Project Title	CHEMICAL DIVERSITY OF MIDGE PHEROMONES
Project number:	CP 38
Project leader:	Prof. David Hall, Natural Resources Institute
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Key staff:	Lakmali Amarawardana, NRI Dr Jerry Cross, East Malling Research
Location of project:	NRI, University of Greenwich at Medway, EMR, East Malling
Project coordinator:	Lindrea Latham, HDC
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Key words:	Pear leaf midge; *Dasineura pyri*; pear midge; *Contarinia pyrivora*; sex pheromone

The results and conclusions in this report are based on a series of experiments conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

I declare that this work was done under my supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

PROF DAVID R HALL
Professor of Chemical Ecology
Natural Resources Institute, University of Greenwich

Signature ..... Date .....

CONTENTS

Grower Summary

Headline

Female sex pheromones of the pear leaf midge and pear midge have been collected and analysed. Two active components have been detected in each and these are being identified and synthesised for evaluation in field trapping experiments.

Background and expected deliverables

The pear leaf midge, *Dasineura pyri*, and pear midge, *Contarinia pyrivora,* are important pests of pear in the UK and elsewhere. Pear leaf midge affects new shoots on young and fruiting trees. Pear midge attacks young fruitlets causing them to swell abnormally and then blacken and die. Female sex pheromones provide a means for monitoring and control of insect pests that have the potential to be more sustainable than control with conventional insecticides. The objective of this project is to study the chemical diversity of the female sex pheromones of midges of importance to UK horticulture, including pear leaf midge and pear midge, and to make them available for use in monitoring and control of the pests.

Summary of the project and main conclusions

Pear leaf midge

Over 6,000 late larvae of *D. pyri* were collected from pear orchards in the UK and reared to adulthood individually. After sexing, volatiles were collected from both males and females by air entrainment. The volatile collections were analysed by gas chromatography (GC) coupled to electroantennographic (EAG) recording from a male antenna, and by GC coupled to mass spectrometry (MS). Male midges responded to two components in the volatile collections from females. The structure of the major component was determined on the basis of mass spectrum and the retention indices as (*Z*)-2,13-diacetoxy-8-heptadecene, a novel pheromone component. There are four different forms (stereoisomers) of this compound, but it is likely that the natural pheromone contains only one of them. When the synthetic compound, containing all four stereoisomers, was tested on males, a strong EAG response was observed. However, no male midges were caught in traps baited with this synthetic material deployed in pear orchards. This is likely to be because the correct, naturally-occurring stereoisomer is required for attraction and/or because the second, minor component is also vital for attraction. During the winter months of 2006 mature midge larvae received from New Zealand were reared in the laboratory and pheromone was collected. Work on determination of the attractive stereoisomer of the major pheromone component and identification of the second, minor pheromone component is in progress.

Pear Midge

Larvae of *Contarinia pyrivora* were collected from pear orchards in Kent and reared to adulthood individually. The midge has only one generation per year and it is difficult to break the winter diapause, so although approximately 3000 larvae were collected only 135 reached adulthood. Males and females were separated based on the antennal morphology and volatiles were collected from both females and males and analysed by GC-EAG and GC-MS. In GC-EAG analysis of volatiles from females, male midges responded to two active components. Tentative structures have been proposed for both components and full identification and synthesis is in progress.

Financial benefits

There are no immediate financial benefits to growers at this stage.

Action points for growers

There are no action points for growers at this early stage of the project.

Glossary

Electroantennography

Insects “smell” volatile olfactory stimulants by means of receptors on their antennae. In electroantennographic (EAG) recording, electrodes attached to either end of the antenna are used to detect a small change in potential of a millivolt or so that occurs when the receptors are stimulated by an active compound.

Gas chromatography

Gas chromatography (GC) is a process for separating mixtures of volatile compounds into the individual components. The mixture is injected onto a long thin column coated with a suitable “stationary phase”. Different components pass through the column at different speeds according to their molecular weight and chemical properties and are detected as they emerge from the end of the column with a suitable detecting device.

Mass spectrometry

Mass spectrometry (MS) is a process used in identification of the chemical structure of molecules. The molecule is ionised and then fragments into smaller fragments which are characteristic of the chemical structure.

