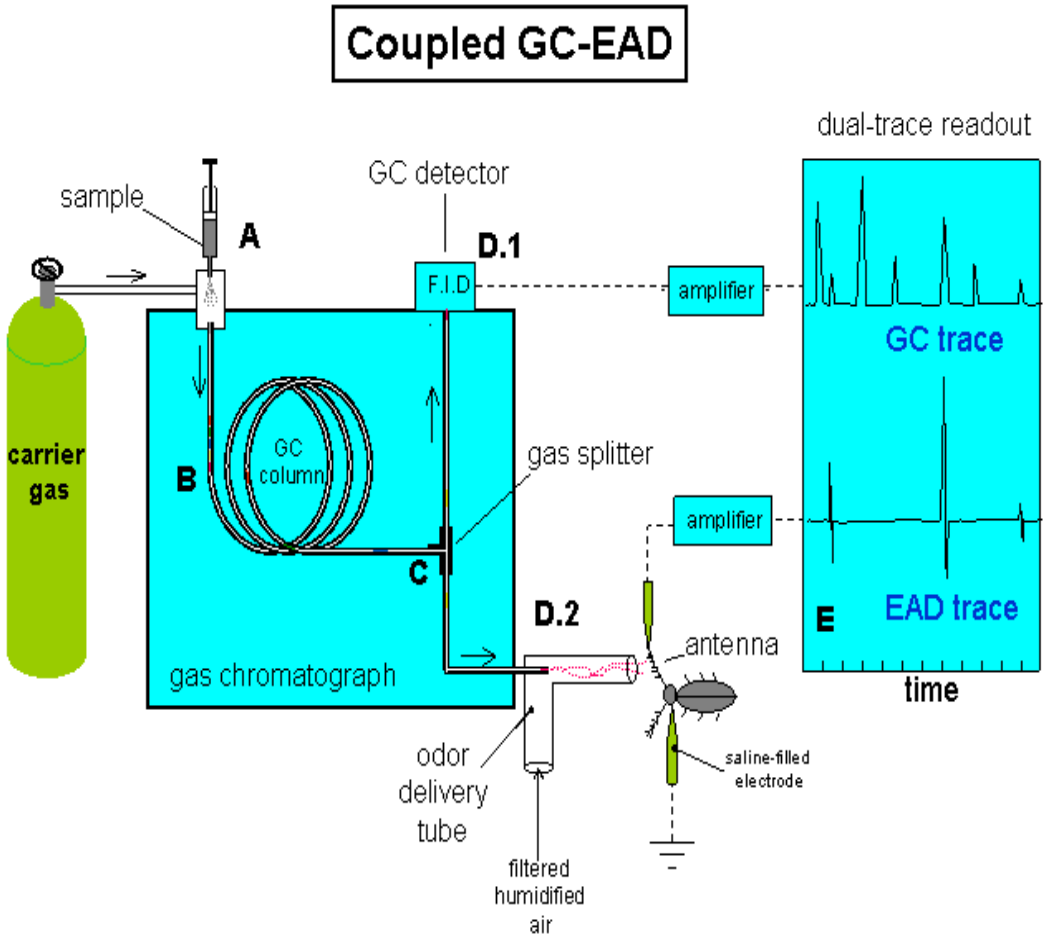


Figure 5 The GC-EAD system that was employed in the detection of electrophysiologically active compounds. The analytes (i.e. samples) made from the insects were injected at point **A**. Helium served as the carrier gas, driving the analytes through the GC column. As the analytes were heated by the oven (**B**), the various individual compounds contained therein separated out from the mixture based on their varying physical and chemical properties. Such unique properties make the compounds differ in their affinities to adsorb to the GC column; some compounds are weakly adsorbed on the column and elute faster than others. At point **C,** the volatile effluent eluting from the column was split into two equal portions; one part went to the F.I.D detector (**D.1**) while the other to insect antenna (**D.2**). Humidified air accelerated rate of flow of effluents to the antenna. Every antennal response to a compound was recorded as an EAD trace (**E**) and that compound simultaneously recorded as the GC trace.

4.5.4 Coupled GC-MS analysis of stink bug extracts.

GC-MS analyses were performed on a Hewlett Packard 5973 mass selective detector (70 eV), coupled to a Hewlett Packard gas chromatograph equipped with a split/splitless programmed temperature vaporization (PTV) injection system (CIS 4; Gerstel, Mülheim an der Ruhr, Germany). One µl of extract from each insect species was submitted to a GC-MS system for analysis. Injections were done in split mode only (1 µl). A 30- m EC-5 fused silica column with a 0.25 mm ID and 0.25 µm film thickness (Alltech/Applied Science BV., Breda, The Netherlands) was employed. The GC-MS system was operated under the following conditions (EC- 5): 50(2)-10-300(20)-15. Helium was the carrier gas, at a constant flow of 1.3 ml/minute.

CHAPTER FIVE

5.0 Results and discussion

5.1 GC-EAD runs of hexane extracts.

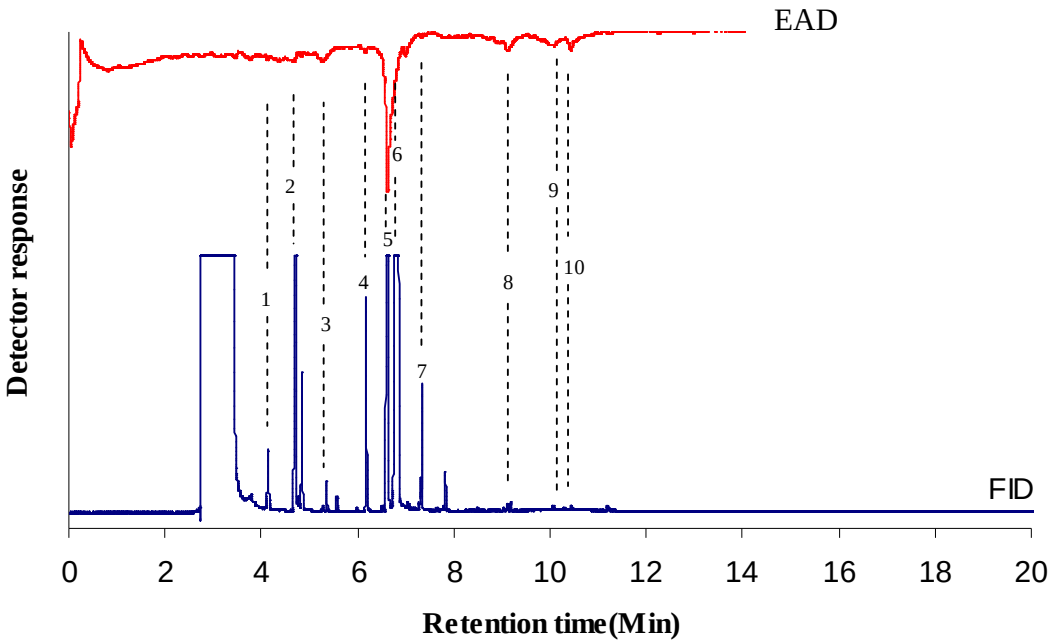
It was found that hexane extract of each of the four species of stink bug contained at least a compound that was detected by an antenna of the same species as the extract source or the three other species (Table 2). Table 2 summarizes results presented in figures 6-53, in which antennal responses of the four insects to various extracts are given. In the graphs, retention times are measured in minutes and centiminutes while detector responses in mV.

Table 2: Results of the GC-EAG runs of the various extracts against antenna of the four species of insects.

		Insect antenna							
		R. clavatus		*D. baccarum*		*P. hybneri*		*H. halys*	
		Male	Female	Male	Female	Male	Female	Male	Female
Solvent extracts injected into GC-EAD.									
R. clavatus	Male	+	+	+	+	+	+	+	+
	Female	+	+	+	+	+	+	+	+
D. baccarum	Male	+	+	+	+	+	+	+	+
	Female	+	+	+	+	+	+	+	+
P. hybneri	Male	+	+	+	+	+	+	+	+
	Female	+	+	+	+	+	+	+	+
H. halys	Male	+	+	+	+	+	+	+	+
	Female	+	+	+	+	+	+	+	+

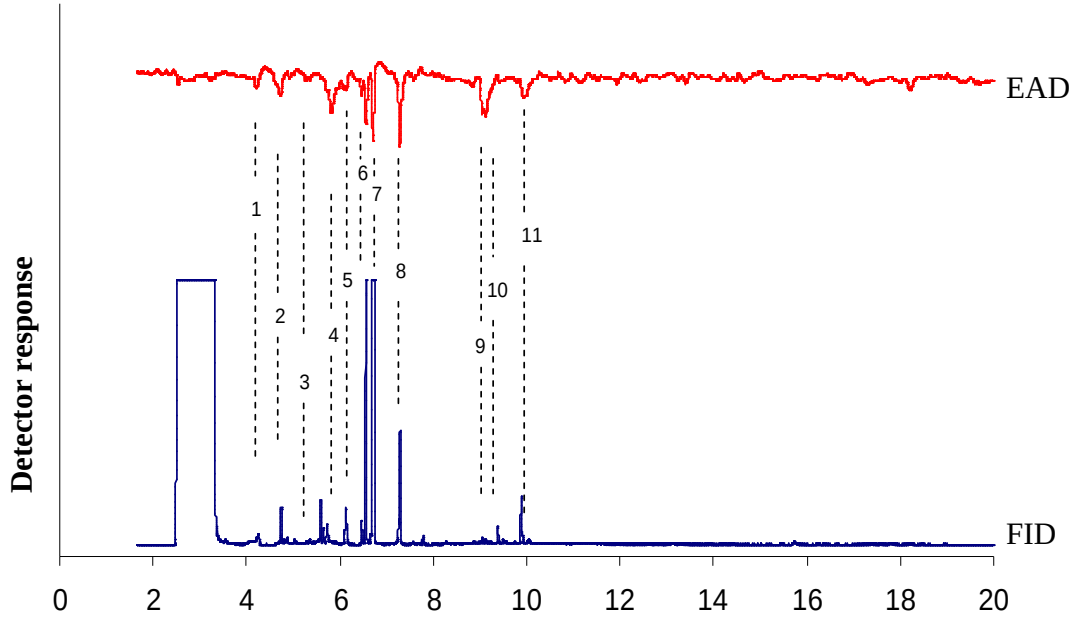
+ means that the extract elicited antennal response. 1µ of hexane extract was used in every injection. The injections were repeated between 4-6 times for very treatment combination. In each run, a new antenna was used.

Figure 6: Response of female *D. baccarum* antenna to extracts from female *H. halys*.



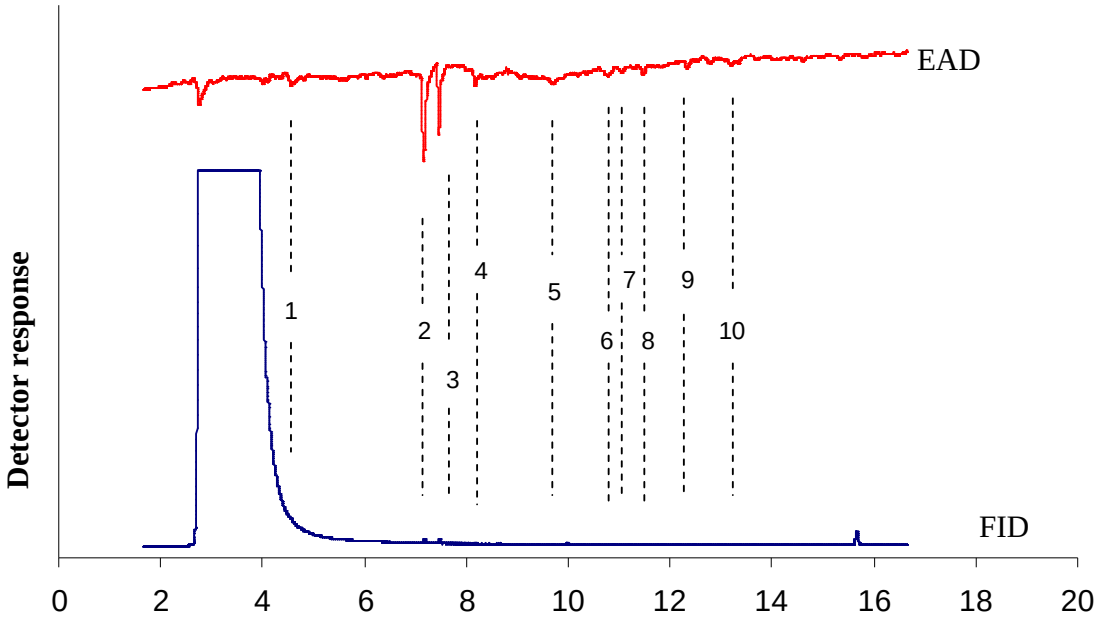
	Retention time	Retention index	Compound
1.	4.13	860	(E)-2-hexenal
2.	4.68	957	4-Oxo-(E)-2-hexenal
3.	5.28	1056	(E)-2-octenal
4.	6.18	1207	Dodecane
5.	6.62	1278	(E)-2-decenal
6.	6.78	1304	Tridecane
7.	7.36	1412	(E)-decenyl acetate
8.	9.11	1776	Unknown
9.	10.03	1956	Unknown
10.	10.43	2028	Unknown

Figure 7: Response of Male *R. clavatus* antenna to female *H. halys* extracts.



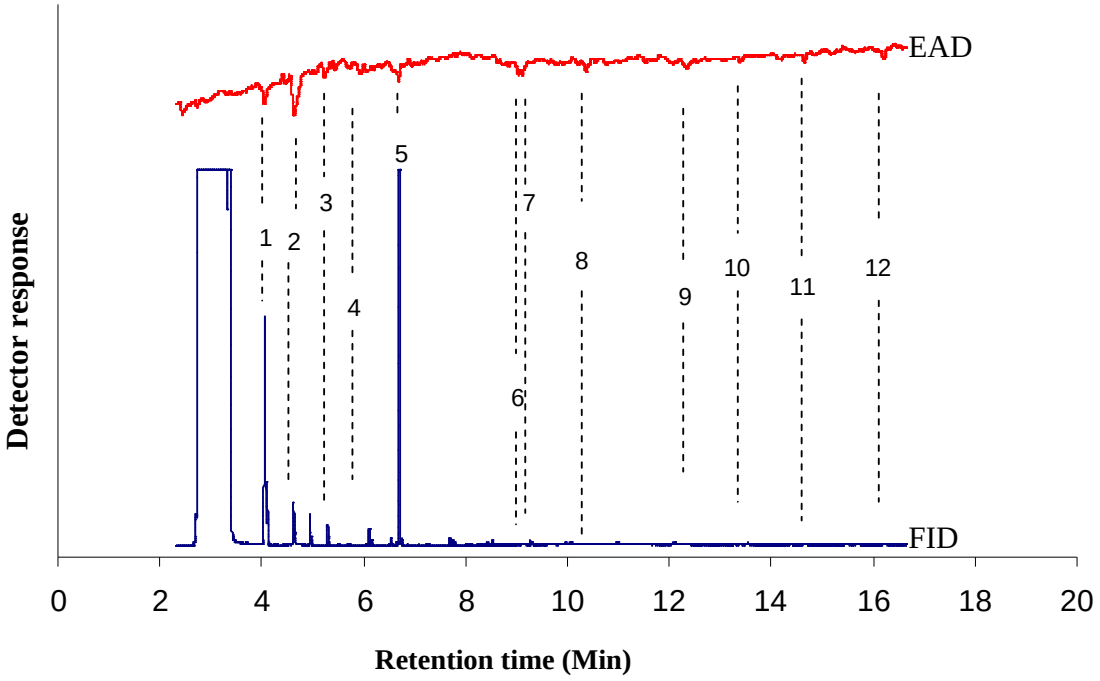
	Retention time	Retention index	Compound
1.	4.32	895	Unknown
2.	4.80	977	Unknown
3.	5.42	1078	Unknown
4.	5.82	1146	Unknown
5.	6.20	1210	Dodecane
6.	6.62	1278	(E)-2-decenal
7.	6.79	1306	Tridecane
8.	7.36	1412	(E)-2-decenyl acetate
9.	9.11	1776	Unknown
10.	9.19	1793	Unknown
11.	9.96	1943	Unknown

Figure 8: Response of Male *R.clavatus* antenna to male *R.clavatus* extract



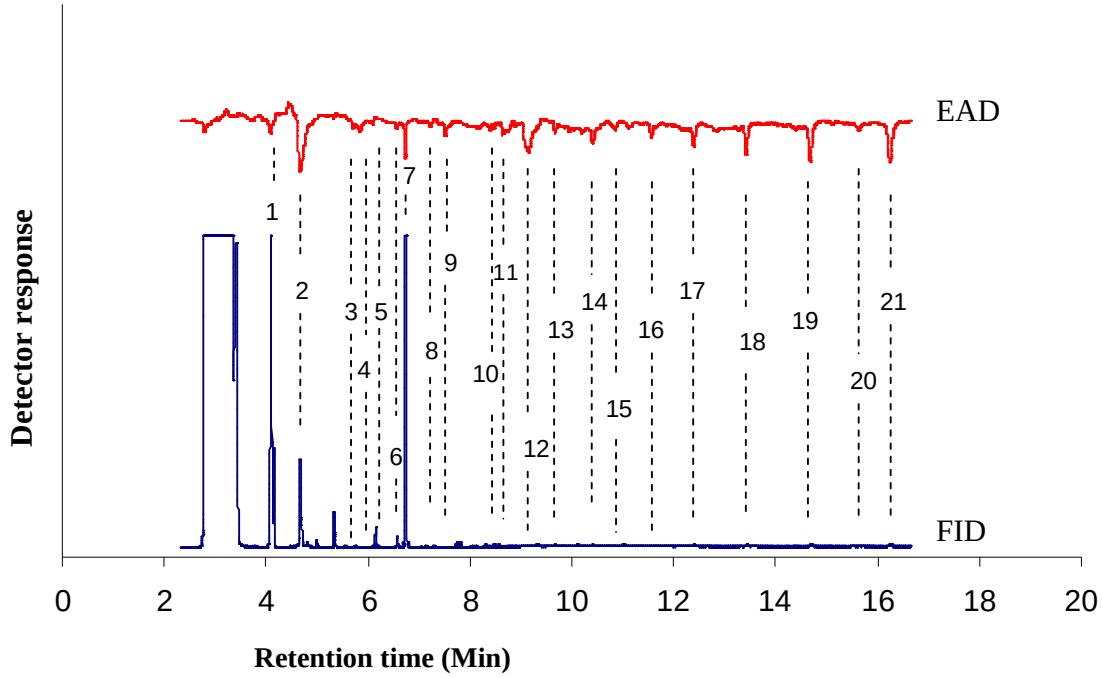
	Retention time	Retention index	Compound
1.	4.70	961	Unknown
2.	7.23	1387	Trans-2-hexenyl-cis-3-hexenoate
3.	7.56	1453	Trans-2-hexenyl-trans-2-hexenoate
4.	8.25	1590	Unknown
5.	8.80	1707	Unknown
6.	10.86	2100	Henicosane
7.	11.10	2134	Unknown
8.	11.54	2195	Unknown
9.	12.38	2297	Unknown
10.	13.25	2381	Unknown

Figure 9: Response of Male *R.clavatus* antenna to male *D.baccarum* extract



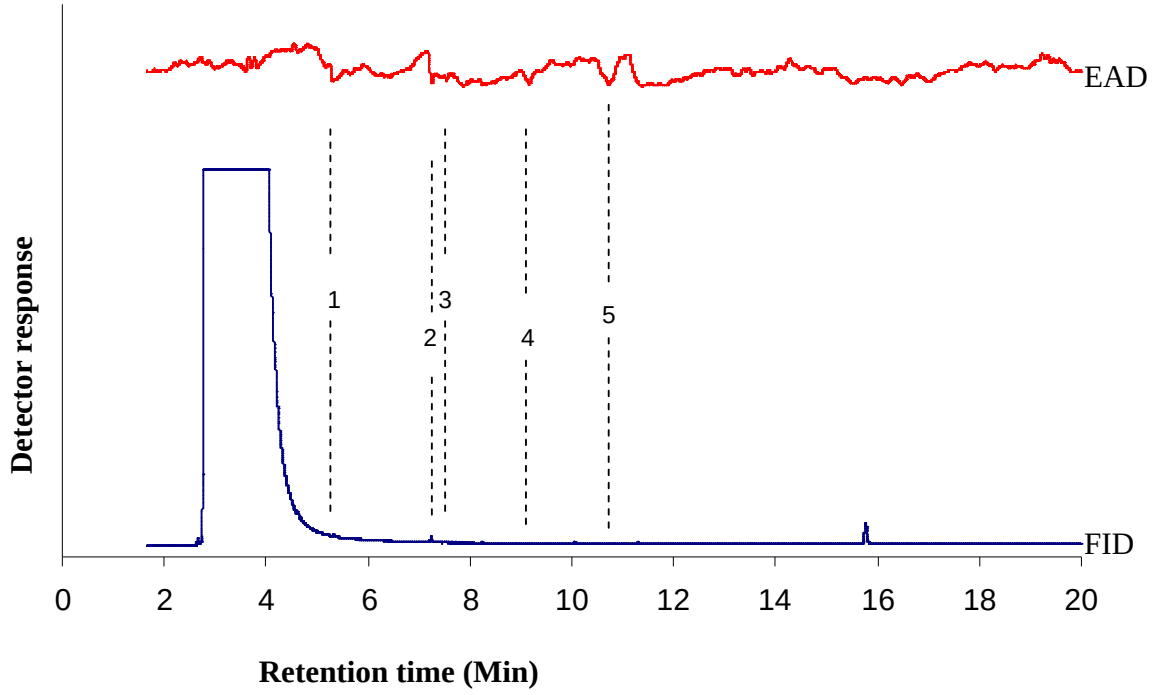
	Retention time	Retention index	Compound
1.	4.11	856	(E)-2-hexenal
2.	4.67	956	4-Oxo-(E)-2-hexenal
3.	5.33	1064	1,4-cyclohex-2-enedione
4.	6.14	1200	Dodecane
5.	6.76	1300	Tridecane
6.	9.11	1776	Unknown
7.	9.20	1796	Unknown
8.	10.45	2031	Unknown
9.	12.39	2298	Tricosane
10.	13.43	2398	Tetracosane
11.	14.70	2499	Pentacosane
12.	16.28	2599	Hexacosane