Sex pheromone

A sex pheromone is a chemical or group of chemicals produced by one sex to attract the other sex of the same species for mating.

Stereoisomer

All molecules have a three-dimensional structure that is not always apparent from formulae drawn on two-dimensional paper. Many molecules can exist in different forms with the same overall formula and identical bonding patterns between the constituent atoms, but in which the relative positions of certain atoms in space are different. These are called stereoisomers, and this phenomenon is of great importance in biology because the active sites in enzymes and receptors generally have a certain shape that will only interact with the "correct" stereoisomer of the active molecule. The "wrong" stereoisomer may be inactive; it may block or inhibit the activity of the correct isomer, or it may even have its own activity – as was the case with thalidomide where one stereoisomer is a sedative but the other causes teratogenic effects in the foetus.

Science Section

Introduction

The pear leaf midge, *Dasineura pyri*, and pear midge, *Contarinia pyrivora,* are important pests of pear in the UK and elsewhere. Pear leaf midge affects new shoots on young and fruiting trees. Pear midge attacks young fruitlets causing them to swell abnormally and then blacken and die; growth of unattacked fruitlets is slowed. Female sex pheromones provide a means for rmonitoring and control of insect pests that have the potential to be more sustainable than control with conventional insecticides. The objective of this project is to study the chemical diversity of the female sex pheromones of midges of importance to UK horticulture and to make them available for use in monitoring and control of the pests. In the first year work has started on the pheromones of pear leaf midge and pear midge.

Materials and Methods

Collection and rearing of pear leaf midge

Late larvae of pear leaf midge, *Dasineura pyri*, were collected in May 2006 from heavily infested pear shoots in pear orchards of Broadwater Farm, West Malling, Kent. Shoots were stored in plastic boxes (19 cm x 10.6 cm x 7.5 cm). Mature larvae crawled out from the leaves for pupation and these were collected and reared individually in clear plastic tubes (1.5 cm i.d. x 2.3 cm) containing a piece of wet filter paper which acted as the substratum for pupation. Tubes were closed with plastic caps and stored under controlled conditions, at 23°-18°C and 16L:8D light cycle. Adults emerged after 7-12 days and males and females were separated on the basis of antennal morphology. Approximately 6,300 larvae were reared, from which 70% emerged as adults.

Collection and rearing of pear midge

Fruitlets infested with pear midge were collected from a pear orchard at Elmstone Farm, Preston, Kent in early June 2006. Mature larvae emerging from the fruitlets were collected and potted individually into tubes containing moistened bulb fibre, cotton wool, wood fibre and paper towel during mid June 2006. The tubes (approximately 3,000) were stored in a box and kept in an outdoor insectary under natural conditions. Tubes were inspected once a month and those that turned mouldy were removed. It was observed that some larvae were pupated by mid February 2007. Pupae were removed from the outdoor insectary and incubated at 20°C under 14:10 hr day:night cycle in the laboratory.

Pheromone collection

Volatiles from male and female midges were collected by air entrainment under the same conditions as used for insect rearing. Midges were placed in a glass vessel (5.3 cm i.d. x 13 cm) with a glass frit at the upwind end. Air was drawn into the vessel (0.5 l/min) with a vacuum pump (M 361C, Charles Austen Pump Ltd, UK) through an activated charcoal filter (20 cm x 2 cm; 10-18 mesh, Fisher Chemicals, UK) and out through a collection filter consisting of a Pasteur pipette (4 mm i.d.) containing Porapak Q (200 mg; 80-100 μm, Waters Associates Inc., USA) held between two plugs of silanised glass wool. The Porapak was purified by Soxhlet extraction with chloroform and prior to use filters were washed with dichloromethane and dried by a stream of nitrogen. Midges were introduced at 24 hr intervals. Every five days the collection filters were extracted with dichloromethane (1.5ml, pesticide grade).

Gas chromatography linked to electroantennography (GC-EAG)

Male antennal responses to female volatiles were analysed by gas chromatography (GC) linked to electroantennography (EAG) (Cork *et al.*, 1990). The GC used was an HP6890 instrument (Aglilent Technologies) with a flame ionisation detector and fused silica capillary columns (30m x 0.32mm x 0.25µm film thickness) coated with polar (Supelcowax-10, Supelco, USA) and non polar (SPB-1, Supelco, USA) phases. The column ends were connected to a push-fit Y-connector, the outlet of which was connected to a second Y-connector. This was connected with identical pieces of deactivated silica capillary column, one going to FID and the other to a glass T piece. A stream of nitrogen (200 ml/min) blew the contents of the T piece directly over the antennal preparation every 3 sec. The oven temperature was maintained at 50°C for 2 min, then programmed at 10°C /min to 250°C and held for 5 min. Injection was splitless at 220°C and helium was used as carrier gas (2.4 ml/min).

EAG responses were recorded using a portable recording unit (INR-2, Synthech, The Netherlands) comprising integrated electrode holders and amplifier. Glass electrodes were pulled and were filled with electrolyte (0.1 M solution of KCl with 1% polyvinylpyrrolidone). The tips of the electrodes were broken off so that both antennae could be inserted into the recording electrode and the abdomen inserted into the reference electrode. Signals were amplified and analysed with EZChrom software (Elite v3.0).

Gas chromatograph linked to mass spectrometry (GC-MS)

GC-MS analyses on a polar GC column were carried out with a fused silica column (30 m x 0.25 mm i.d.) coated with Supelcowax10 linked directly to an ion trap detector (ITD700; Thermo Instruments, Hemel Hempstead, Herts., UK) operated in electron impact (EI) mode (trap temperature 170°C; transfer line 240°C). For GC-MS analyses on a non-polar column, the GC (HP6890 Agilent Technologies) was fitted with a fused silica capillary column (30 m x 0.25 mm i.d.) coated with SPB1 or SPB5 coupled to a mass selective detector (HP 5973, Agilent Technologies). For both machines, carrier gas was helium (1.0 ml/min) and injection was splitless (220°C). The temperature of the oven was held at 60°C for 2 min then programmed at 6°C/min to 250°C and held for 5 min. GC retention times were converted to Retention Indices (RI) relative to those for straight-chain acetates.

Microhydrogenation

An aliquot (20µl) of the collection of volatiles from female *D. pyri* was dried under a stream of nitrogen and dissolved in petroleum spirit (60°-80°; 20µl). A small amount of 10% palladium on charcoal was added and hydrogen bubbled in through a length of fused silica capillary for 1 min. The reaction mixture was analysed by GC-MS.

Field assessment

Preliminary field tests with the synthetic pear leaf midge pheromone were carried out using white delta traps (28 cm long × 20 cm sides; Agrisense-BCS, Pontypridd, Wales,) with sticky bases baited with rubber septa containing 10 µg of the synthetic pheromone. These were deployed in four separate plots of pears in East Malling Research Station and Broadwater Farm, West Malling, Kent, UK. In each plot an unbaited control and a pheromone-baited trap were hung on the 10th and the 20th pear trees on the centre row. The sizes of the plots varied from 7 ha to 0.25 ha. Insect counts were taken once a week for a period of one month during August 2006.

Results and Discussion

Pear leaf midge

Isolation and identification of pheromone

Three sets of volatiles from both males and females of *D. pyri* were collected with batches of between 300 to 1,160 midges. GC-EAG analyses of volatiles from female midges on both polar and non-polar GC columns indicated the presence of two compounds eliciting consistent EAG responses from male antennae. The minor component (EAG response with lower intensity) appeared at 18.42 and 19.26 min on polar and non polar columns respectively while the major component (EAG response with higher intensity) appeared at 20.92 and 20.37 min respectively (Figure 1).