Figure 10: Response of Male *R.clavatus* antenna to female *D.baccarum* extract



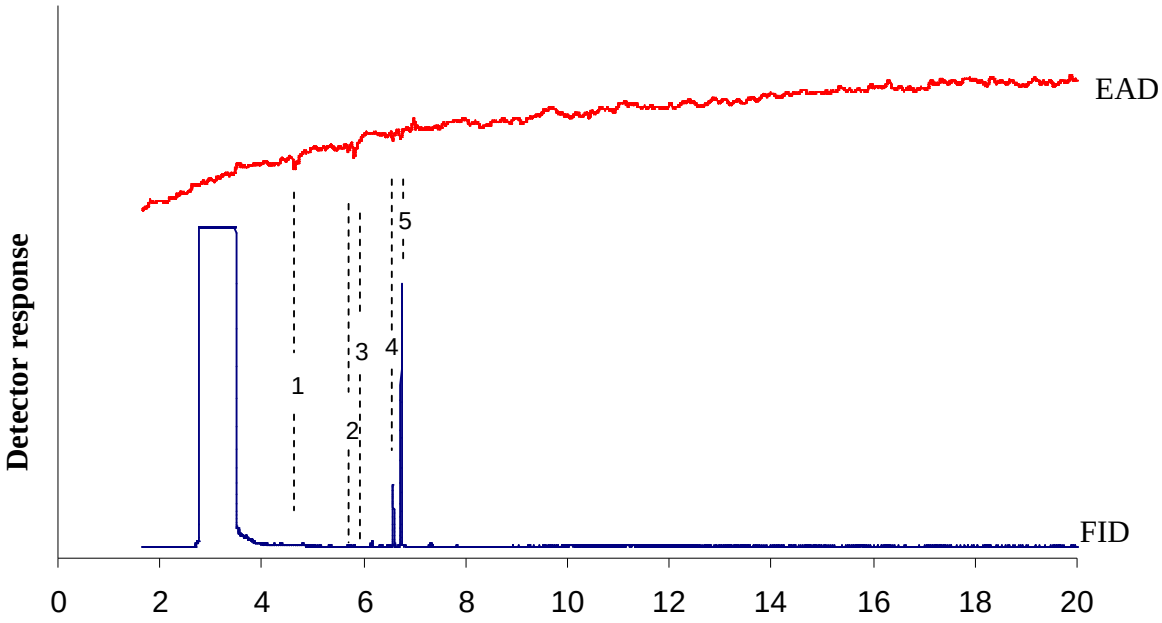
	Retention time	Retention index	Compound
1.	4.11	856	(E)-2-hexenal
2.	4.67	956	4-Oxo-(E)-2-hexenal
3.	5.33	1064	(E)-2-octenal
4.	5.75	1134	Unknown
5.	6.14	1200	Dodecane
6.	6.57	1270	(E)-2-decenal
7.	6.76	1300	Tridecane
8.	7.25	1391	Unknown
9.	7.56	1453	Unknown
10.	8.41	1624	Unknown
11.	8.49	1641	Unknown
12.	9.11	1776	Unknown
13.	9.68	1890	Unknown
14.	10.20	1987	Unknown
15.	10.43	2028	Unknown
16.	11.58	2200	Docosane
17.	12.42	2301	Tricosane
18.	13.43	2398	Tetracosane
19.	14.70	2499	Pentacosane
20.	15.67	2562	Unknown
21.	16.27	2599	Hexacosane

Figure 11: Response of female *R.clavatus* antenna to female *R. clavatus* extract



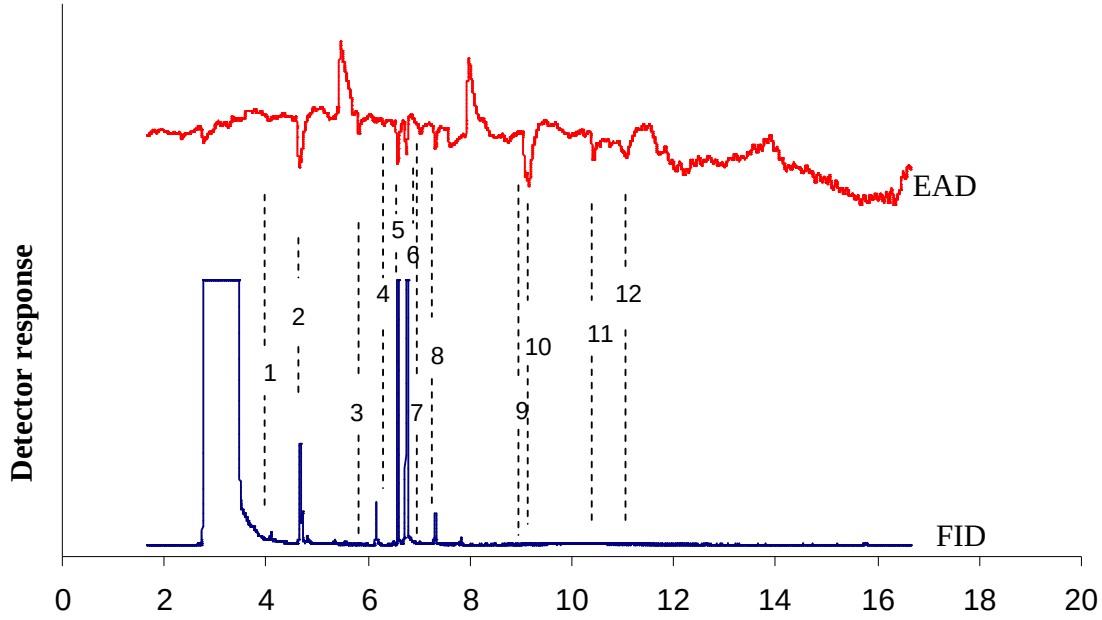
	Retention time	Retention index	Compound
1.	5.33	1064	Unknown
2.	7.23	1387	Trans-2-hexenyl-cis-3-hexenoate?
3.	7.54	1449	Trans-2-hexenyl-trans2-hexenoate?
4.	9.20	1796	Unknown
5.	10.75	2082	Unknown

Figure 12: Response of female *R.clavatus* antenna to female *H.halys* extract



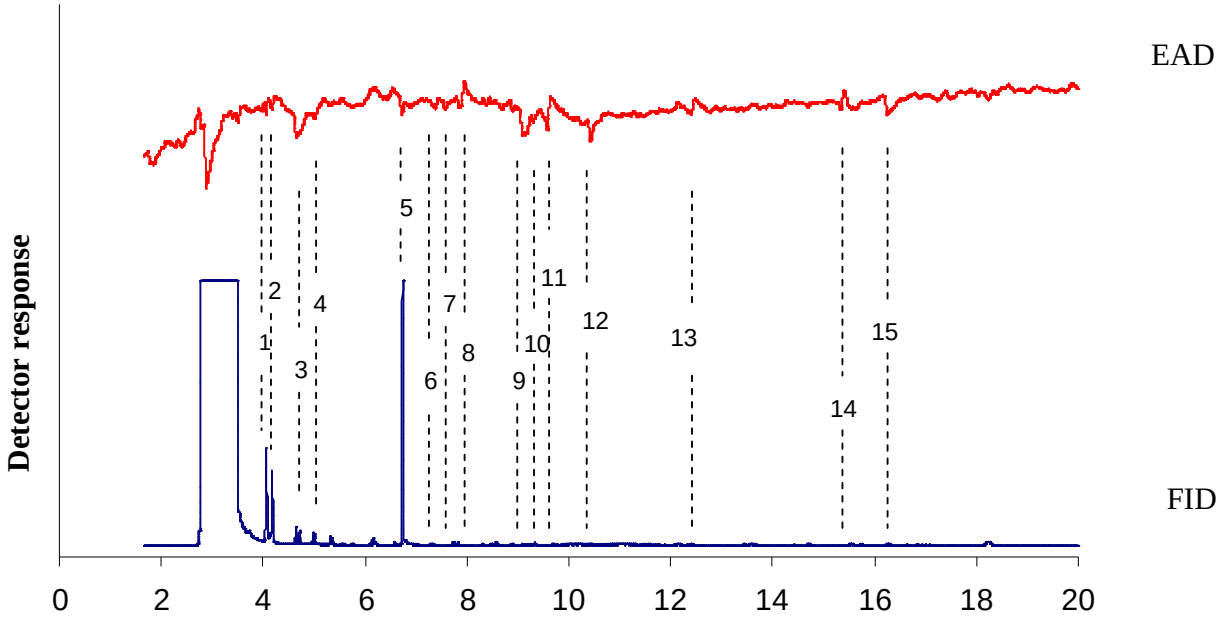
	Retention time	Retention index	Compound
1.	4.65	952	4-Oxo-(E)-2-hexenal
2.	5.71	1127	Unknown
3.	5.83	1148	Unknown
4.	6.58	1272	(E)-2-decenal
5.	6.75	1298	Tridecane

Figure 13: Response of female *R.clavatus* antenna to male *H.halys* extract



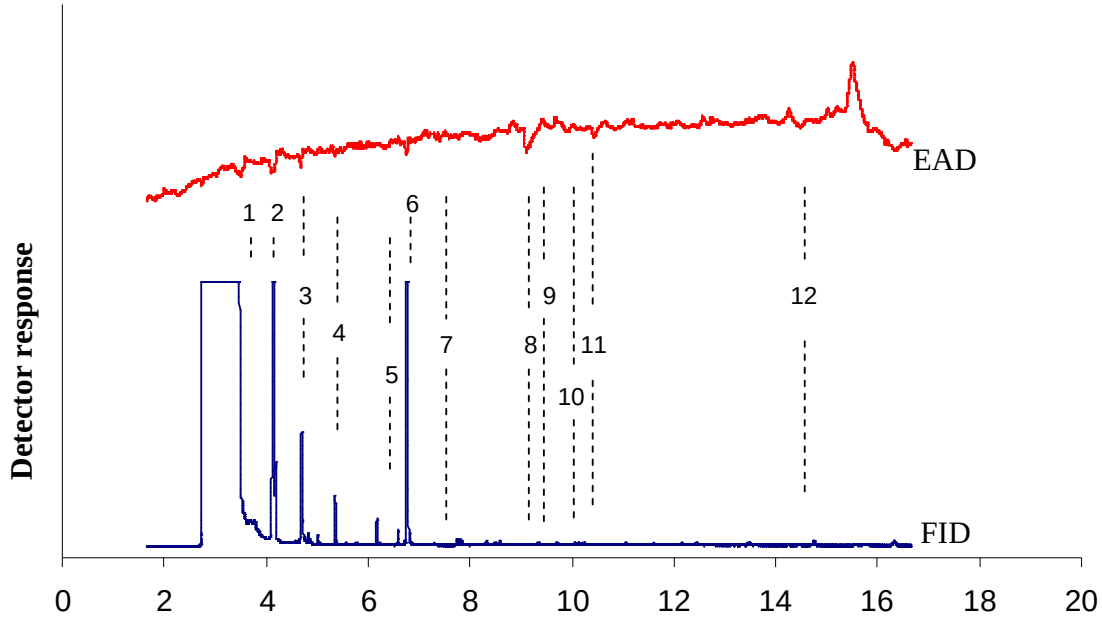
	Retention time	Retention index	Compound
1.	4.11	856	(E)-2-hexenal
2.	4.68	957	4-Oxo-(E)-2-hexenal
3.	5.84	1150	Unknown
4.	6.16	1203	Dodecane
5.	6.60	1275	(E)-2-decenal
6.	6.78	1304	Tridecane
7.	7.02	1349	Unknown
8.	7.33	1406	(E)-2-decenyl acetate
9.	9.11	1776	Unknown
10.	9.19	1793	Unknown
11.	10.43	2028	Unknown
12.	11.08	2131	Unknown

Figure 14: Response of Female *R.clavatus* antenna to male *D.baccarum* extract



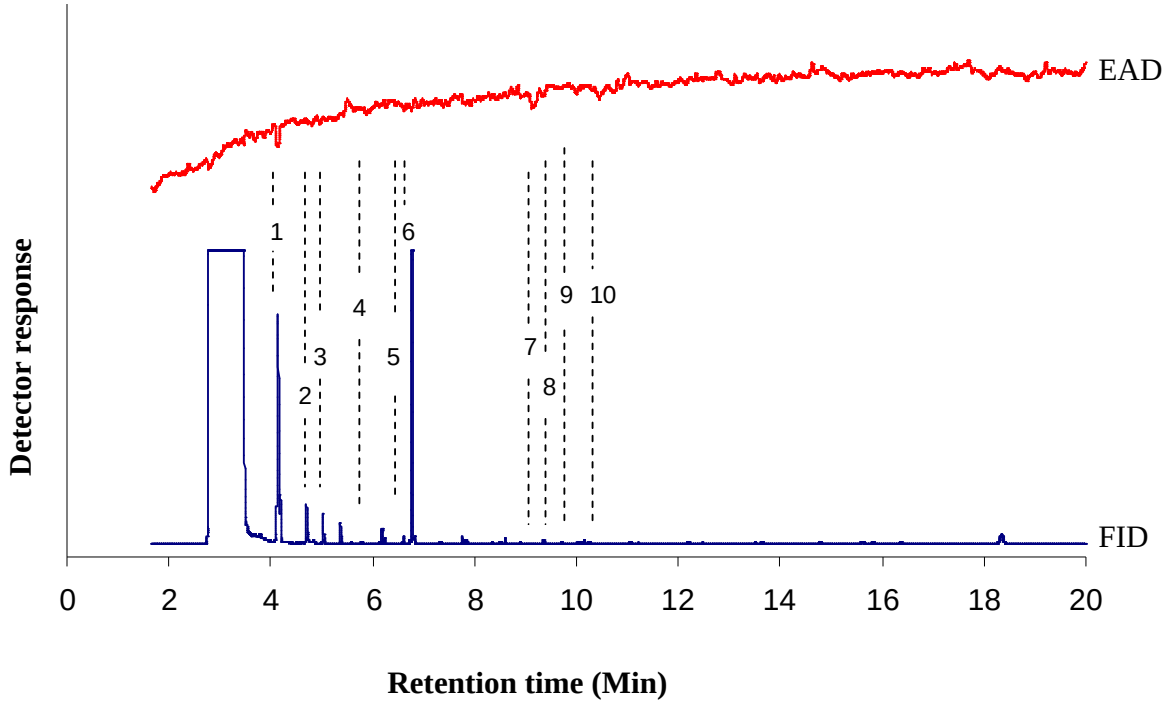
Retention time	Retention index	Compound
1. 4.06	847	Unknown
2. 4.17	867	(E)-2-hexenal
3. 4.65	952	4-Oxo-(E)-2-hexenal
4. 5.01	1012	(E)-2-hexenyl acetate
5. 6.77	1302	Tridecane
6. 7.38	1416	(E)-2-decenyl acetate
7. 7.58	1457	Unknown
8. 7.85	1510	Unknown
9. 9.11	1776	Unknown
10. 9.20	1796	Unknown
11. 9.60	1875	Unknown
12. 10.44	2029	Unknown
13. 12.42	2301	Tricosane
14. 15.35	2542	Unknown
15. 16.24	2597	Hexacosane

Figure 15: Response of male *D.baccarum* antenna to female *D.baccarum* extract



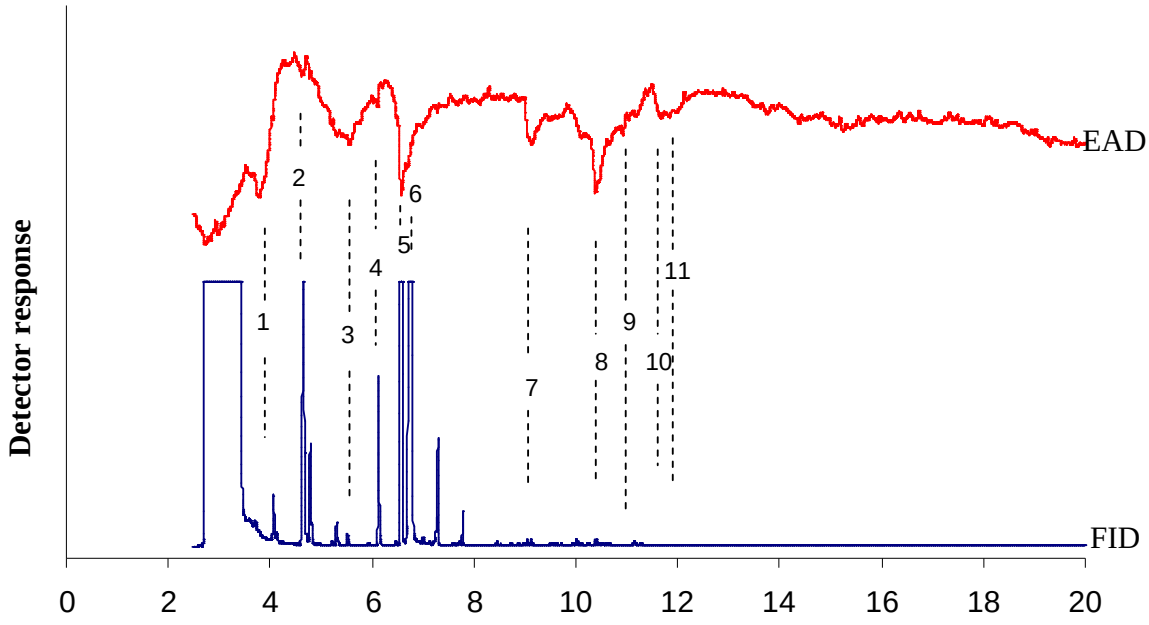
	Retention time	Retention index	Compound
1.	3.57	?	
2.	4.12	858	(E)-2-hexenal
3.	4.70	961	Unknown
4.	5.36	1069	(E)-2-octenal
5.	6.60	1275	(E)-2-decenal
6.	6.77	1302	Tridecane
7.	7.82	1504	Pentadecane
8.	9.14	1783	Unknown
9.	9.20	1796	Unknown
10.	9.96	1943	Unknown
11.	10.44	2029	Unknown
12.	14.52	2485	Unknown

Figure 16: Response of male *D.baccarum* antenna to male *D.baccarum* extract



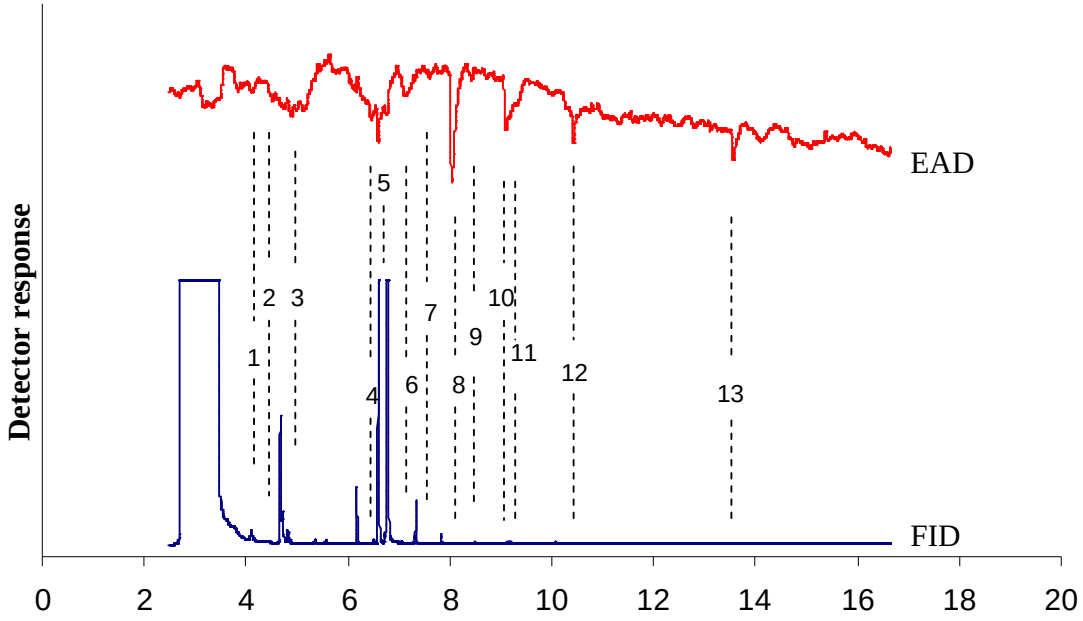
	Retention time	Retention index	Compound
1.	4.11	856	(E)-2-hexenal
2.	4.69	959	4-Oxo-E-2-hexenal
3.	5.01	1012	Unknown
4.	5.89	1158	Cyclopentasiloxane, decamethyl
5.	6.62	1278	(E)-2-decenal
6.	6.76	1300	Tridecane
7.	9.12	1778	Unknown
8.	9.20	1796	Unknown
9.	10.01	1953	Unknown
10.	10.47	2035	Unknown

Figure 17: Response of male *D.baccarum* antenna to female *H.halys* extract



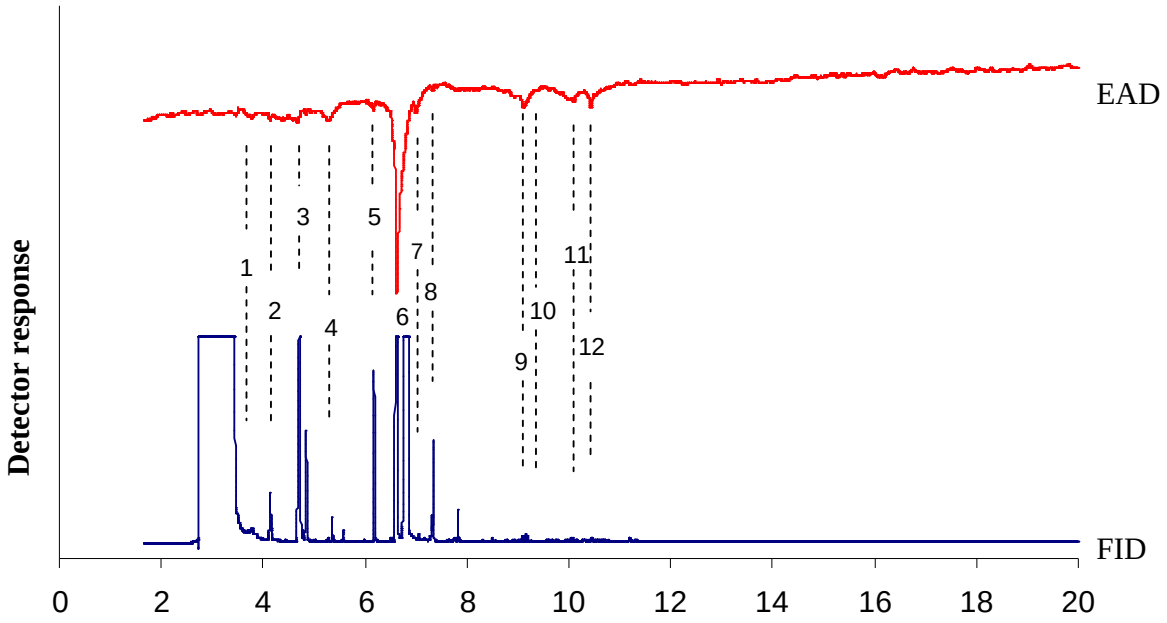
	Retention time	Retention index	Compound
1.	4.12	858	(E)-2-hexenal
2.	4.68	957	4-Oxo-(E)-2-hexenal
3.	5.62	1111	Unknown
4.	6.18	1207	Dodecane
5.	6.63	1280	(E)-2-decenal
6.	6.75	1298	Tridecane
7.	9.11	1776	Unknown
8.	10.45	2031	Unknown
9.	10.99	2119	Unknown
10.	11.10	2134	Unknown
11.	11.74	2220	Unknown

Figure 18: Response of male *D.baccarum* antenna to male *H.halys* extract



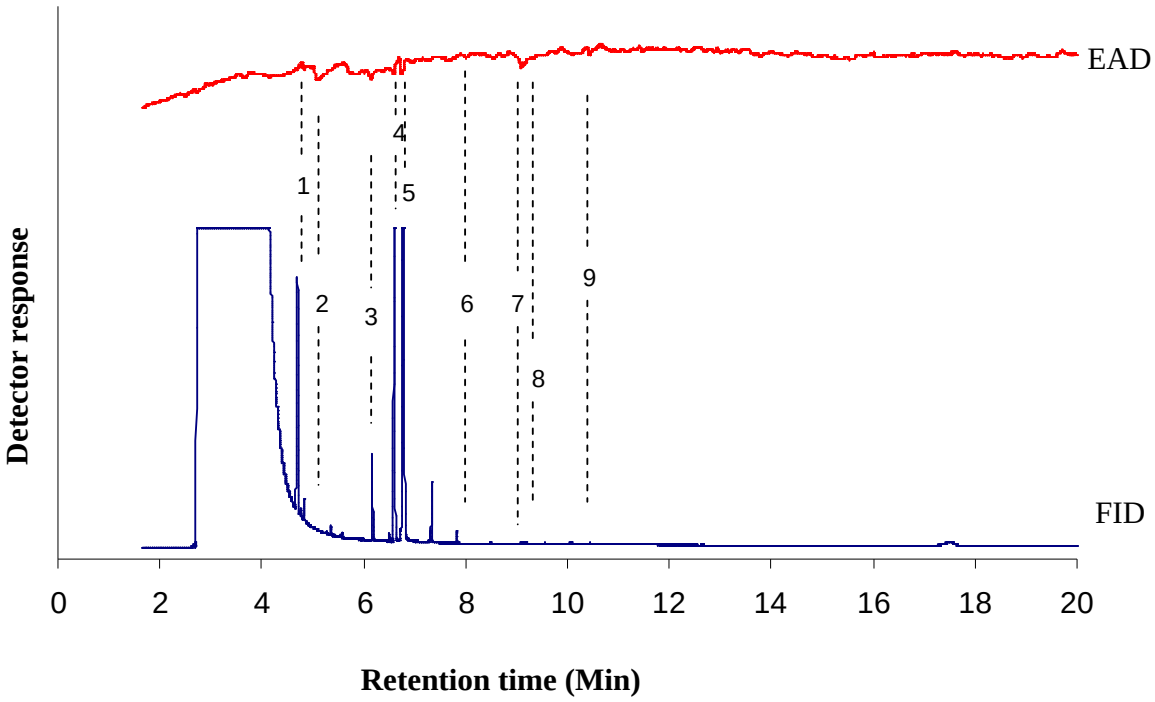
	Retention time	Retention index	Compound
1.	4.09	851	(E)-2-hexenal
2.	4.53	932	Unknown
3.	4.69	959	4-Oxo-(E)-2-hexenal
4.	6.49	1258	Unknown
5.	6.62	1278	(E)-2-decenal
6.	6.78	1303	Tridecane
7.	7.34	1408	(E)-2-decenyl-acetate
8.	8.06	1553	Unknown
9.	8.45	1633	Unknown
10.	9.12	1778	Unknown
11.	9.20	1796	Unknown
12.	10.45	2031	Unknown
13.	13.59	2412	Unknown

Figure 19: Response of female *D.baccarum* antenna to female *H.halys* extract



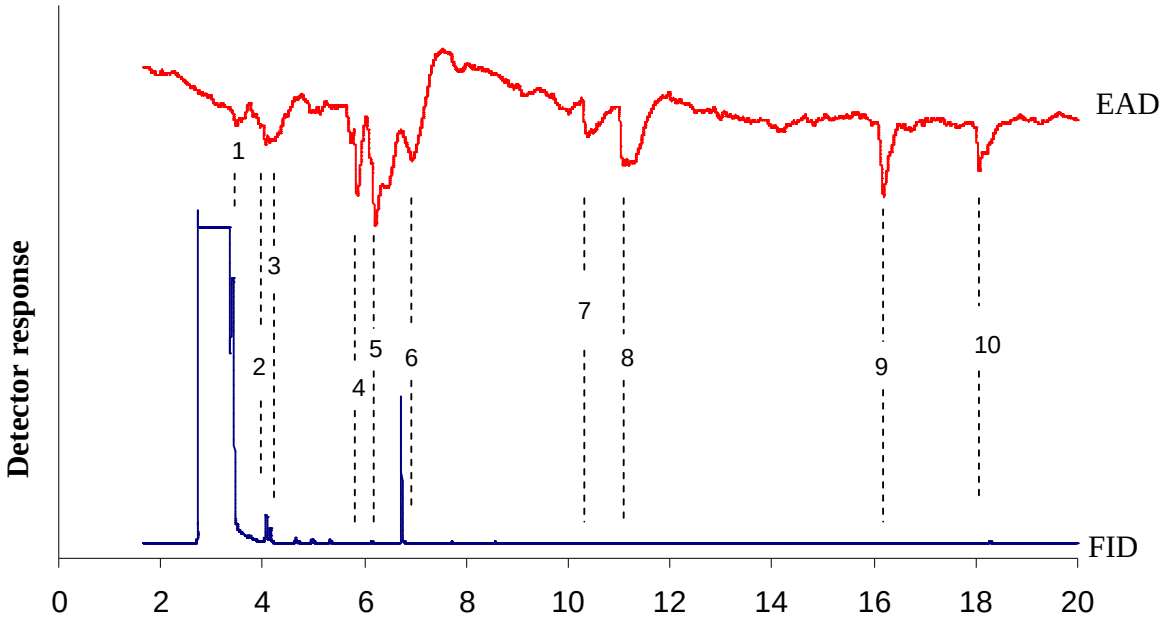
	Retention time	Retention index	Compound
1.	3.78	?	
2.	4.13	860	(E)-2-hexenal
3.	4.69	959	4-Oxo-(E)-2-hexenal
4.	5.30	1059	(E)-2-Octenal
5.	6.20	1210	Dodecane
6.	6.63	1280	(E)-2-decenal
7.	7.03	1351	Unknown
8.	7.36	1412	Unknown
9.	9.11	1776	Unknown
10.	9.16	1787	Unknown
11.	10.13	1975	Unknown
12.	10.45	2031	Unknown

Figure 20: Response female *D.baccarum* antenna to male *H.halys* extract



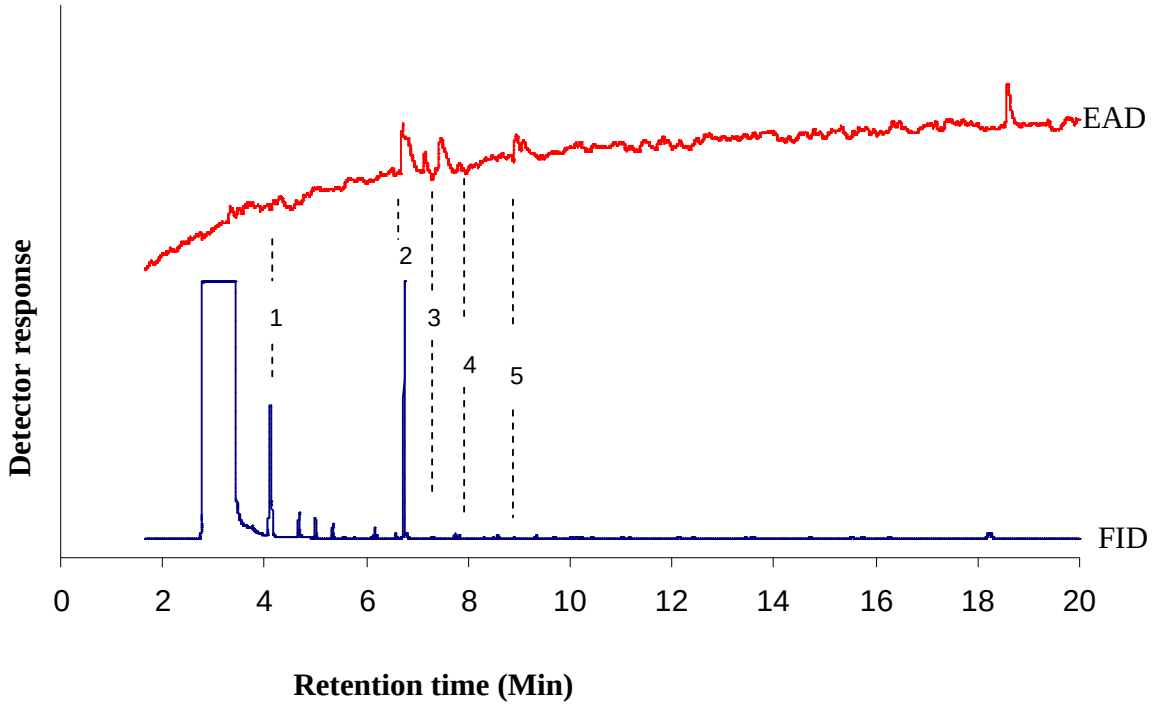
	Retention time	Retention index	Compound
1.	4.68	957	4-Oxo-(E)-2-hexenal
2.	5.10	1027	1,4-cyclohex-2-enedione
3.	6.18	1207	Dodecane
4.	6.60	1275	(E)-2-decenal
5.	6.76	1300	Tridecane
6.	8.03	1547	Unknown
7.	9.10	1774	Unknown
8.	9.16	1787	Unknown
9.	10.45	2031	Unknown

Figure 21: Response of female *D.baccarum* antenna to male *D.baccarum* extract



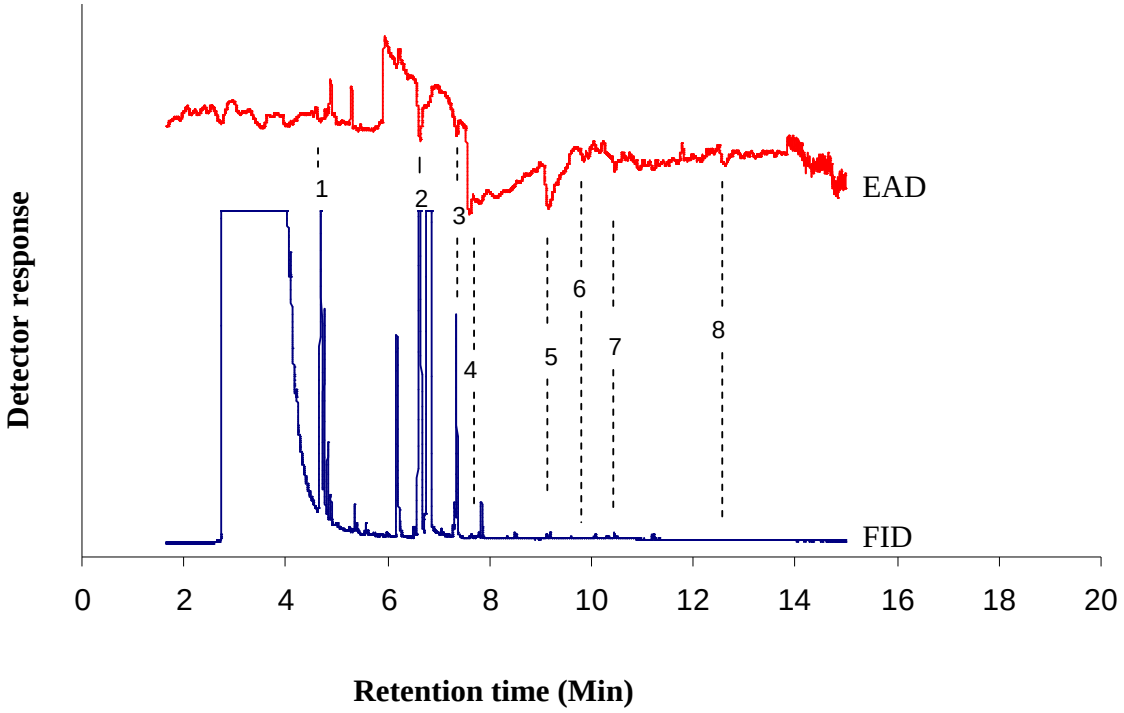
	Retention time	Retention index	Compound
1.	3.66	?	Unknown
2.	4.13	860	(E)-2-hexenal
3.	4.22	877	Unknown
4.	5.94	1167	Unknown
5.	6.28	1223	Unknown
6.	7.02	1349	Unknown
7.	10.48	2036	Unknown
8.	11.18	2145	Unknown
9.	16.27	2599	Hexacosane
10.	18.14	2694	Heptacosane

Figure 22: Response of female *H.halys* antenna versus male *D.baccarum* extract



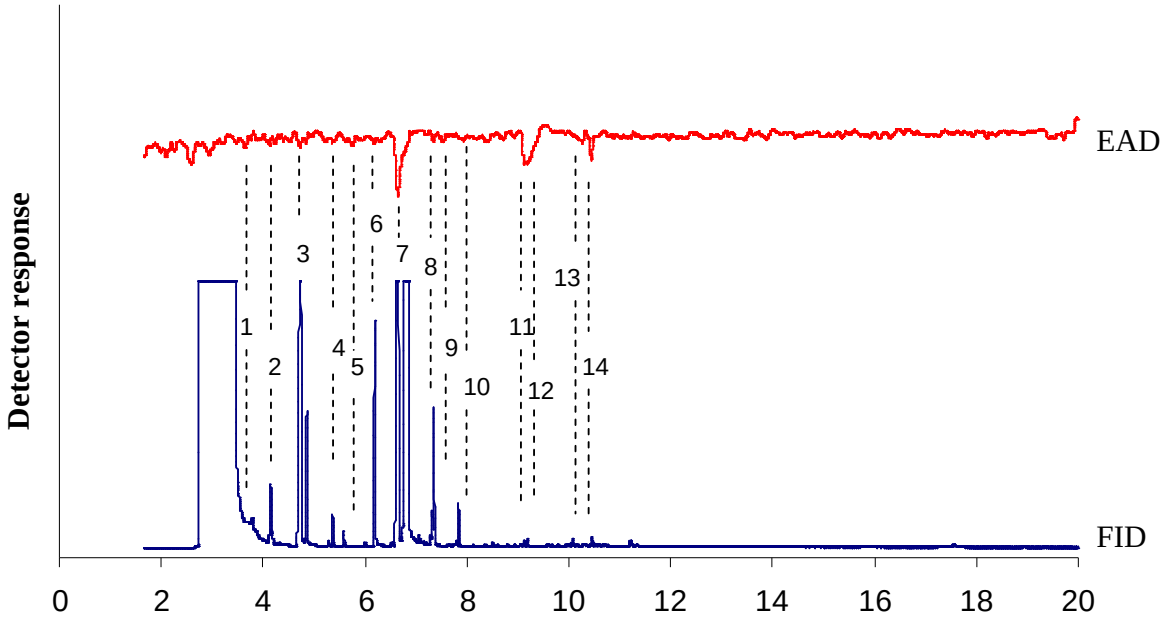
	Retention time	Retention index	Compound
1.	4.13	860	(E)-2-hexenal
2.	6.73	1295	Tridecane
3.	7.33	1406	(E)-2-decenyl acetate
4.	7.94	1529	Unknown
5.	8.91	1732	Unknown

Figure 23: Response of female *H.halys* antenna to male *H.halys* extract



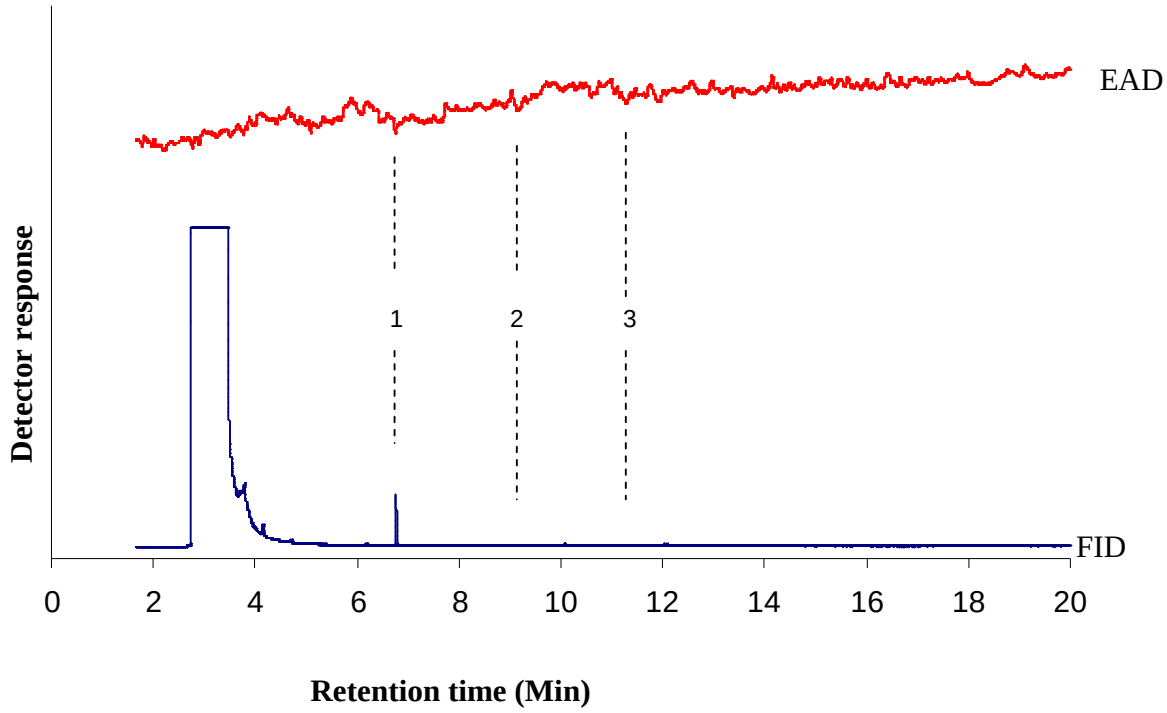
	Retention time	Retention index	Compound
1.	4.72	964	Unknown
2.	6.65	1283	(E)-2-decenal
3.	7.37	1414	(E)-2-decenyl acetate
4.	7.63	1467	Unknown
5.	9.17	1789	Unknown
6.	9.96	1943	Unknown
7.	10.46	2033	Unknown
8.	12.64	2323	Unknown

Figure 24: Response of female *H.halys* antenna to female *H.halys* extract



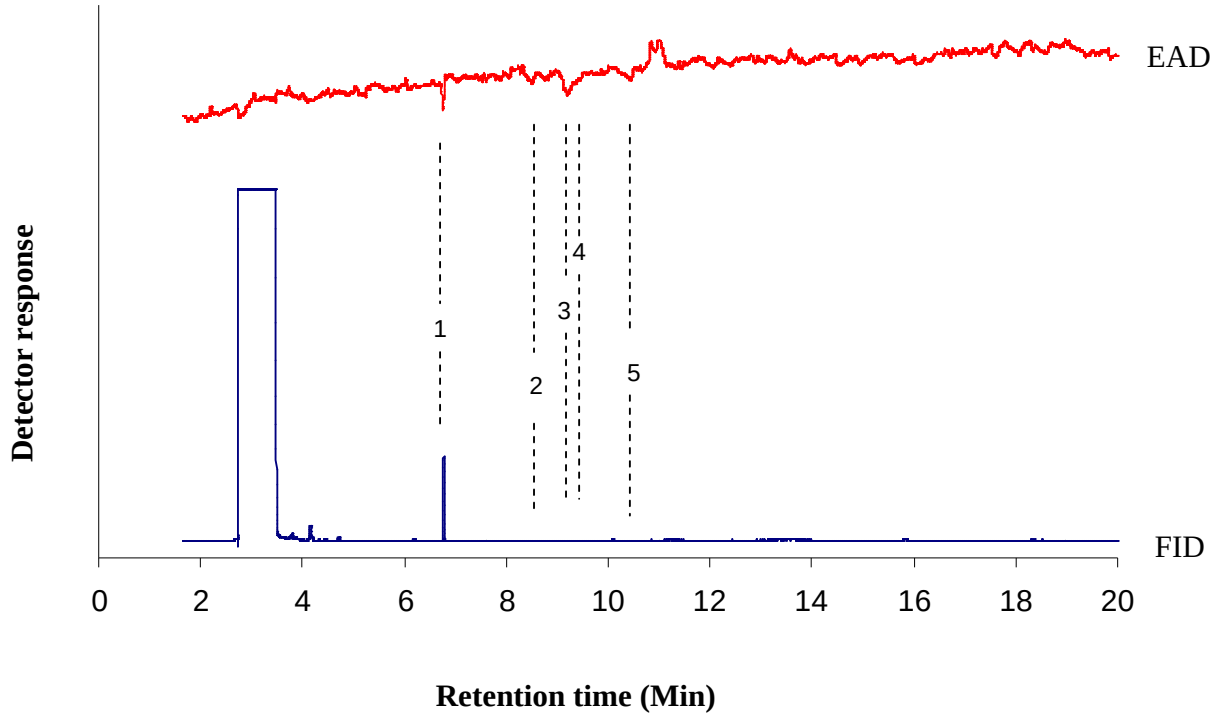
	Retention time	Retention index	Compound
1.	3.67	?	Unknown
2.	4.14	862	(E)-2-hexenal
3.	4.73	966	Unknown
4.	5.36	1069	(E)-2-octenal
5.	5.76	1136	Unknown
6.	6.20	1210	Dodecane
7.	6.67	1286	(E)-2-decenal
8.	7.34	1408	(E)-decenyl acetate
9.	7.54	1449	Unknown
10.	8.45	1633	Unknown
11.	9.15	1785	Unknown
12.	9.20	1796	Unknown
13.	10.29	2003	Icosane
14.	10.45	2031	Unknown