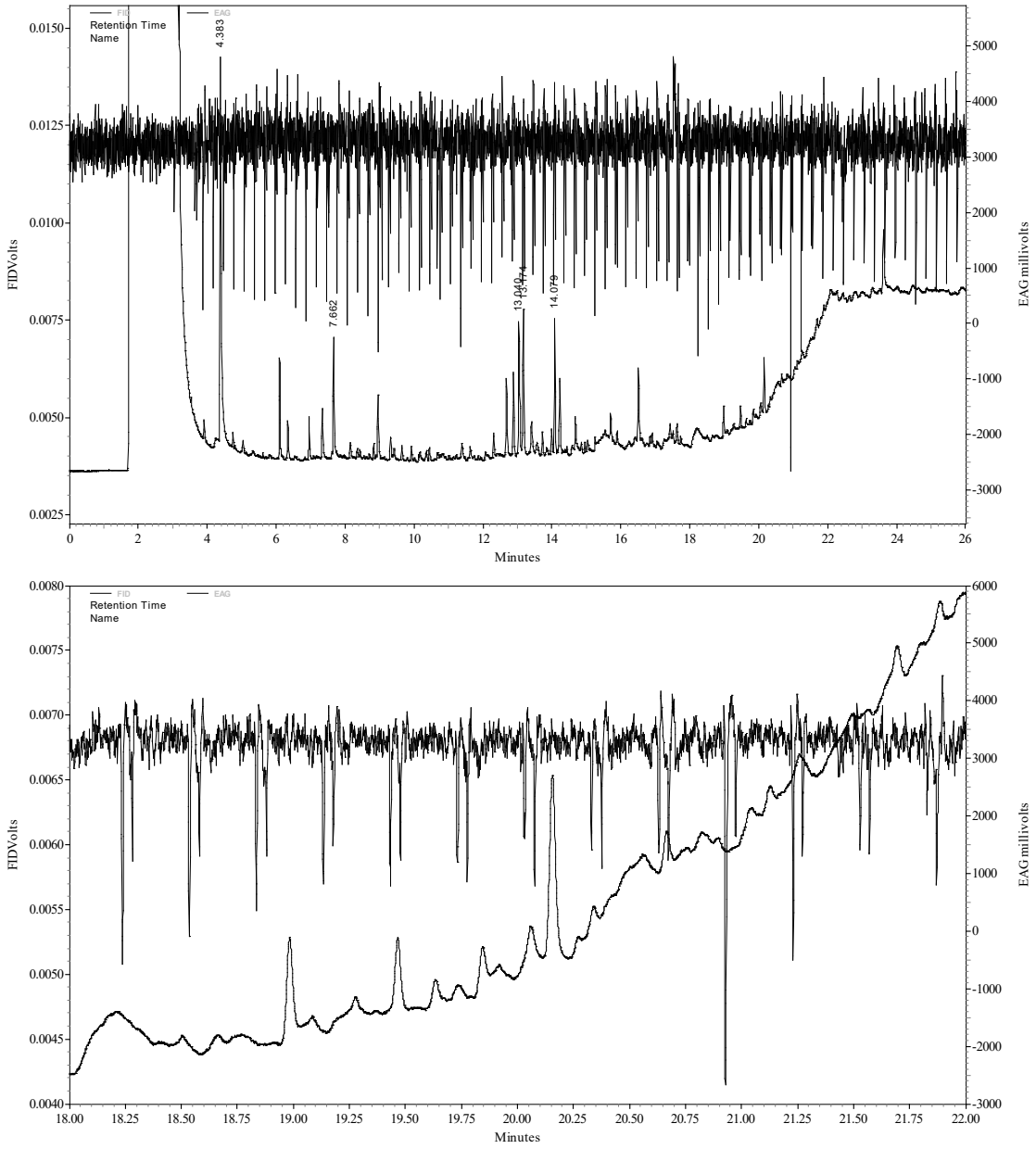

Figure 1. GC-EAG analysis of female volatile extract on polar column (upper- full GC-EAG trace, lower-expanded; EAG responses at 18.25 min and 20.95 min).

Although only tiny amounts of the pheromone components were present (approx. 4.3 pg per female), a mass spectrum was obtained on the major component and the MS fragmentation pattern and GC retention indices were compared with those of known compounds. The mass spectrum (Figure 2) showed ions at *m/z* 43 and *m/z* 61, which are diagnostic peaks for acetate esters. The fragment of low intensity at *m/z* 234 is consistent with loss of two acetoxy groups from a 17-carbon diacetate with one double bond (molecular weight 354). The rest of the MS fragmentation pattern was difficult to interpret.