Figure 25: Response of female *H.halys* antenna to male *P.hybneri* extract



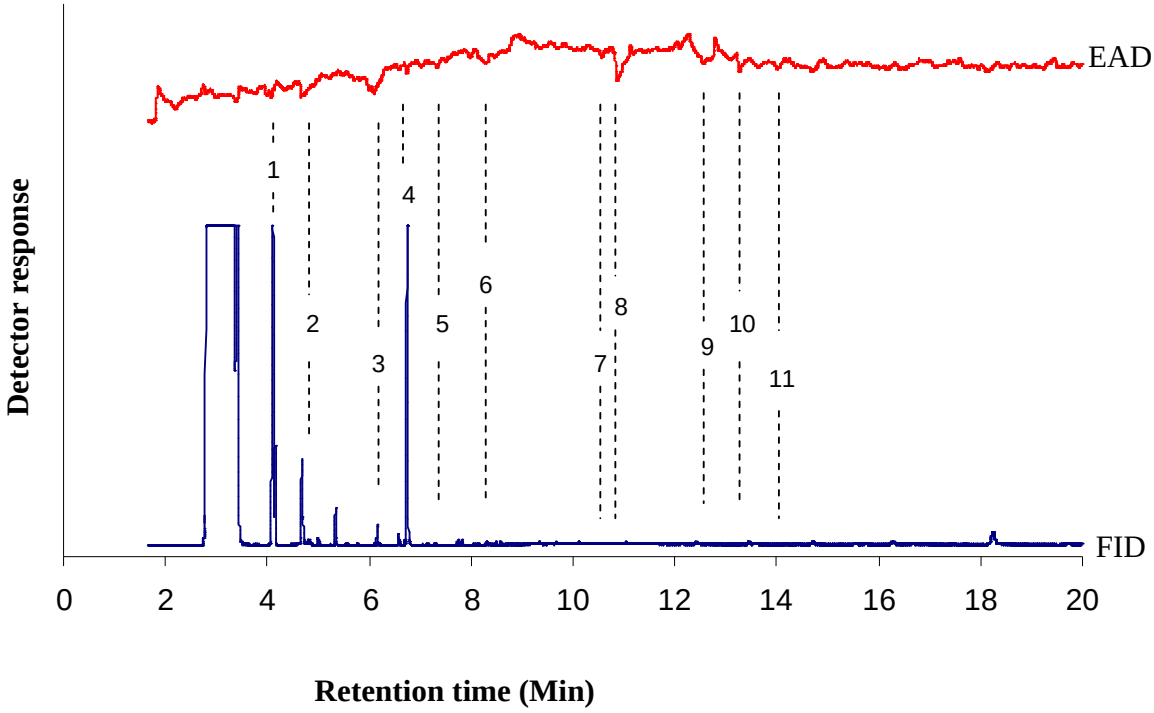
	Retention time	Retention index	Compound
1.	6.77	1302	Tridecane
2.	9.20	1796	Unknown
3.	11.43	2180	Unknown

Figure 26: Response of female *H.halys* antenna to female *P.hybneri* extract



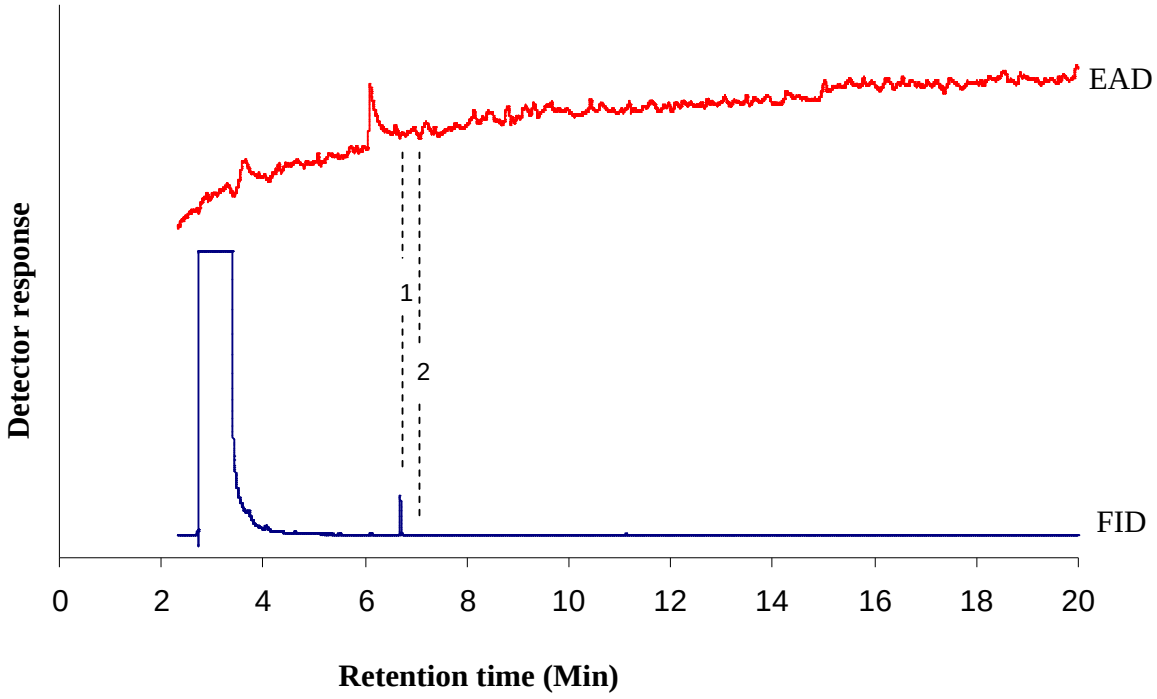
	Retention time	Retention index	Compound
1.	6.77	1302	Tridecane
2.	8.54	1652	Unknown
3.	9.13	1780	Unknown
4.	9.21	1798	Unknown
5.	10.45	2031	Unknown

Figure 27: Response of male *H.halys* antenna to female *D.baccarum* extract



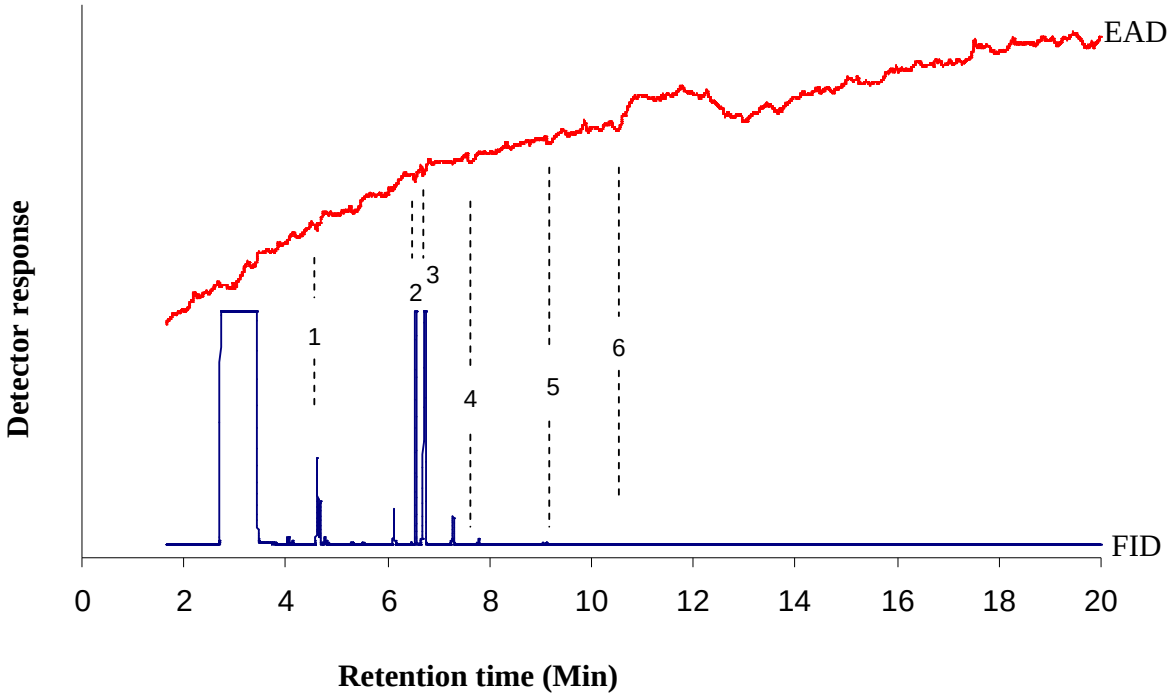
	Retention time	Retention index	Compound
1.	4.10	854	(E)-2-hexenal
2.	4.68	957	4-Oxo-(E)-2-hexenal
3.	6.14	1200	Dodecane
4.	6.75	1298	Tridecane
5.	7.32	1404	(E)-2-decenyl acetate
6.	8.29	1598	Hexadecane
7.	10.58	2053	Unknown
8.	10.88	2103	Henicosane
9.	12.63	2322	Unknown
10.	13.29	2385	Unknown
11.	14.08	2450	Unknown

Figure 28: Response of male *H.halys* antenna to female *P.hybneri* extract



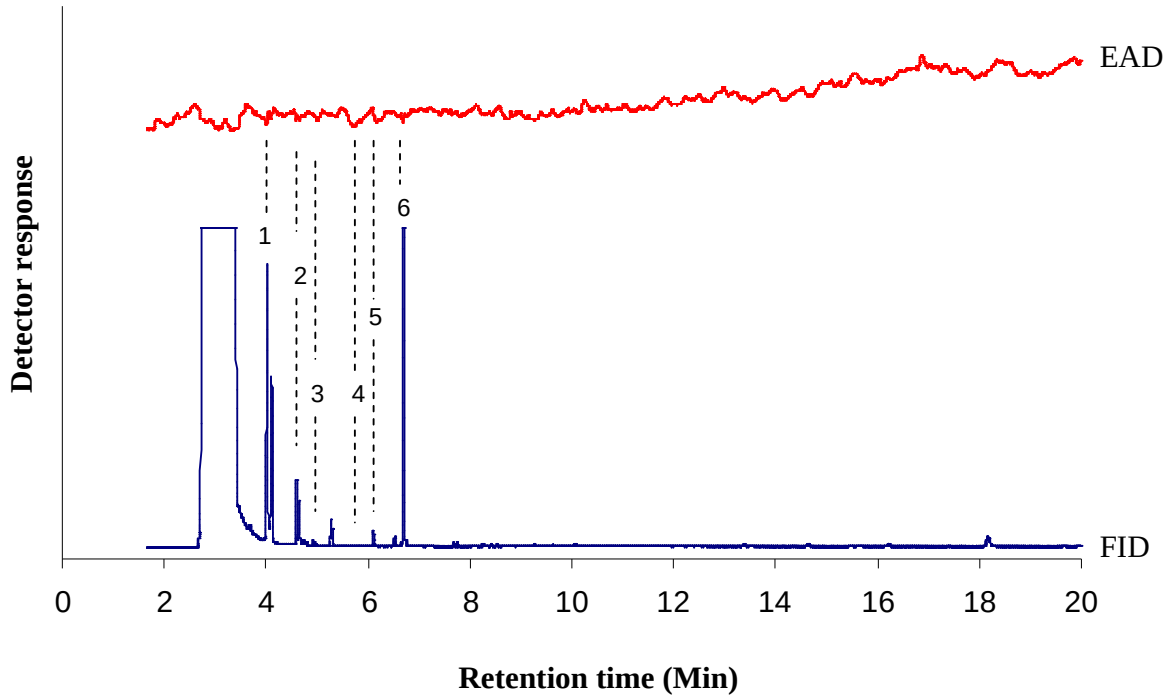
	Retention time	Retention index	Compound
1.	6.74	1296	Tridecane
2.	7.12	1368	Unknown

Figure 29: Response of male *H.halys* antenna to male *H.halys* extract



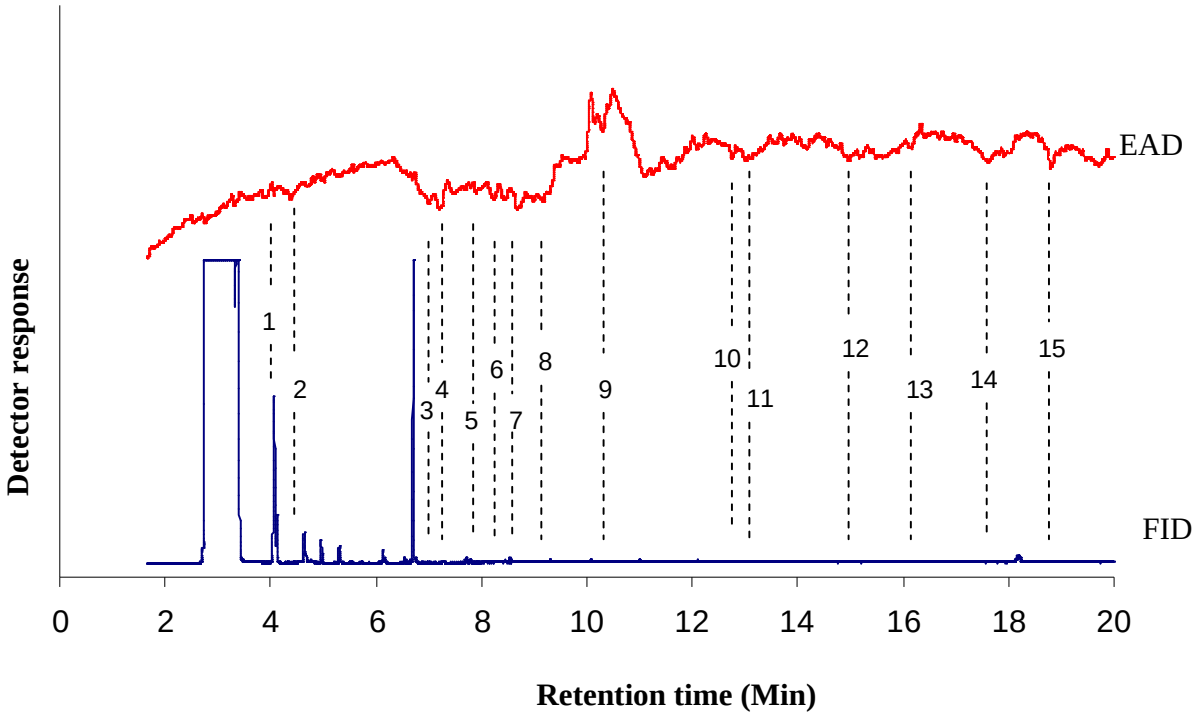
	Retention time	Retention index	Compound
1.	4.68	957	4-Oxo-(E)-hexenal
2.	6.58	1272	(E)-2-decenal
3.	6.73	1295	Tridecane
4.	7.70	1481	Unknown
5.	9.15	1785	Unknown
6.	10.51	2041	Unknown

Figure 30: Response of male *H.halys* antenna to female *D.baccarum* extract



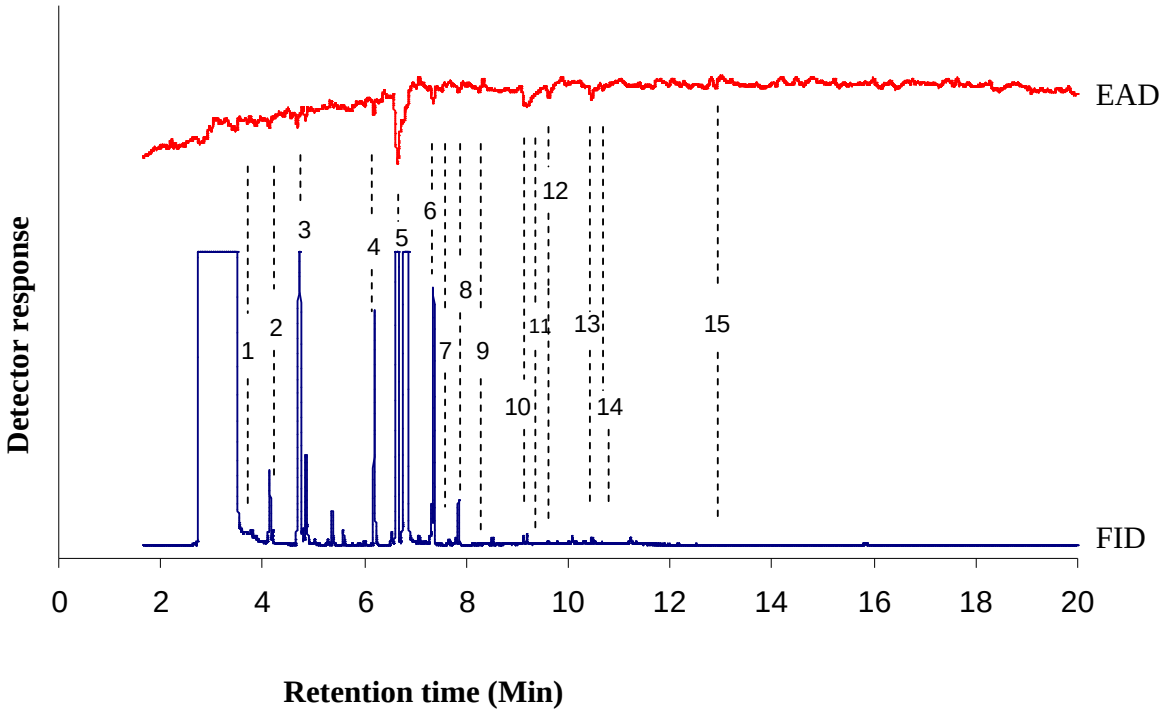
	Retention time	Retention index	Compound
1.	4.16	866	(E)-2-hexenal
2.	4.71	963	Unknown
3.	5.05	1019	2-hexen-1-ol, acetate
4.	5.80	1143	Unknown
5.	6.24	1217	Dodecane
6.	6.75	1298	Tridecane

Figure 31: Response of male *H.halys* antenna to male *D.baccarum* extract



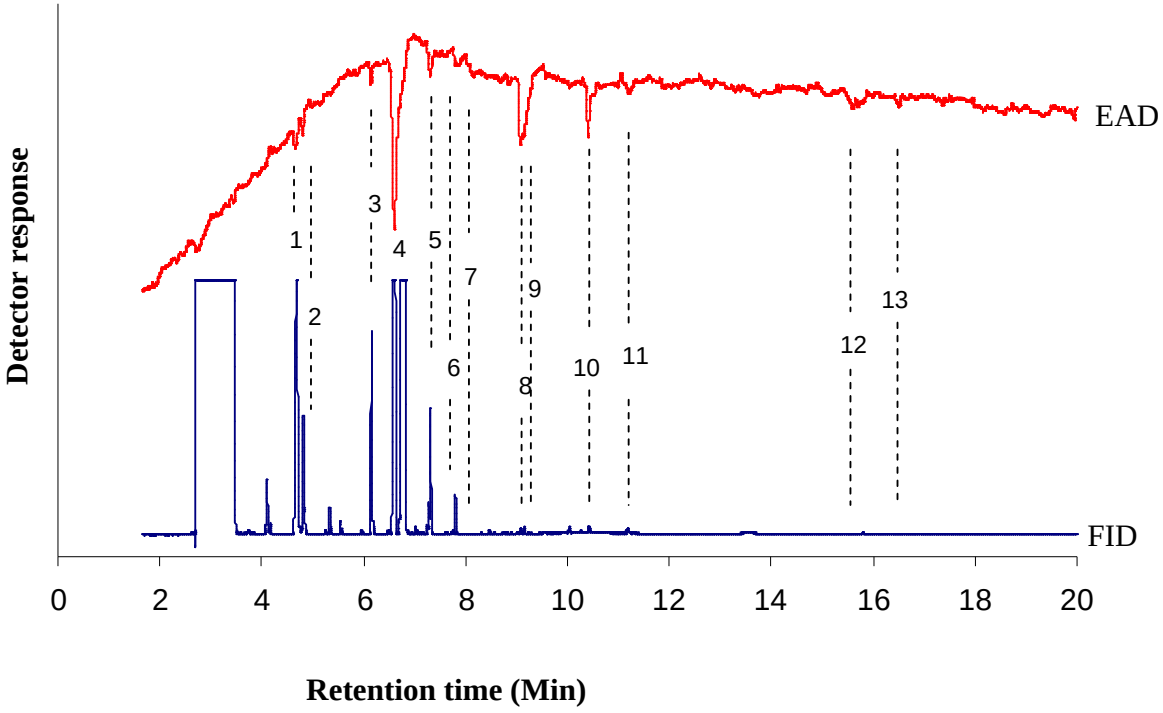
	Retention time	Retention index	Compound
1.	4.11	856	(E)-2-hexenal
2.	4.42	913	Unknown
3.	7.07	1358	Unknown
4.	7.25	1391	Unknown
5.	7.94	1529	Unknown
6.	8.29	1598	Hexadecane
7.	8.71	1688	Unknown
8.	9.19	1793	Unknown
9.	10.35	2014	Unknown
10.	12.81	2339	Unknown
11.	13.08	2365	Unknown
12.	15.03	2521	Unknown
13.	16.20	2595	Hexacosane
14.	17.64	2669	Unknown
15.	18.83	?	