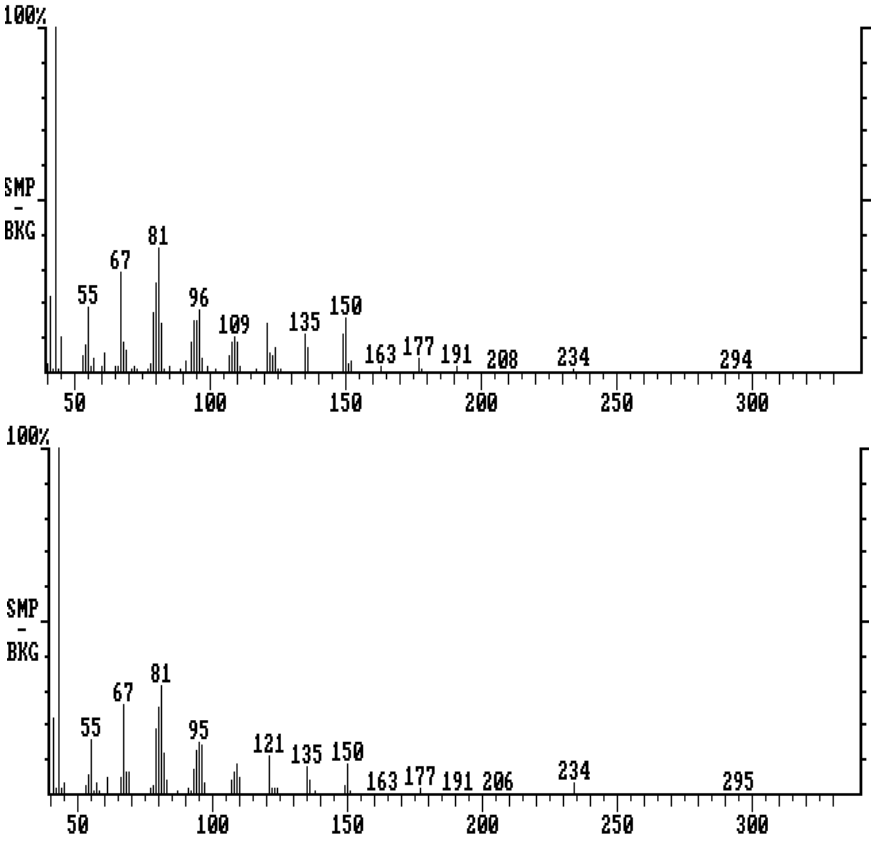

Figure 2. Mass spectrum of major pheromone component (upper) and synthetic (*Z*)-2,13-diacetoxy-8-heptadecene (lower).

Microhydrogenation of the major component showed 2 *m/z* unit increase in fragments in the mass spectrum (fragment ions at m/z 234 and 150 converted to m/z 236 and 152), confirming the presence of a single double bond.

Comparison of GC retention times (Table 1) and mass spectra (Figure 2) with those of synthetic standards showed that the data for the naturally-occurring compound were identical with those for (*Z*)-2,13-diacetoxy-8-heptadecene (Figure 3) prepared during previous work on the pheromone of the closely related apple leaf curling midge, *D. mali*.

Table 1. Retention times and retention indices (RI) of pheromone components of *D. pyri* and synthetic (*Z*)-2,13-diacetoxy-8-heptadecene on non-polar and polar GC columns in GC-EAG and GC-MS.

		Non-polar GC column		Polar GC column	
	Compound	RT (min)	RI	RT (min)	RI
GC-EAG	Major component	20.37	1854	20.92	2087
GC-MS	Major component	33.86	1854	24.33	2082
GC-EAG	Minor component	19.26	1770	18.42	1727
GC-MS	(*Z*)-2,13-diacetoxy-8-heptadecene	33.88	1857	24.34	2083

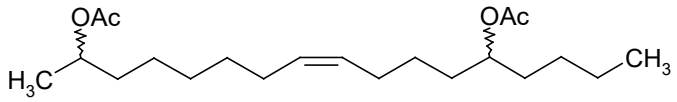

Figure 3. Structure of (*Z*)-2,13-diacetoxy-8-heptadecene

The synthetic compound elicited a strong EAG response from the antenna of a male *D. pyri* midge (Figure 4).