Figure 32: Response of male *H.halys* antenna to male *H.halys* extract



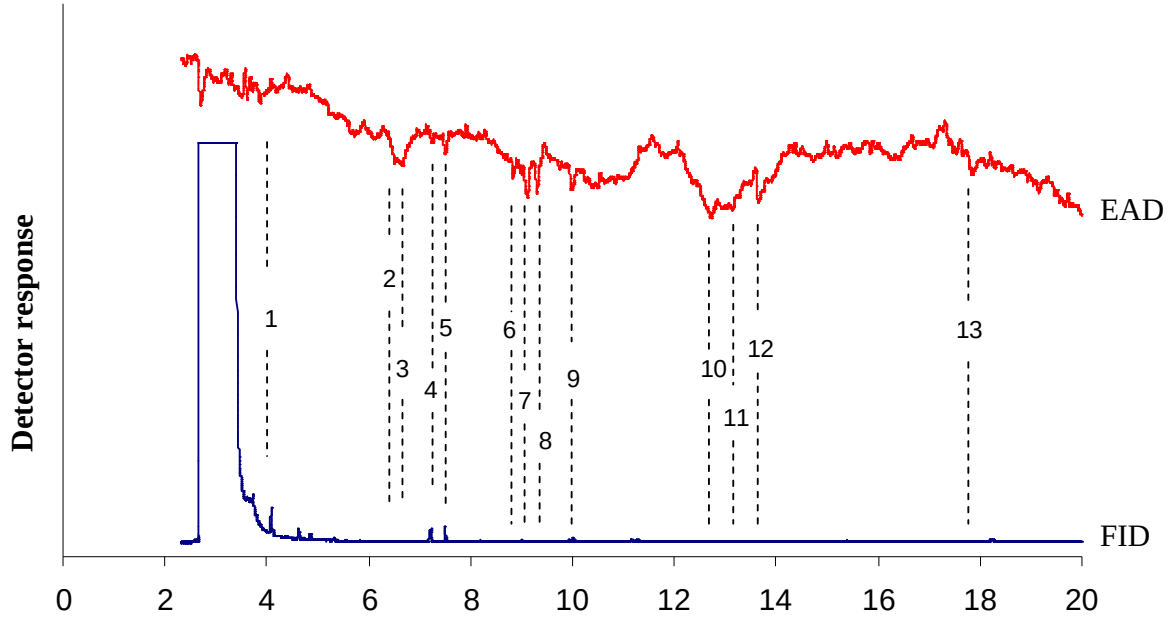
	Retention time	Retention index	Compound
1.	3.88	812	Unknown
2.	4.15	864	(E)-2-hexenal
3.	4.70	961	Unknown
4.	6.20	1210	Dodecane
5.	6.68	1288	(E)-2-decenal
6.	7.34	1408	(E)-2-deceny acetate
7.	7.62	1465	Unknown
8.	7.86	1512	Unknown
9.	8.26	1592	Hexadecane
10.	9.14	1783	Unknown
11.	9.21	1798	Unknown
12.	9.64	1883	Unknown
13.	10.47	2035	Unknown
14.	10.69	2072	Unknown
15.	12.91	2349	Unknown

Figure 33: Response of male *H.halys* antenna to female *H.halys* extract



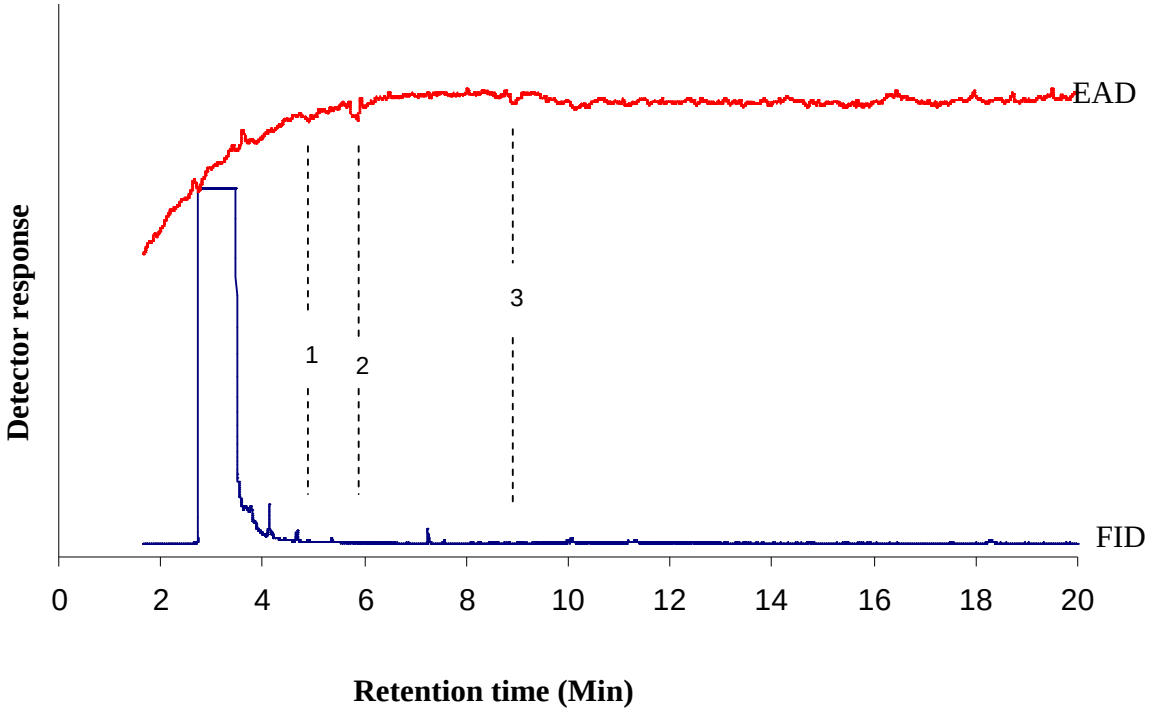
	Retention time	Retention index	Compound
1.	4.72	964	Unknown
2.	4.85	986	Unknown
3.	6.20	1210	Dodecane
4.	6.67	1286	(E)-2-decenal
5.	7.37	1414	(E)-2-decenyl acetate
6.	7.85	1510	Unknown
7.	8.23	1586	Hexadecane
8.	9.12	1778	Unknown
9.	9.19	1793	Unknown
10.	10.47	2035	Unknown
11.	11.22	2151	Unknown
12.	15.66	2561	Unknown
13.	16.59	2616	Unknown

Figure 34: Response of male *P.hybneri* antenna to male *R.clavatus* extract



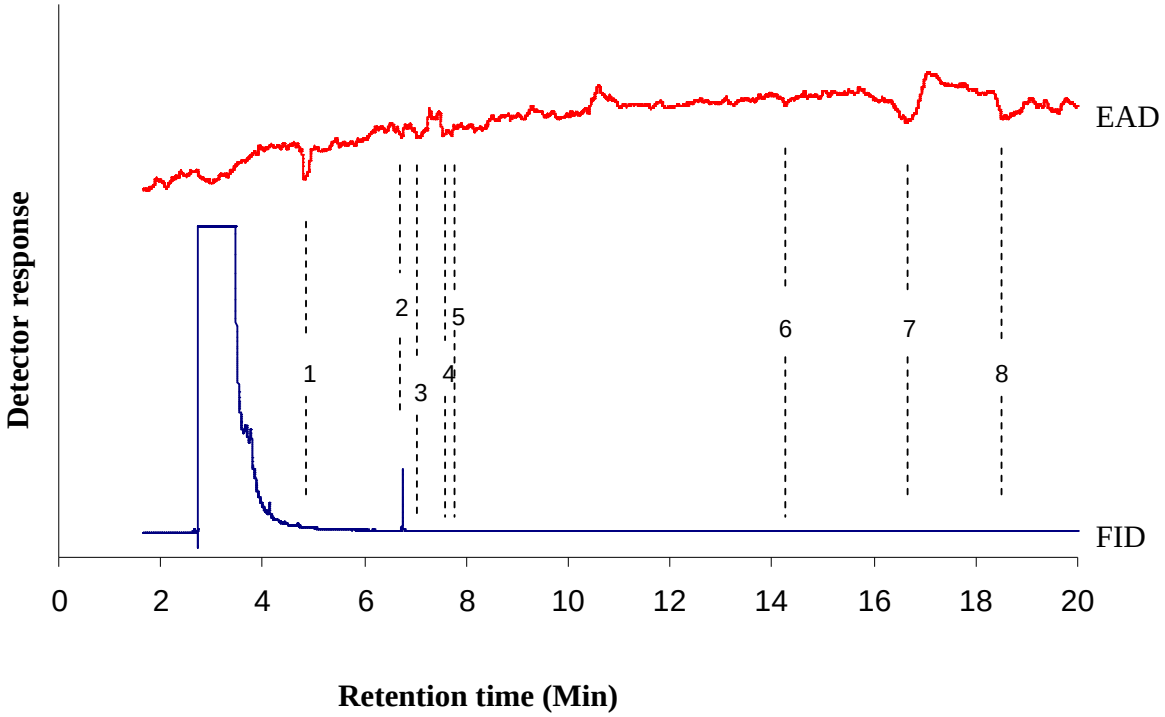
	Retention time	Retention index	Compound
1.	3.77	?	
2.	6.60	1275	(E)-2-decenal
3.	6.75	1298	Tridecane
4.	7.23	1387	Trans-2-hexenyl-cis-3-hexenoate
5.	7.56	1453	Trans-2-hexenyl-trans-2-hexenoate
6.	8.91	1732	Unknown
7.	9.19	1793	Unknown
8.	9.36	1828	Unknown
9.	9.84	1921	Unknown
10.	12.80	2338	Unknown
11.	13.23	2380	Unknown
12.	13.73	2423	Unknown
13.	17.91	2683	Unknown

Figure 35: Response of male *P.hybneri* antenna to female *R.clavatus* extract



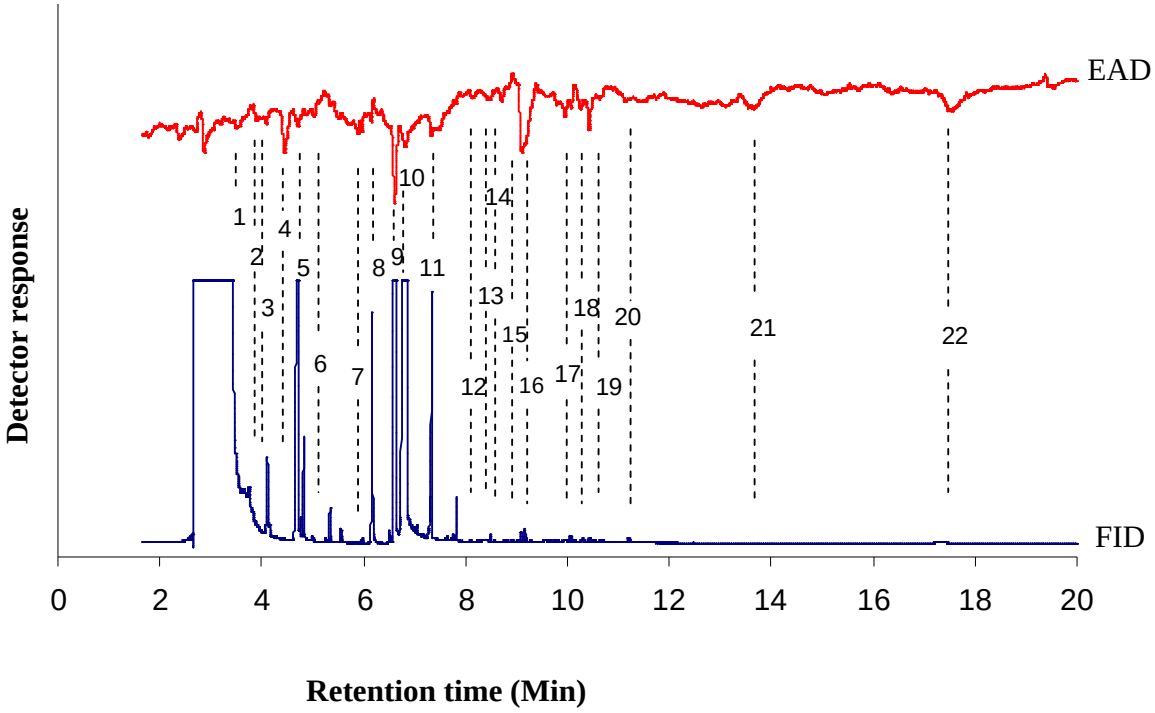
	Retention time	Retention index	Compound
1.	4.92	997	Decane
2.	5.88	1156	Unknown
3.	8.89	1727	Unknown

Figure 36: Response of male *P.hybneri* antenna to female *P.hybneri* extract



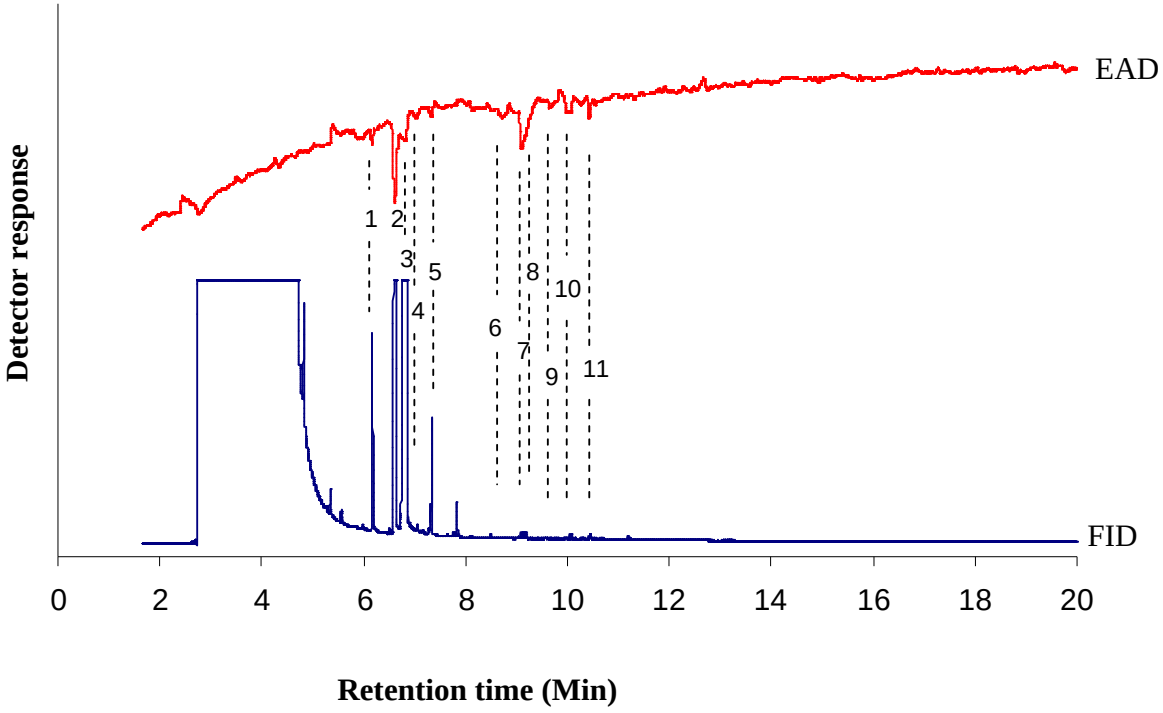
	Retention time	Retention index	Compound
1.	4.85	986	Unknown
2.	6.77	1302	Tridecane
3.	7.13	1369	Unknown
4.	7.56	1453	Trans-2-hexenyl-trans-2-hexenoate?
5.	7.76	1492	Unknown
6.	14.30	2468	Unknown
7.	16.67	2620	Unknown
8.	18.56	?	

Figure 37: Response of male *P.hybneri* antenna to male *H.halys* extract



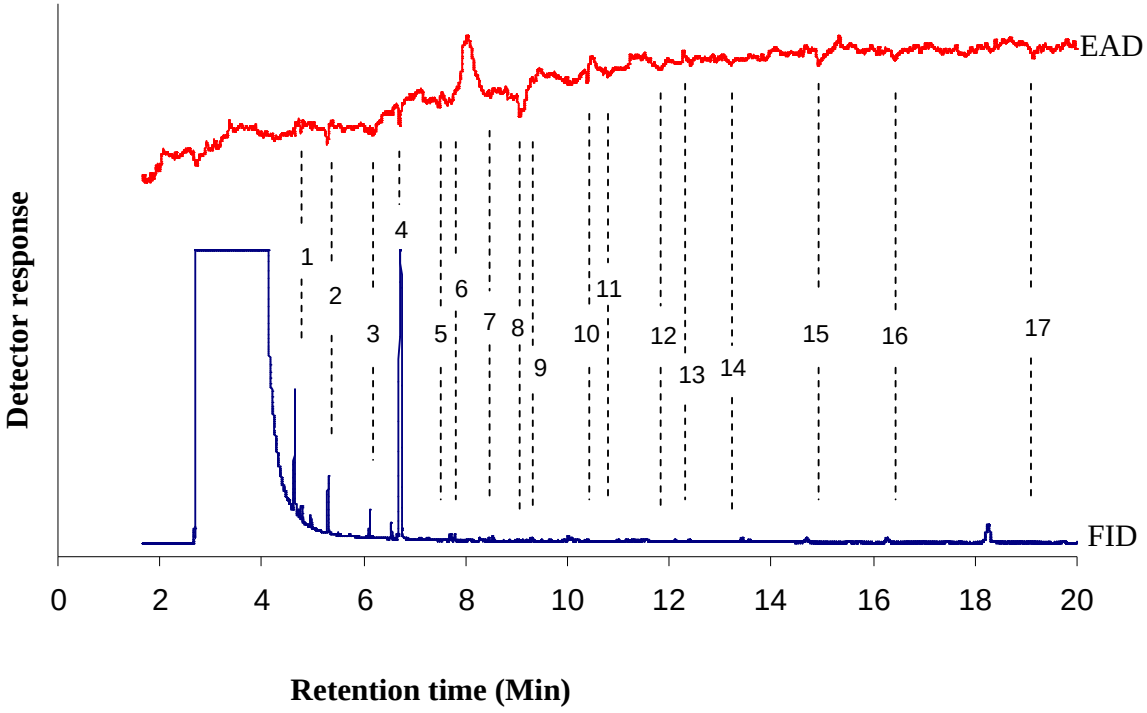
Retention time	Retention index	Compound
1. 3.64	?	Unknown
2. 4.02	839	Unknown
3. 4.12	858	(E)-2-hexenal
4. 4.45	918	Unknown
5. 4.72	962	Unknown
6. 5.04	1017	1,4-cyclohex-2-enedione
7. 5.92	1163	Unknown
8. 6.16	1203	Dodecane
9. 6.65	1283	(E)-2-decenal
10. 6.80	1308	Tridecane
11. 7.34	1408	(E)-2-decenyl acetate
12. 8.12	1565	Unknown
13. 8.52	1647	Unknown
14. 8.72	1690	Unknown
15. 9.12	1778	Unknown
16. 9.19	1793	Unknown
17. 9.96	1943	Unknown
18. 10.28	2002	Icosane
19. 10.45	2031	Unknown
20. 11.16	2142	Unknown
21. 13.67	2418	Unknown
22. 17.58	2666	Unknown

Figure 38: Response of male *P.hybneri* antenna to female *H.halys* extract



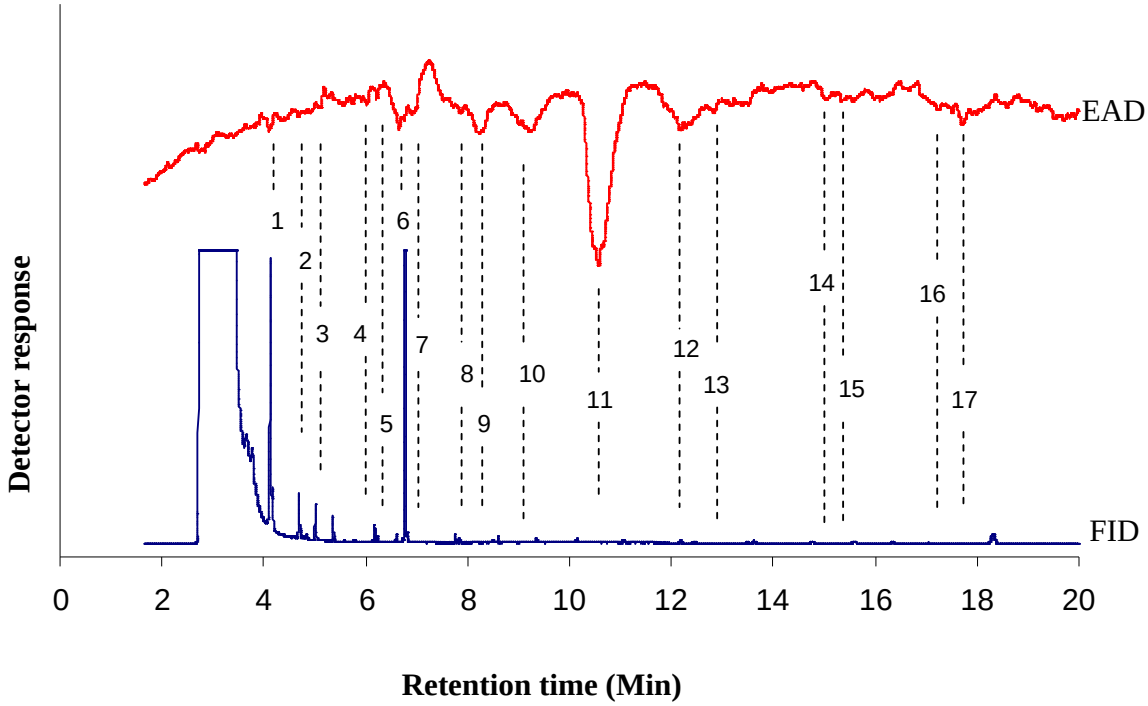
	Retention time	Retention index	Compound
1.	6.19	1208	Dodecane
2.	6.63	1280	(E)-2-decenal
3.	6.83	1313	Unknown
4.	7.05	1355	Unknown
5.	7.34	1408	(E)-2-decenyl acetate
6.	8.73	1692	Unknown
7.	9.11	1776	Unknown
8.	9.16	1787	Unknown
9.	9.65	1885	Unknown
10.	10.07	1964	Unknown
11.	10.44	2029	Unknown

Figure 39: Response of male *P.hybneri* antenna to female *D.baccarum* extract



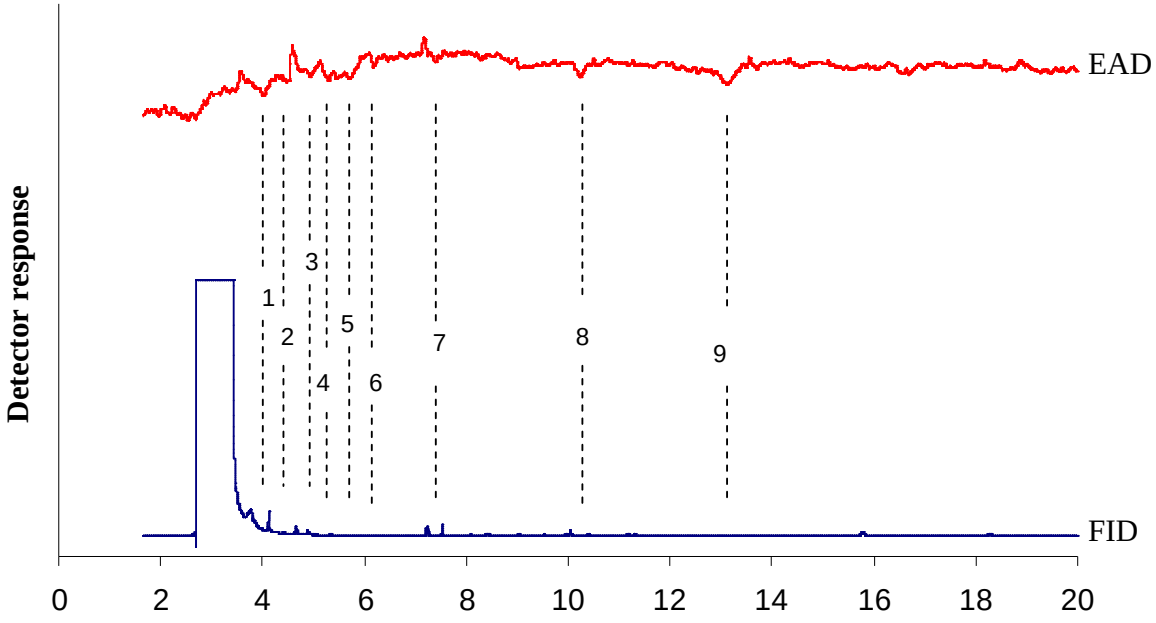
	Retention time	Retention index	Compound
1.	4.71	963	Unknown
2.	5.35	1067	(E)-2-octenal
3.	6.18	1207	Dodecane
4.	6.77	1302	Tridecane
5.	7.56	1453	Unknown
6.	7.75	1490	Unknown
7.	8.48	1639	Unknown
8.	9.12	1778	Unknown
9.	9.20	1796	Unknown
10.	10.45	2031	Unknown
11.	10.86	2100	Henicosane
12.	11.86	2235	Unknown
13.	12.45	2304	Tricosane
14.	13.25	2381	Unknown
15.	14.99	2518	Unknown
16.	16.50	2611	Unknown
17.	19.21	?	Unknown

Figure 40: Response of male *P.hybneri* antenna to male *D.baccarum* extract



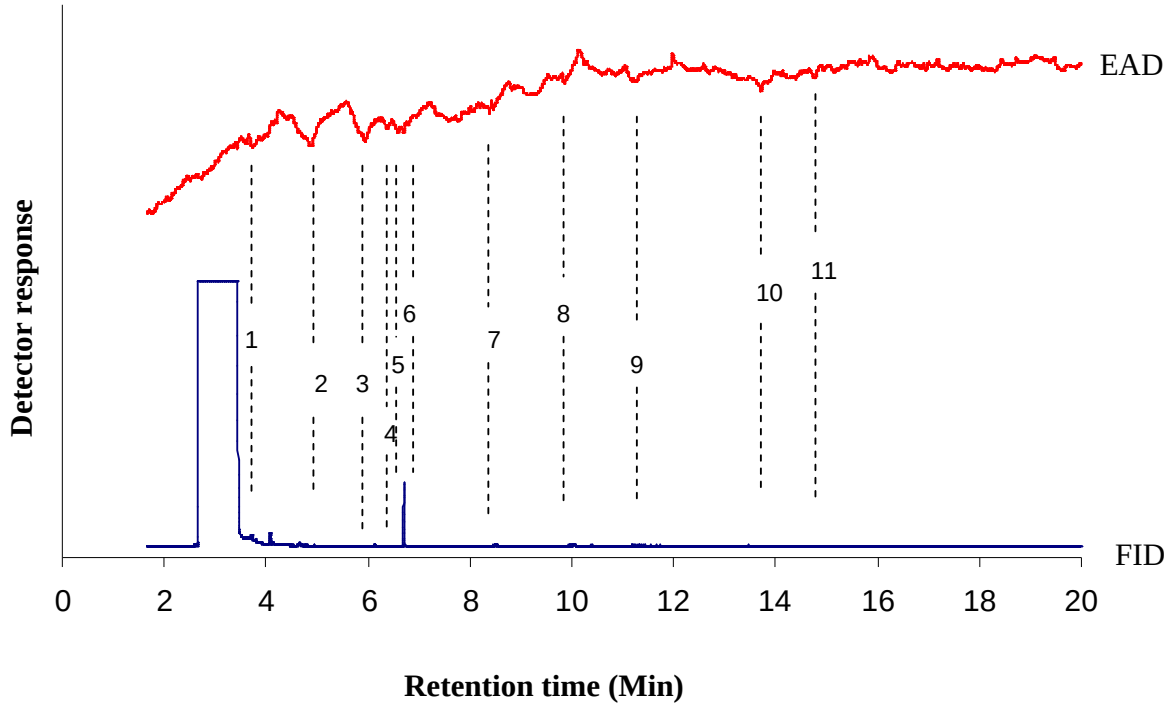
	Retention time	Retention index	Compound
1.	4.12	858	(E)-2-hexenal
2.	4.68	957	4-Oxo-(E)-2-hexenal
3.	5.28	1056	(E)-2-octenal
4.	6.03	1182	Unknown
5.	6.23	1215	Dodecane
6.	6.66	1285	(E)-2-decenal
7.	6.78	1304	Tridecane
8.	8.04	1549	Unknown
9.	8.25	1590	Hexadecane
10.	9.25	1806	Unknown
11.	10.60	2057	Unknown
12.	12.18	2273	Unknown
13.	12.84	2342	Unknown
14.	15.36	2542	Unknown
15.	15.58	2556	Unknown
16.	17.27	2651	Unknown
17.	17.72	2672	Unknown

Figure 41: Response of female *P.hybneri* antenna to male *R.clavatus* extract



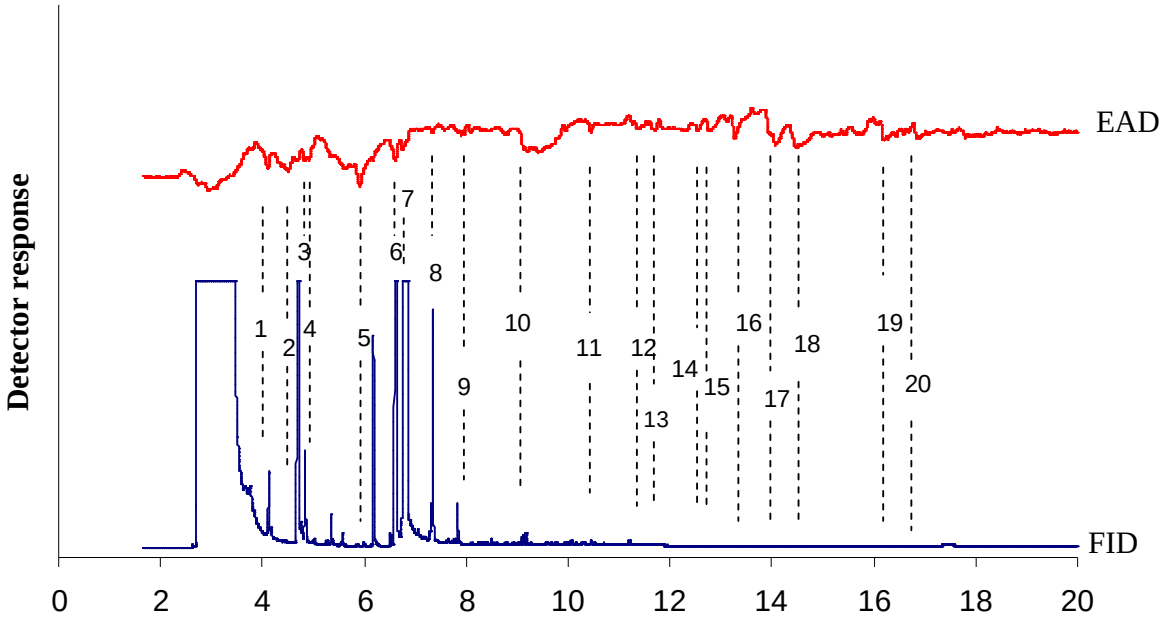
	Retention time	Retention index	Compound
1.	4.10	854	(E)-2-hexenal
2.	4.60	944	Unknown
3.	4.99	1009	Decane
4.	5.35	1067	Unknown
5.	5.75	1134	Unknown
6.	6.22	1213	Unknown
7.	7.41	1423	Unknown
8.	10.29	2003	Icosane
9.	13.14	2371	Unknown

Figure 42: Response of female *P.hybneri* antenna to female *P.hybneri* extract



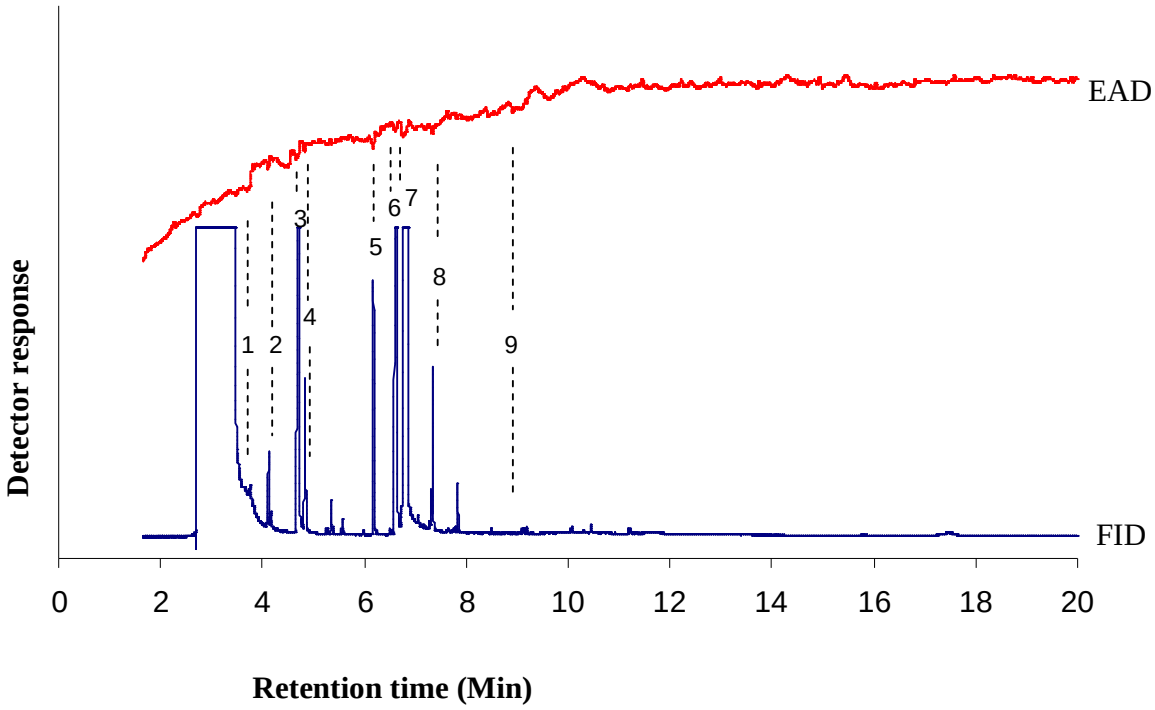
	Retention time	Retention index	Compound
1.	3.82	800	Octane
2.	4.93	998	Decane
3.	6.03	1182	Unknown
4.	6.46	1253	Unknown
5.	6.67	1286	(E)-2-decenal
6.	6.78	1304	Tridecane
7.	8.47	1637	Unknown
8.	9.91	1934	Unknown
9.	11.27	2158	Unknown
10.	13.80	2429	Unknown
11.	14.85	2509	Unknown

Figure 43: Response of female *P.hybneri* antenna to male *H. halys* extract



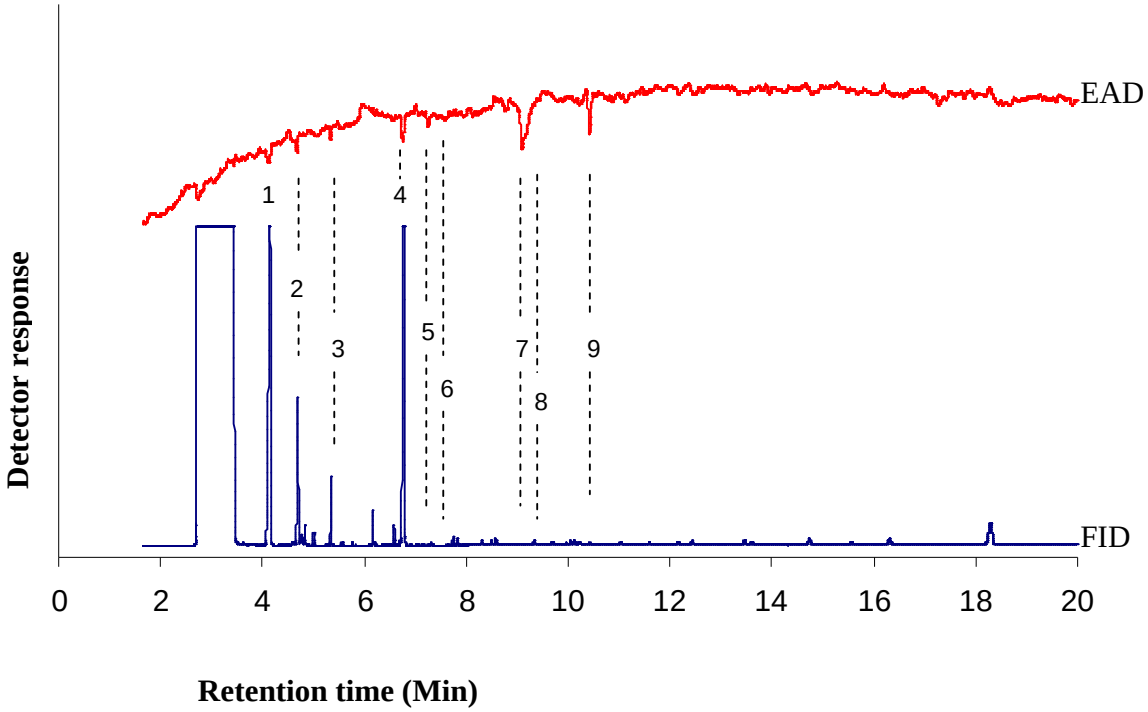
	Retention time	Retention index	Compound
1.	4.12	858	(E)-2-hexenal
2.	4.51	928	Unknown
3.	4.83	982	Unknown
4.	4.92	997	Decane
5.	5.92	1163	Unknown
6.	6.62	1278	(E)-2-decenal
7.	6.77	1302	Tridecane
8.	7.35	1410	(E)-2-decenyl acetate
9.	7.92	1525	Unknown
10.	9.12	1778	Unknown
11.	10.46	2033	Unknown
12.	11.41	2177	Unknown
13.	11.73	2219	Unknown
14.	12.57	2316	Unknown
15.	12.80	2338	Unknown
16.	13.28	2384	Unknown
17.	14.07	2450	Unknown
18.	14.62	2493	Pentacosane
19.	16.22	2596	Hexacosane
20.	16.85	2629	Unknown

Figure 44: Response of female *P.hybneri* antenna to female *H.halys* extract



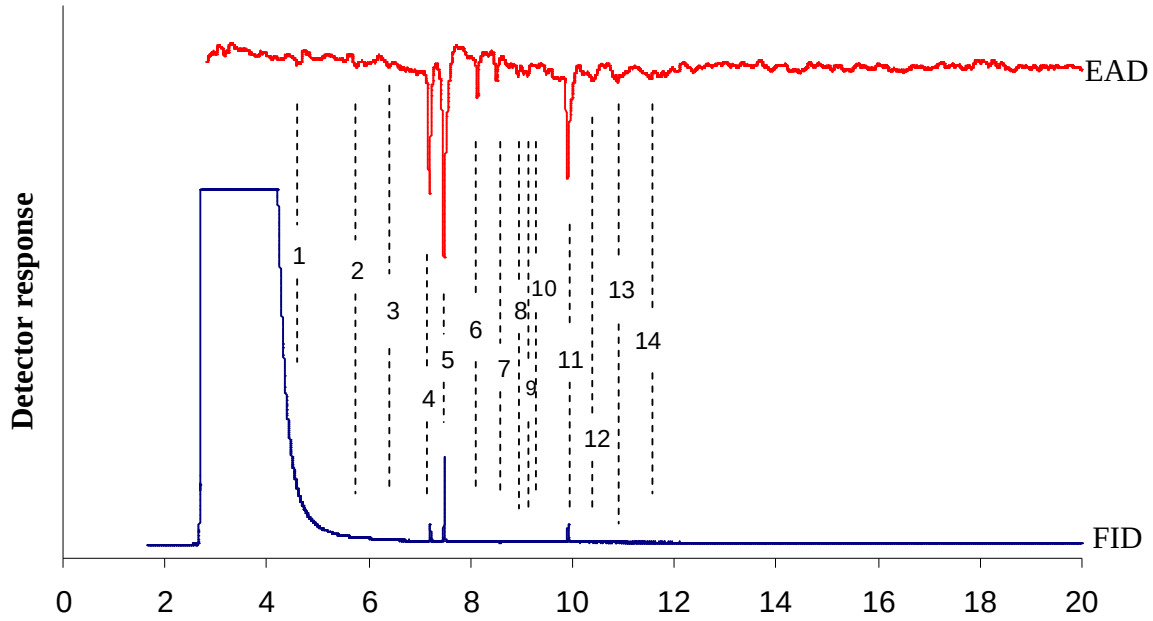
	Retention time	Retention index	Compound
1.	3.74	?	Unknown
2.	4.13	857	(E)-2-hexenal
3.	4.68	957	4-Oxo-(E)-2-hexenal
4.	4.84	984	Unknown
5.	6.18	1207	Decane
6.	6.62	1278	(E)-2-decenal
7.	6.75	1298	Tridecane
8.	7.36	1412	(E)-2-decenyl acetate
9.	9.09	1771	Unknown

Figure 45: Response of female *P.hybneri* antenna to female *D.baccarum* extract



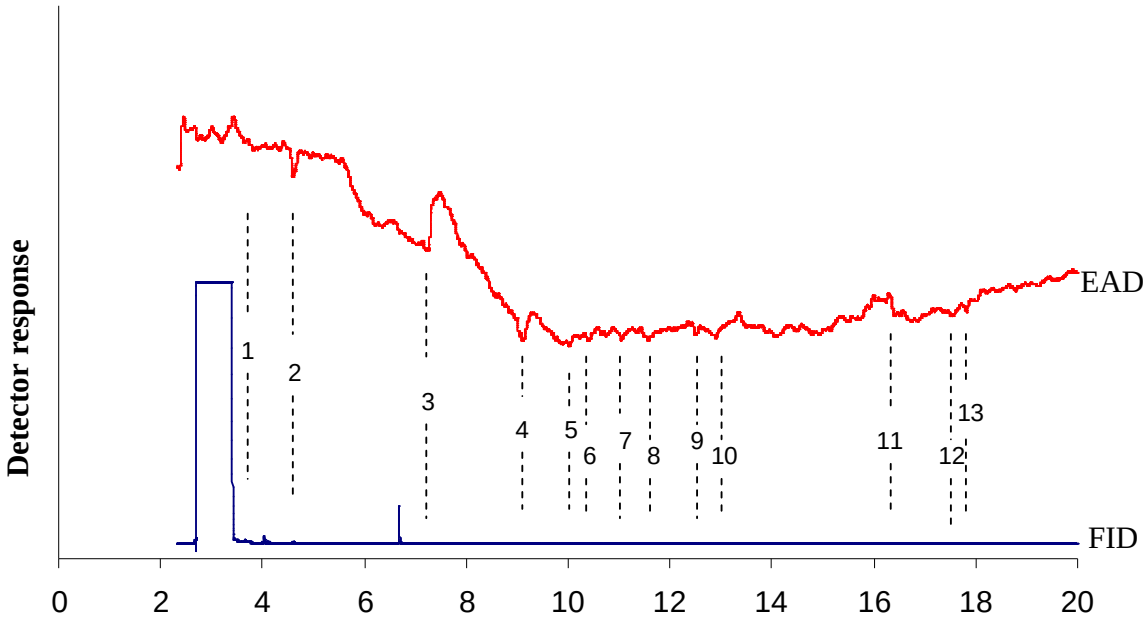
	Retention time	Retention index	Compound
1.	4.13	857	(E)-2-hexenal
2.	4.72	964	Unknown
3.	5.35	1067	(E)-2-octenal
4.	6.78	1304	Tridecane
5.	7.30	1400	Tetradecane
6.	7.61	1463	Unknown
7.	9.12	1778	Unknown
8.	9.19	1793	Unknown
9.	10.44	2029	Unknown

Figure 46: Response of female *R.clavatus* antenna versus male *R.clavatus* extract



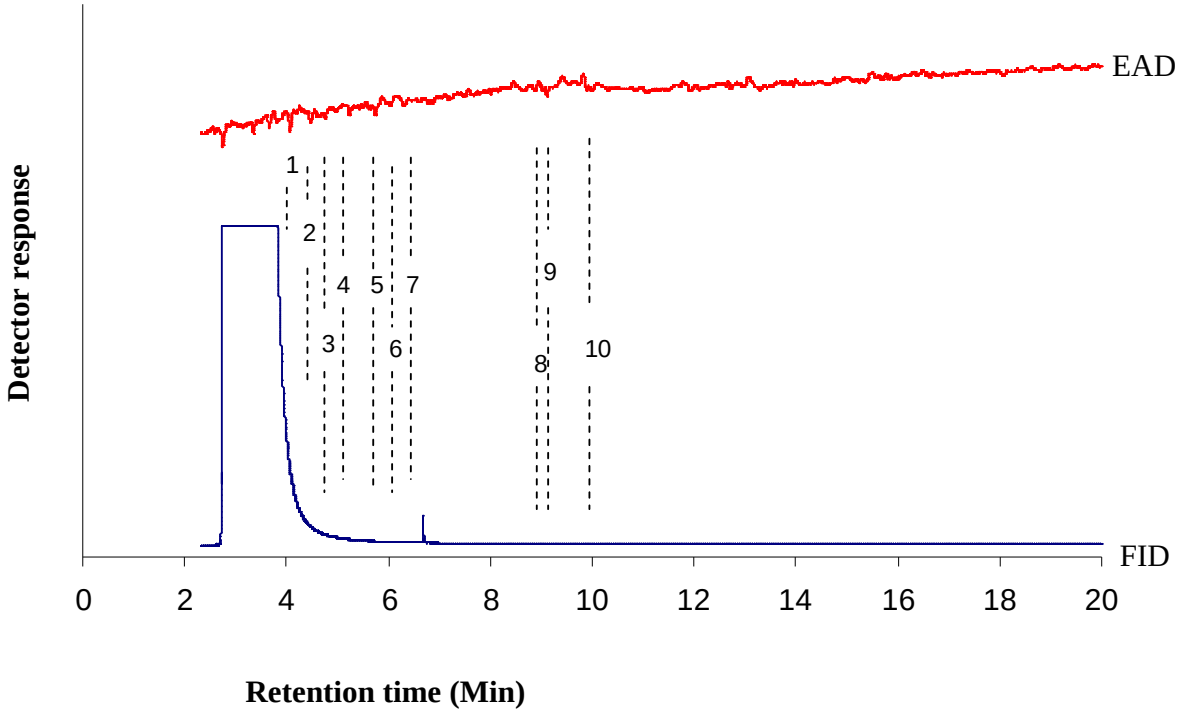
	Retention time	Retention index	Compound
1.	4.65	952	4-Oxo-(E)-2-hexenal
2.	5.88	1156	Unknown
3.	6.47	1254	Unknown
4.	7.26	1393	Trans-2-hexenyl-cis-3-hexenoate
5.	7.56	1453	Trans-2-hexenyl-trans-2-hexenoate
6.	8.20	1582	Unknown
7.	8.57	1659	Unknown
8.	9.00	1752	Unknown
9.	9.11	1776	Unknown
10.	9.18	1791	Unknown
11.	9.96	1943	Tetradecyl isobutyrate
12.	10.47	2035	Unknown
13.	10.94	2111	Unknown
14.	11.59	2199	Docosane

Figure 47: Response of female *R.clavatus* antenna to female *P.hybneri* extract



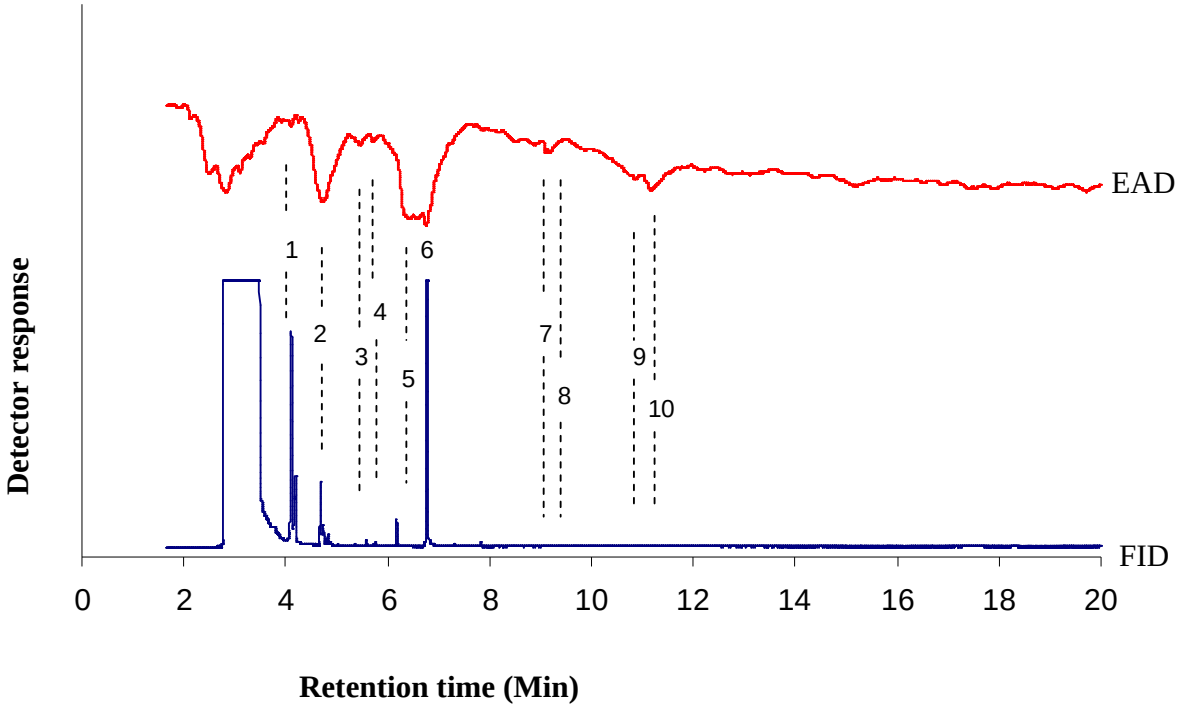
	Retention time	Retention index	Compound
1.	3.89	814	Unknown
2.	4.68	957	4-Oxo-(E)-2-hexenal
3.	7.36	1412	(E)-2-decenyl acetate
4.	9.18	1791	Unknown
5.	10.08	1965	Unknown
6.	10.44	2029	Unknown
7.	11.12	2137	Unknown
8.	11.65	2209	Unknown
9.	12.55	2316	Unknown
10.	12.98	2357	Unknown
11.	16.47	2609	Unknown
12.	17.58	2666	Unknown
13.	17.89	2683	Unknown

Figure 48: Response of female *R.clavatus* antenna to male *P.hybneri* extract



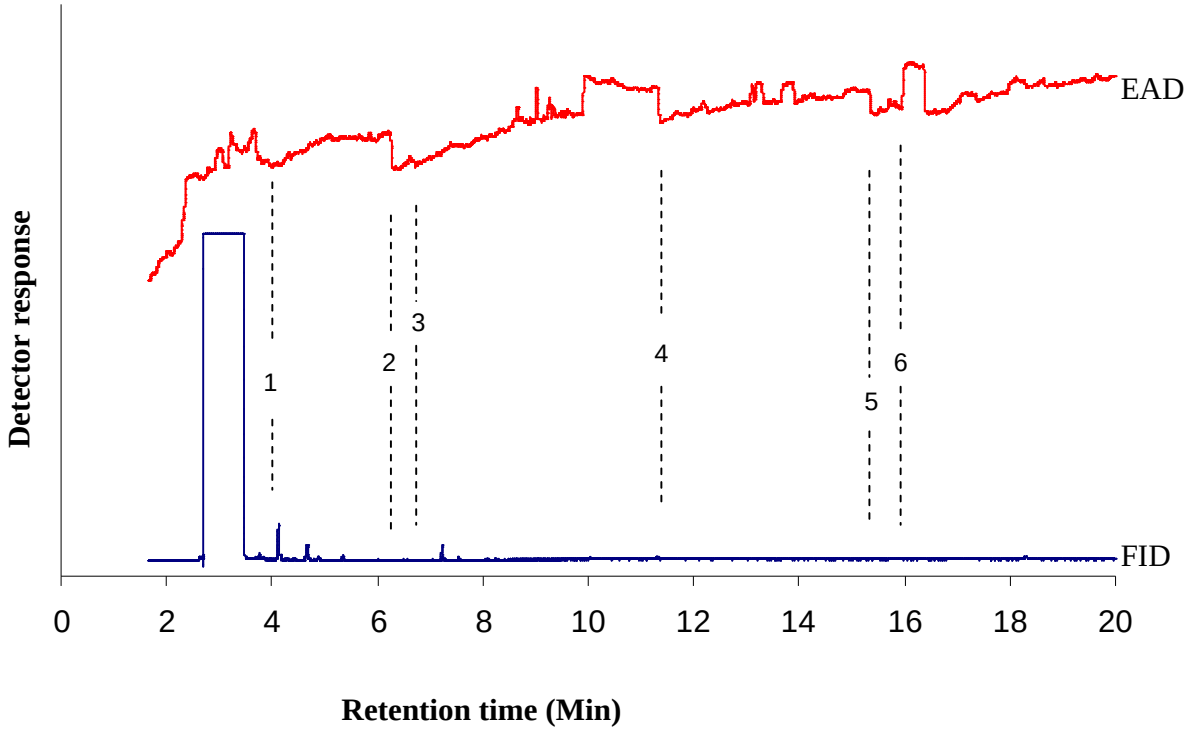
	Retention time	Retention index	Compound
1.	4.12	856	(E)-2-hexenal
2.	4.55	935	Unknown
3.	4.65	952	4-Oxo-(E)-2-hexenal
4.	5.31	1061	Unknown
5.	5.82	1145	Unknown
6.	6.11	1195	Unknown
7.	6.30	1226	Unknown
8.	9.09	1772	Unknown
9.	9.18	1791	Unknown
10.	10.03	1956	Unknown

Figure 49: Response of male *D.baccarum* antenna to male *P.hybneri* extract



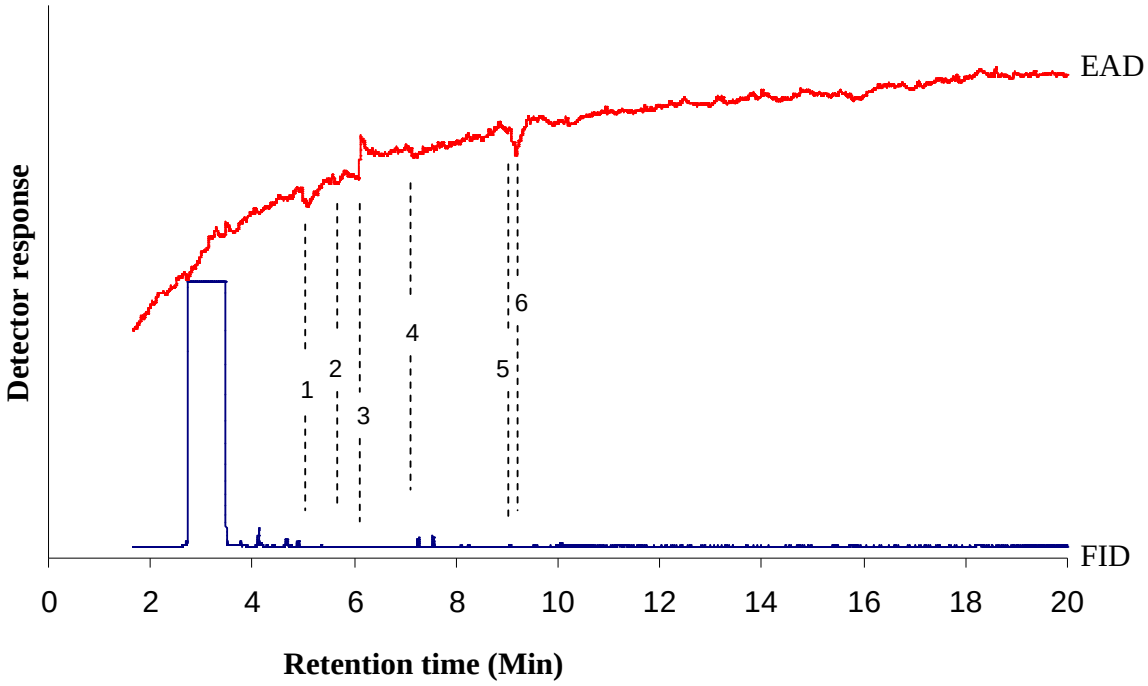
	Retention time	Retention index	Compound
1.	4.12	856	(E)-2-hexenal
2.	4.68	956	4-Oxo-(E)-2-hexenal
3.	5.48	1085	Unknown
4.	5.74	1130	Unknown
5.	6.41	1245	Unknown
6.	6.78	1304	Tridecane
7.	9.12	1773	Unknown
8.	9.20	1790	Unknown
9.	10.88	2100	Henicosane
10.	11.19	2145	Uknown

Figure 50: Response of male *D.baccarum* antenna to female *R.clavatus* extract



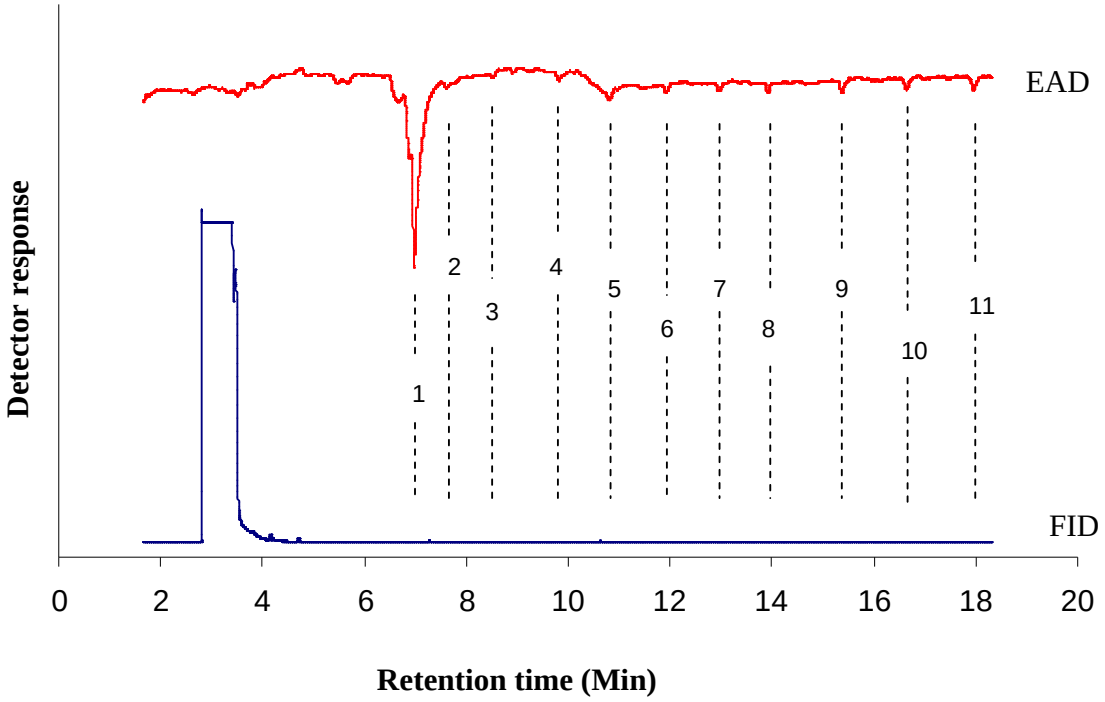
	Retention time	Retention index	Compound
1.	4.12	856	(E)-2-hexenal
2.	6.34	1233	Unknown
3.	6.76	1300	Tridecane
4.	11.42	2178	Unknown
5.	15.49	2551	Unknown
6.	15.97	2580	Unknown

Figure 51: Response of male *D.baccarum* antenna to male *R.clavatus* extract



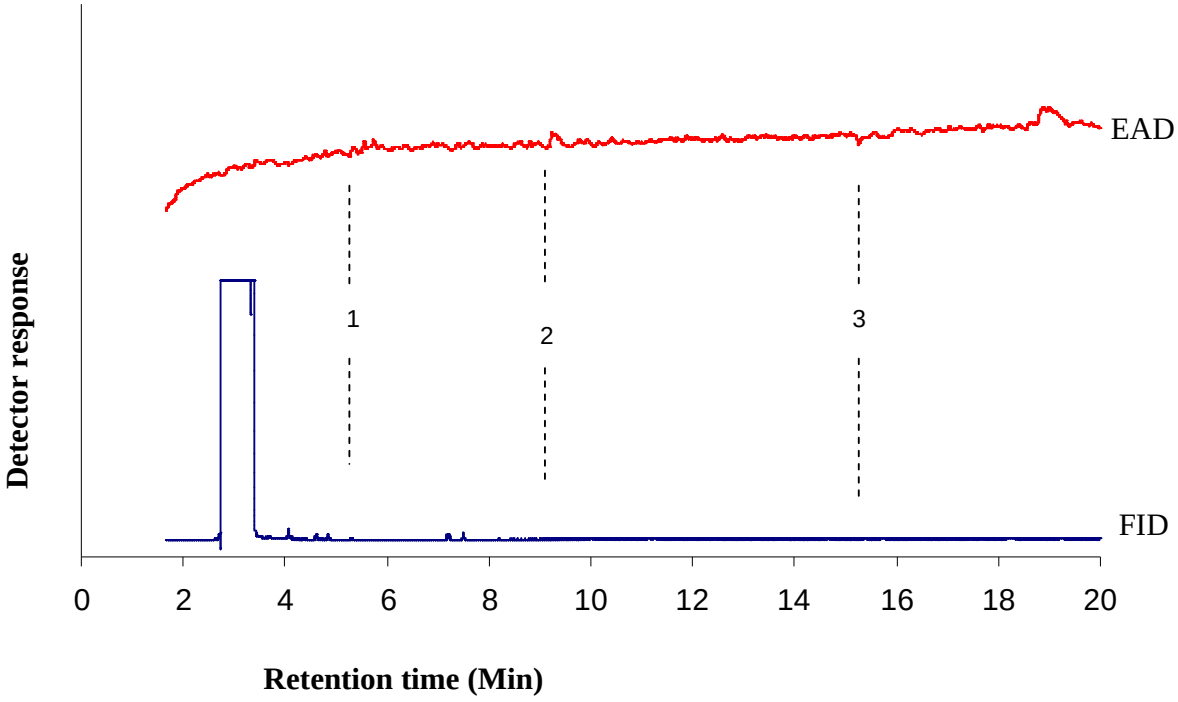
	Retention time	Retention index	Compound
1.	4.12	856	(E)-2-hexenal
2.	5.70	1125	Unknown
3.	6.10	1193	Dodecane
4.	7.25	1391	Trans-2-hexenyl-cis-3-hexenoate
5.	9.12	1773	Unknown
6.	9.20	1790	Unknown

Figure 52: Response of female *D.baccarum* antenna to female *R.clavatus* extract



	Retention time	Retention index	Compound
1.	6.99	1342	Unknown
2.	7.60	1455	Unknown
3.	8.52	1642	Unknown
4.	9.83	1913	Unknown
5.	10.82	1922	Unknown
6.	11.94	2240	Unknown
7.	12.96	2349	Unknown
8.	13.97	2437	Unknown
9.	15.37	2540	Unknown
10.	16.66	2617	Unknown
11.	17.97	2681	Unknown

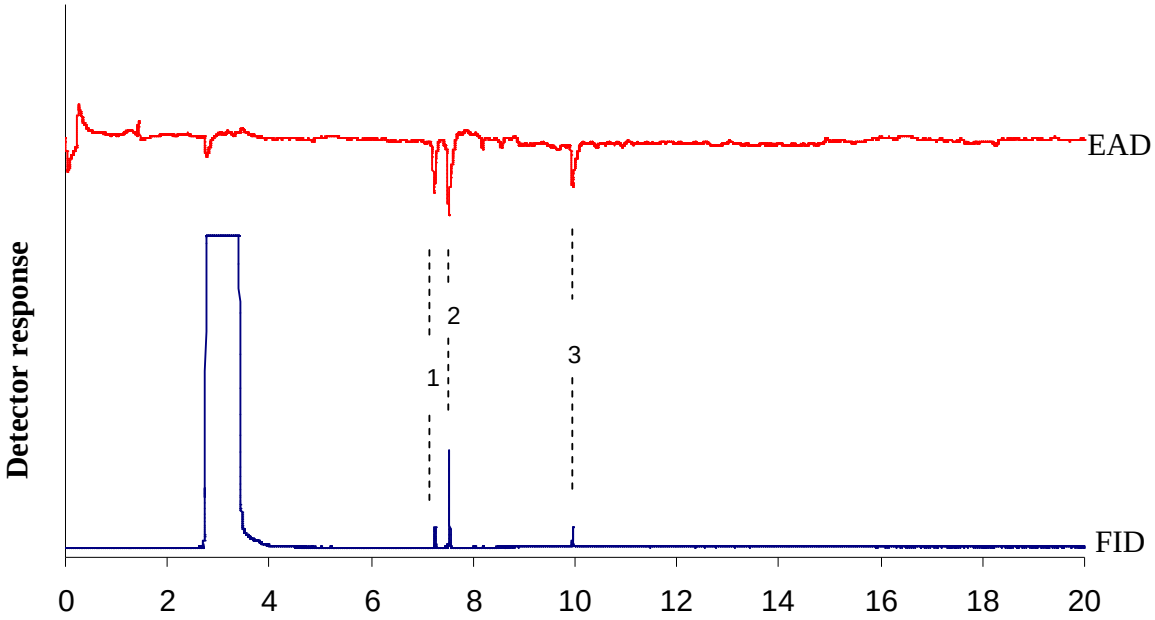
Figure 53: Response of female *H.halys* antenna to male *R.clavatus* extract



	Retention time	Retention index	Compound
1.	5.35	1066	Unknown
2.	9.20	1796	Unknown
3.	15.32	2539	Unknown

A positive control

Figure 54: Response of female *R.clavatus* antenna to synthetic version of male *R. clavatus*-produced aggregation pheromone.



Retention time	Retention index	Compound
7.24	1389	Trans-2-hexenyl-cis-3-hexenoate
7.56	1453	Trans-2-hexenyl-trans-2-hexenoate
9.97	1945	Tetradecyl isobutyrate

A negative control.

Figure 55: Response of female *R.clavatus* antenna to hexane.

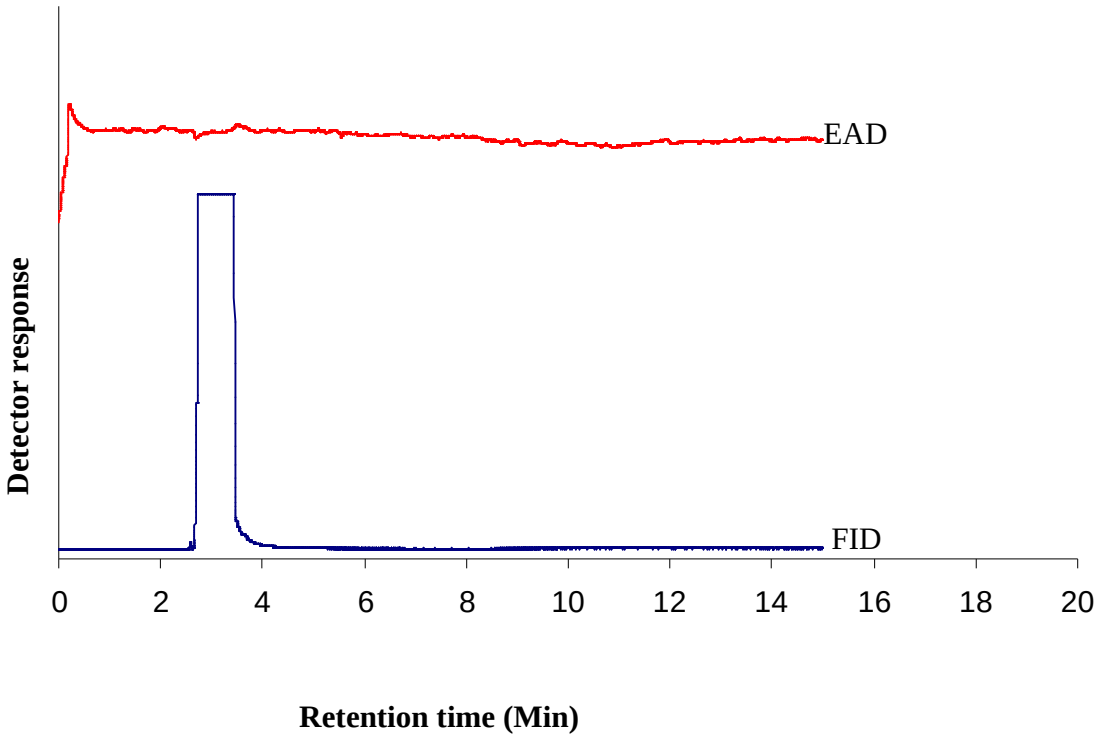
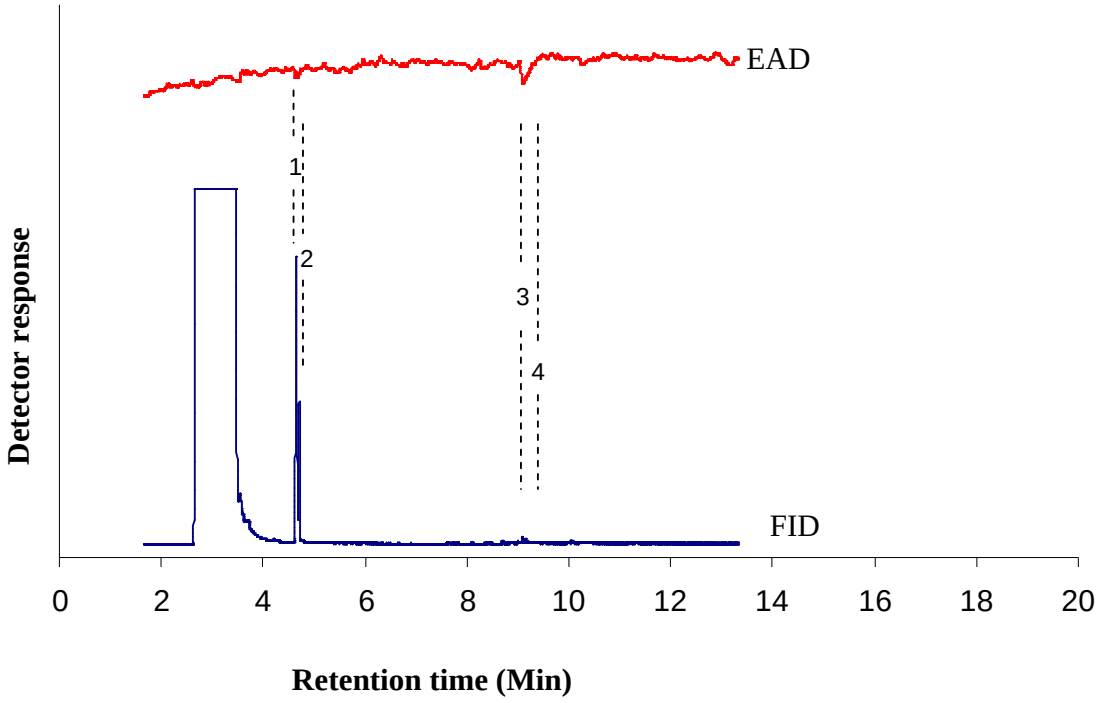


Figure 56: Response of male *P.hybneri* antenna to 4-Oxo-(E)-hexenal and its unidentified dimers (0.01mg/ml)



Retention time	Retention index	Compound
4.68	957	4-Oxo-(E)-2-hexenal
4.72	964	Unknown
9.11	1776	Unknown
9.20	1796	Unknown

5.2 Compounds identified from the hexane extracts.

Compounds contained within the extracts that elicited antennal responses from the four insects were identified through GC-MS analyses. To chemically identify the compounds, the retention times of flame ionization detector (FID) peaks that corresponded to antennal (EAD) peaks were converted into retention indices. The GC-EAD-derived retention indices were compared with those calculated from the same hexane extracts injected into GC-MS system. When the retention indices derived from GC-EAD perfectly or very closely matched (at least 80% match) those obtained from the GC-MS, then the respective compounds were identified from the GC-MS library based on mass spectrum matches. As such, identifications of EAD-active compounds from the hexane extracts were provisionally based on their closeness of match in retention indices and mass spectra with compounds already present in the library. On the basis of this, various

compounds were identified from the crude extracts of the four species of stink bug (Figs.6-53; Table 3). Nevertheless, many electrophysiologically-active compounds remained unknown because they were in too low concentrations (as measured by their peak heights) to be identified by the GC-MS machine.

In this study, an aggregation pheromone of *R.clavatus* (a mixture of trans-2-hexenyl-cis-3-hexenoate, trans-2-hexenyl-trans-2-hexenoate and tetradecyl isobutyrate) was used as a positive control to check if the GC-EAD system was functioning correctly (Fig. 54). On the other hand, hexane was used as a negative control (Fig 55) since antennae of most insects do not respond to this compound.

All the three components of an aggregation pheromone of *R. clavatus* (i.e. trans-2-hexenyl-cis-3-hexenoate, trans-2-hexenyl-trans-2-hexenoate and tetradecyl isobutyrate) were identified in male *R.clavatus* extracts (Fig. 46). This observation is in line with findings of Leal et al., (1995) who were the first to report identification of the compounds. It is known that all the three components of aggregation pheromone of *R.clavatus* are emitted by males (Leal et al., 1995). However, results in figure 11 seem to suggest that female *R.clavatus* can also produce two (i.e. trans-2-hexenyl-cis-3-hexenoate and trans-2-hexenyl-trans-2-hexenoate) out of the three compounds. These could not, however, be confirmed since the compounds were in too low a concentration to be identified by the GC-MS. Hu et al., (2006) reported that *P. hybneri* can respond to a component (i.e. Trans-2-hexenyl-trans-2-hexenoate) of aggregation pheromone of *R. clavatus*. Here, it is further reported that male *P. hybneri* antenna responded to trans-2-hexenyl-cis-3-hexenoate, another of the three components of aggregation pheromone (Fig. 34). Unlike extracts from other species, male and female *P. hybneri* extracts did not contain many identifiable compounds (see for example, Figs. 28, 42, and 47-48), not even any one of the already three known pheromone compounds of this species. Figures 36, 10 and 39 respectively show that female *P. hybneri* and female *D. baccarum* extract contained an unidentified compound eluting at a time similar to that of trans-2-hexenyl-trans-2-hexenoate and trans-2-hexenyl-cis-3-hexenoate. Female *D. baccarum* extract also had an unknown compound eluting at 9.96 minutes, same elution time as that of tetradecyl isobutyrate (Fig.15). It is reported for the first time here that male *D.baccarum* antenna responded to trans-2-hexenyl-cis-3-hexenoate (an aggregation pheromone

component of *R. clavatus*) (Fig. 51). Male *D. baccarum* extract also had an unidentified compound eluting at a time similar to that of trans-2-hexenyl-cis-3-hexenoate (Fig. 31). Crude extracts from male (Figs. 23, 37) and female (Figs. 7, 24) *H. halys* contained an unidentified compound eluting at a time, 9.96 minutes, which was the same as that of tetradecyl isobutyrate, a component of the aggregation pheromone of *R. clavatus* (Figs 46 & 54).

A summary of all compounds identified from the crude extracts made from all the four species of stink bugs is given in table 3 below. From this list, a number of compounds were common to all the extracts (Table 4). These compounds belonged to one of various chemical groups: alkanes, aldehydes, acetates, and Oxo-(E)-2-Alkenal. Table 4 shows the antennal responses to synthetic versions of these chemicals.

Of all the compounds identified, three, *viz*., 4-oxo-(E)-2-hexenal, (E)-2-hexenal and tridecane were found in extracts from nearly every male and female of all the four species (Table 3). Whenever tridecane was detected, it occurred in the highest proportion (indicated by FID peak height) relative to other compounds in the extracts (Figs.6-53). Of the three compounds, 4-Oxo-(E)-2-hexenal, which eluted at 4.68 minutes, was notable in the sense that it had three unidentified dimers. These eluted at minutes 4.72, 9.11 and 9.20 (Figs.6-53 & 56). The latter two are of interest; though they were contained in very negligible quantities within the extracts (as indicated by their peak heights), the antennal responses they evoked were, in most cases, some of the strongest in terms of peak height as measured in mV (Figs.6-53 & 56). Some clues as to the possible chemical structure of these dimers exist (Griepink pers. com.). However, it is still very difficult to synthesize and use them individually in GC-EAD and behavioral studies owing to their high instability (Griepink pers. com.). Figure 56 shows an example of antennal responses evoked by a synthetic version of 4-Oxo-(E)-2-hexenal containing the three dimers.

Table 3: Summary of compounds identified from hexane extracts of the four species of stink bug.

Compounds identified in *H. halys* extract

Male	**Female**
4-Oxo-(E)-2- hexenal	4-Oxo-(E)-2 hexenal
(E)-2-decenal	(E)-2-decenal
Tridecane	Tridecane
(E)-2-decenyl acetate	(E)-2-decenyl acetate
Icosane	Icosane
Hexacosane	Hexadecane
Pentacosane	Dodecane
Dodecane	(E)-2-octenal
(E)-2-hexenal	(E)-2-hexenal
Hexadecane	
1,4,cyclohex-2-enedione	
Decane	

Compounds identified in *D. baccarum* extract

Male	**Female**
4-Oxo-(E)-2-hexenal	4-Oxo-(E)-2 hexenal
(E)-2 hexenal	(E)-2 hexenal
(E)-2-decenal	(E)-2-decenal
Tridecane	Tridecane
(E)-2-octenal	(E)-2-octenal
Hexadecane	Hexadecane
(E)-2-decenyl acetate	(E)-2-decenyl acetate
Dodecane	Dodecane
Hexacosane	Undecane
Heptacosane	Tetradecane
Tricosane	Tricosane
Tetracosane	Henicosane
Pentacosane	Pentadecane
Hexacosane	Hexacosane
(E)-2-hexenyl acetate	Pentacosane
Cyclopentasiloxane, decamethyl	Docosane
1, 4, cyclohex-2-enedione	Tetracosane
	2-hexen-1-ol, acetate

Table 3 continued.

Compounds identified in *P. hybneri* extract	
Male	**Female**
(E)-2 hexenal	(E)-2 hexenal
4-Oxo-(E)-2 hexenal	4-Oxo-(E)-2 hexenal
Tridecane	Tridecane
Henicosane	(E)-2-decenyl acetate
	(E)-decenal
	Octane
	Decane

Compounds identified in *R. clavatus* extract	
Male	**Female**
Trans-2-hexenyl-cis-3-hexenoate	Trans-2-hexenyl-cis-3-hexenoate?
Trans-2-hexenyl-trans-2-hexenoate	Trans-2-hexenyl-trans-2-hexenoate?
Tetradecyl isobuytyrate	(E)-2-hexenal
(E)-2-hexenal	Tridecane
Tridecane	Decane
Decane	
Icosane	
Docosane	
(E)-2-decenal	
Henicosane	
4-Oxo-(E)-2-hexenal	

Table 4: Synthetic versions of some of the compounds identified from hexane extracts of the four species of stink bug and the antennae that responded to the chemicals.

	Insect Antenna					
	R. clavatus		*D. baccarum*		*P. hybneri*	
Compound	Male	Female	Male	Female	Male	Female
Aldehydes:						
E2-octenal	+	+	-	-	-	+
Z2-octenal	+	+	+	+	+	-
E2-hexenal	+	+	+	+	-	+
Z2-hexenal	-	-	-	-	-	-
E3-hexenal	+	+	+	-	-	-
Z3-hexenal	+	+	+	-	-	-
E2-decenal	+	+	+	+	+	+
Z2-decenal	+	+	+	+	+	-
Alkanes:						
Undecane	+	+	-	-	-	-
Dodecane	+	+	+	-	+	+
Tridecane	+	+	+	+	+	+
Acetates:						
Z3-hexenyl acetate	+	+	+	+	-	-
E2-hexenyl acetate	+	+	+	+	-	-
EZ-decenyl acetate	+	+	+	+	+	-
E2-decenyl acetate	+	+	+	+	+	+
Oxo-(E)-2-Alkenal:						
4-oxo-E2-hexenal & **Dimer**	+	+	+	+	+	+
EEZ-246-9: CooMe	+	+	-	*	-	-

Compounds in bold are the same as those listed in table 3. The rest were used either because they are isomers of the compounds identified from hexane extracts or they have been shown to be emitted by other stink bug species. GC-EAD runs were made four times for every compound. In each run, a new antenna was used. 4-Oxo-E2-hexenal was used in combination with the dimer because the latter was too unstable to exist on its own. ***** Compound not tested against the antenna.

Some of the electrophsiologically-active compounds that were identified from hexane extracts of the stink bugs (Table 3) have been reportedly found in members of heteroptera including the species that were used in our current study (see reviews by Aldrich, 1988 and Millar, 2005a; Borges and Aldrich, 1992; Pavis et al., 1994; Krall et al., 1999; Zarbina et al., 2000). Tridecane is often produced in relatively larger quantities than other alkanes common among pentatomidae (Pavis et al., 1994). Our current data presented in the various figures 6-53 are in agreement with this. Dodecane was found to exist in relatively smaller quantities among the various extracts we analyzed (Figs. 6-53). Pavis et al., (1994) report that other authors have isolated dodecane in heteropterans belonging to the families Coreidae and Pyrrhocoridae, and that in those cases the compound was in relatively small quantities. Zarbina et al., (2000) also identified dodecane alongside undecane in metathoracic scent glands of *Piezodorus guildinii* (Heteroptera: Pentatomidae). 4-oxo-(E)-2-hexenal has previously been identified from whole body extracts of nymphal *R. clavatus* (Leal et al., 1995). (E)-2-hexenal is also commonly produced by pentatomids (James at al., 1996). (E)-2-decenyl acetate is found in defensive secretions of heteroptera (Krall et al., 1999).

Insects may still biosynthesize and emit pheromones even soon after mating. This can occur when, for example, a mated female insect wants to attract conspecifics to a food source so that they can colonize the host by collectively overwhelming its defense (Walgenbach et al., 1983). This way the female may eventually enhance her reproductive success since an increased availability of food to herself may enable her lay more viable eggs. For the insects that lay eggs inside food host (e.g. grain weevils), this strategy of attracting conspecifics may enable them to optimize their egg–deposition ability since by acting together, the insects may hollow into a food with much less energy spent (Walgenbach et al., 1983; Fadamiro and Wyatt, 1996). Mated males may also continue emitting aggregation pheromones when they want to increase their reproductive success by mating with many other females. This tactic is common among polygamous insects (Walgenbach et al., 1983). Therefore, our finding that all the three aggregation pheromone compounds of *R. clavatus* were identified from extracts made from mated males could point to a strategy of the insect to maximize its reproductive success.

Although a three-component sex pheromone of *P. hybneri* has been reported before (Leal et al., 1998), not even one of these compounds was identified in our study. It is possible the compounds were detected by the insect antennae but could not be identified by the GC-MS due to their low concentrations. Alternatively, the compounds were absent from the crude extracts altogether. Since the crude extracts were obtained from mated adults, it could be that the insects had not synthesized the sex pheromone at the time immediately preceding the extract preparation. Leal et al., (1998) succeeded in identifying male- released sex pheromones of *P.hybneri* by collecting headspace volatiles of mature adults on an adsorbent column. They then washed the entire column with hexane. The hexane wash (i.e. crude extract) was subjected to a flash column chromatography in hexane: ether mixtures of 100:0, 95: 5, 90: 10, 80: 20, 50:50 and 0: 100 in that order before doing GC-EAD runs. In their GC-EAD experiments, it is only the compounds that eluted in the hexane: ether eluants (90: 10) that elicited antennal responses in male and female *P. hybneri* (Leal et al., 1998). Therefore, it could be the case that by making whole body extracts and not collecting headspace volatiles, our method missed to capture the pheromone compounds. Furthermore, while Leal et al., (1998) collected headspace volatiles from sensible insects, anaesthetized insects were used in our method of extraction of compounds from the stink bugs. It could be that anaesthetizing the insects prevented them from emitting sex pheromones. In addition, authors who have reportedly identified the pheromone compounds of stink bugs from hexane extracts have concentrated the crude extracts before injecting them into GC-EAD and GC-MS systems (e.g. Leal, et al., 1995; Yasuda et al., 2007). The fact that the crude extracts used in our studies were not concentrated prior to GC-EAD and GC-MS analyses could also explain why we did not succeed in identifying pheromone compounds of species whose pheromones are known already.

Various factors such as age, mating status, diet quality, and feeding status are known to influence the physiological status of insects and hence synthesis of, and response to pheromones (Pierce et al., 1983; Walgenbach & Burkholder,1986; Fadamiro et al., 1996; Fadamiro and Wyatt, 1996). How these factors could interact to influence biosynthesis of and sensitivity to pheromones by the stink bugs, particularly those whose pheromones have not been identified, would be a subject of future study.

Production and emission of alarm pheromones by stink bugs may overshadow biosynthesis, emission and/or detection of other pheromones like the sex and aggregation ones. As such the process of identifying stink bug pheromones other than the alarm still remains a challenge. This difficulty is made much more so due to lack of knowledge of hormonal messengers that mediate pheromone biosynthesis in stink bugs. Presently, knowledge on hormones [juvenile hormones (JH), *p*heromone *b*iosynthesis *a*ctivating *n*europeptides (PBAN) and ecdysteroids] that regulate pheromone biosynthesis exist with respect to only a few insect orders, namely, Blattodeans, Coleopterans, Dipterans and Lepidoptera (Tillman et al., 1999). With the help of such hormones, the difficulty in obtaining pheromone compounds in quantities sufficient for chemical identification could easily be solved. For instance, Dickens et al., (2002) when trying to identify pheromone compounds from Colorado potato beetle observed a male- specific compound that was EAD active. However, the chemical identity of that compound could not be immediately ascertained because the hexane extract contained it in quantity not sufficient for chemical identification by the GC-MS. The authors solved that problem by making use of a juvenile hormone III (JH III). Through a topical application of this chemical on Colorado potato beetle, they improved emission of the male-produced aggregation pheromone of the insect, which subsequently led to identification of the compound. There are only a few reported instances where some of the already known hormones have been used in studying reproduction biology in stink bugs. As an example, Adams et al., (2002) observed that treatment of a female *Perillus bioculatus* (F.) (a stink bug predator of Colorado potato beetle) with JH III stimulated ovarian maturation. This suggests that JH III could be used to manipulate reproduction in female stink bugs. However, no reports exist on how the hormone affects pheromone synthesis in the stink bugs. It would be curious to find out if JH III (or PBAN/ ecdysteroids) can induce pheromone biosynthesis in stink bugs. And although such a study could be driven by pure curiosity, other methodical investigations should focus on identifying pheromone biosynthesis-mediating hormones that are specific to stink bugs.

5.3 Functions of some of the compounds identified.

Table 4 shows the results of GC-EAD tests of synthetic compounds against insect antennae. Population size of *H. halys* went so low in the insect rearing unit that there were not enough of them for testing against the synthetic compounds. Therefore, *H. halys* were not used further in these experiments. Of all the alkanes tested, tridecane evoked antennal responses in both males and females of *P.hybneri*, *D. baccarum and R. clavatus*. Undecane stimulated antennal response in *R. clavatus* only. Alkanes e.g. undecane, dodecane and tridecane are known to be released by stink bugs when molested (Zarbina et al., 2000). It is thought that the alkanes may act as solvents for the alarm pheromones produced by the hemipterans (Pavis et al., 1994). The alkanes may also boost toxicity and repellent effects of the defensive compounds produced by heteroptera (Pavis et al., 1994; Zarbina et al., 2000). As such they are generally regarded as defense-related compounds. It is interesting to note, however, that tridecane consistently attracted *D. baccarum* when tested against other alkanes and acetates in a y-tube olfactometer (Kim, unpublished data).

(E)-2-hexenal and (E)-2-decenal were both detected by antenna from each of the three stink bugs (Table 4). Preliminary results of behavioral assays conducted by Kim (unpublished data) showed that (E)-2-decenal was highly attractive to *D. baccarum*. Generally (E)-2-hexenal is a defensive compound for the heteropterans including stink bugs (James et al., 1996). The compound is generally produced by nymphs of hemipterans (Blatt et al., 1998). Blatt et al., (1998) isolated (E)-2-hexenal from abdomen of nymphal *Leptoglossus occidentalis,* a heteropteran belonging to a family known as coreidae. This (E)-2-hexenal caused dispersal of nymphs of the same species in the field (Blatt et al., 1998). In certain cases, however, (E)-2-hexenal can also serve as an attractant pheromone compound. The bifunctional property of (E)-2-hexenal is determined by its concentration. At low concentrations, (E)-2-hexenal can attract conspecifics to the emitter insect, while at high concentrations they cause dispersal of the insects (Aldrich et al., 1995). James et al., (1996) found that (E)-2-hexenal was attractive to reproductive *Biprorulus bibax* (Hemiptera: Pentatomidae) in citrus orchards.

The bifunctional property of (E)-2-hexenal is also true for tridecane; at low concentrations, the latter compound elicits an aggregation behavior in first instar nymphs of *N. viridula* while at high concentration, it is an alarm pheromone to the same insect (Lockwood and Story (1985), cited by Blatt et al., 1998). 4-oxo-(E)-2-hexenal also elicited antennal responses from all the three species (Table 4). 4-oxo-(E)-2-hexenal and (E)-2-octenal have been identified from whole body extracts of nymphal *R. clavatus* and are thought to be employed by the nymphs for self defense (Leal et al., 1995).

Some of the compounds we identified have been shown to influence not only behaviors of the stink bugs but also their natural enemies. For instance, Mattiacci et al., (1993) found that (E)-2-decenal, which is a defensive compound in many heteropterans stimulated oviposition-related behaviors in *Trissolcus basalis*, a parasitoid of *Nezara viridula* eggs. The same study discovered as well that (E)-2-hexenal elicited antennation from the same parasitoid (Mattiacci et al., 1993).

Various EAD-active long chain alkanes (C14-C27) were present in some of the hexane extracts (Table 3). Generally, such alkanes are known to serve defensive functions for the stink bugs (Aldrich, 1988).

5.4 Conclusion from the study

A number of compounds were chemically identified that evoked cross EAD responses in both males and females of the four species of stink bug: *P. hybneri*, *D. baccarum*, *H. halys* and *R. clavatus*. Most of the compounds fall into one of four chemical groups: aldehydes, acetates, alkanes and oxo-(E)-2-alkenals. Save for the aggregation pheromone compounds of *R.clavatus* (esters), most of these compounds have been reported before as common defensive chemicals in heteroptera. Some of them are said to be alarm pheromones for the stink bugs at certain concentrations, while also serving as attractants to conspecifics and/or heterospecifics at other concentrations. A few new EAD-active compounds, *viz,* 1, 4, cyclohex-2-enedione, cyclopentasiloxane- decamethyl, and 2-hexen-1-ol (Table 3) were identified alongside the ones already discussed above.

5.4.1 Future prospects

The synthetic versions of newly identified compounds: 1, 4, cyclohex-2-enedione, cyclopentasiloxane- decamethyl, and 2-hexen-1-ol were not used in the GC-EAD study.

They should be used in such a study to confirm if indeed the insects can detect and respond to them.

Not every compound that is EAD- active can stimulate behavioral responses from insects (Blatt et al., 1998). Hence the next logical step in dissecting the biological relevance of the EAD-active compounds is to subject them to behavioral tests in olfactometer and flight chamber settings. However, it is sometimes difficult to get behaviorally- consistent results in bioassays involving heteropterans, perhaps because they are easily irritated. In the face of this difficulty, some investigators who have positively identified the biological relevance of EAD-active compounds have skipped the olfactometer and flight chamber assays and instead proceeded to study the compounds of interest directly in the field (e.g. Aldrich et al., 1995; Yasuda et al., 2007). Therefore, it would be important to test all the compounds that were shown to evoke antennal responses in the various stink bugs not only under laboratory conditions but also in the field. Such studies can be done sequentially or in parallel. In the field, the compounds should be tested where the insects whose antennae were sensitive to the compounds naturally occur at various growth and development stages during the growing season of host crops. The compounds should be tested at various concentrations, ranging from progressively low to high concentrations. This would elucidate which of these compounds are attractive to both nymphs and /or adults. It would also be useful to study how these compounds affect the behaviors of the natural enemies of these insects.

Even though some of the compounds that were identified to be electrophysiologically-active have been reported to serve defensive functions in other members of heteroptera, this should not discourage their use in field tests. As noted by various authors already cited above, those defensive functions of the compounds are determined by such factors as physiological status and age of the target insects and also concentrations of the compounds. A compound that is regarded as an alarm pheromone for an insect species can fail to stimulate an alarm pheromone-related behavior in insects of the same species but at different physiological statuses. This is evident from the study by Blatt et al., (1998) in which they found that (E)-2-hexenal, hexanal, and hexyl acetate caused dispersal of *L. occidentalis* reared under summer conditions. When they tested the same compounds against individuals of *L. occidentalis* collected from the field during fall

season, the repulsion was not significantly different from that of the control (i.e. heptane used as a solvent for the compounds) (Blatt et al., 1998). Blatt et al., (1998) hypothesized that the significant reduction in dispersal caused by the alarm pheromone compounds was due to a change in physiological conditions of the insects during fall season. In fall, the insects are migrating away from the field in search of warm places for overwintering. Accordingly, they tend to aggregate and even alarm pheromones would not break up such aggregations. Therefore, it could be that during those harsh weather conditions at fall time, aggregation of the insects can be mediated by even the compounds that have been shown to be alarm pheromones. Thus the EAD-active compounds we identified should be used in field tests during various seasons of the year. Furthermore, it has been reported that some defensive compounds can be exploited by natural enemies of the stink bugs to locate their hosts. So it is necessary to test such compounds in the field to see if they can attract the natural enemies. Attracting natural enemies to a field where their hosts abound can be useful in reducing the population levels of the pests.

Future studies aiming to identify EAD-active compounds other than the defense-related ones (particularly the sex and aggregation pheromones) of stink bugs in which such compounds have not been reported should use other approaches than the ones we used. For example, they could adopt volatile collection from active insects feeding on their hosts. They could also use insects in various physiological statuses e.g. mated / unmated and fed/unfed insects. Studies should also be initiated that aim to identify hormones that mediate pheromone biosynthesis in stink bugs since such hormones can be used to manipulate pheromone biosynthesis by the insects.

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