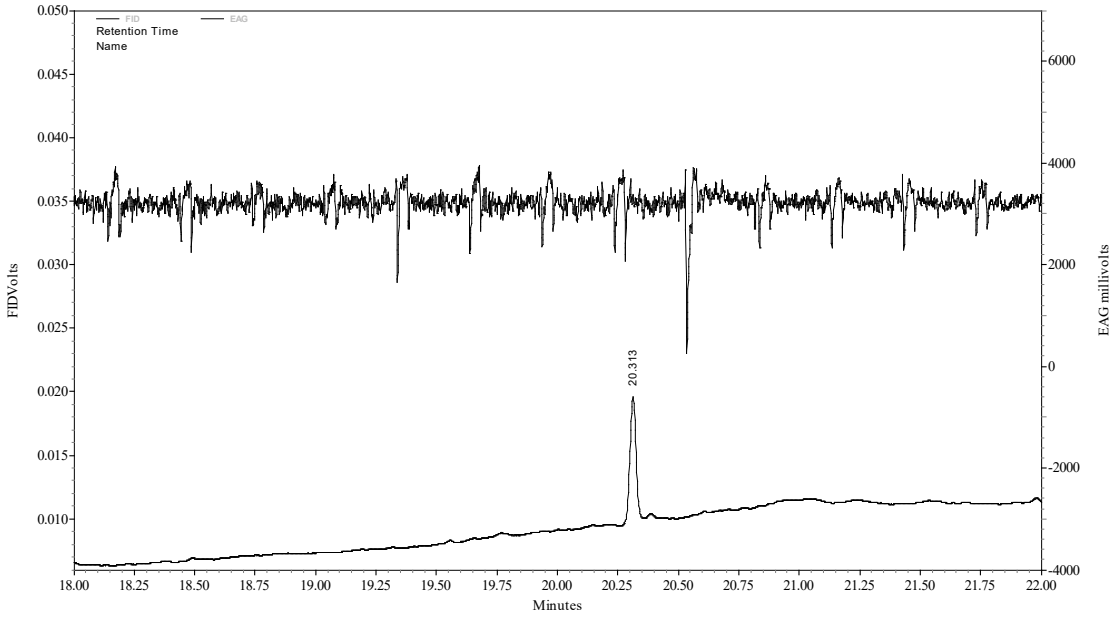

Figure 4. GC-EAG analysis of synthetic (*Z*)-2,13-diacetoxy-8-heptadecene on polar column.

Work is in progress on the identification of the minor pheromone component. It has proved very difficult to obtain a mass spectrum due to the small amount present. Candidate structures have been proposed, based on the GC retention times (Table 1).

Field trapping

Traps baited with the synthetic, racemic (*Z*)-2,13-diacetoxy-8-heptadecene did not catch males of *D. pyri* even though an EAG response was observed for this compound (Figure 4). This may be because only one of the four stereoisomers of *(Z)*-2,13-diacetoxy-8-heptadecene is attractive and one or more of the others inhibits attraction. The synthetic compound also contained 5% of the (*E*) isomer and this may be inhibitory. Furthermore, the presence of the minor component in the pheromone blend may be required for attraction.

Pear midge

Isolation and identification of pheromone

Only 135 adult *C. pyrivora* midges were derived from the 3,000 larvae collected. Volatiles were collected from four batches of virgin females (total 65) and two batches of males (total 34).

In GC-EAG analyses of volatiles from females using a male midge for EAG recording, two responses were observed on both polar (14.73 and 14.33 min for major and minor responses respectively) and non-polar (15.22 and 13.56 min) GC columns (Table 2 and Figure 5). These responses were not observed in analyses of volatiles from male midges.

GC-MS traces of volatiles from male and female midges were compared and two peaks were found that coincided with the retention indices of the EAG responses and were only present in volatiles collected from female midges.

Table 2. Retention times (RT) and retention indices (RI) of pheromone components of *C. pyrivora* pheromone on non-polar and polar GC columns in GC-EAG and GC-MS.

	Compound	Non-polar GC column		Polar GC column	
		RT (min)	RI	RT (min)	RI
GC-EAG	Major component	15.22	13.11	14.73	14.77
GC-MS	Major component	22.92	12.96	15.95	14.62
GC-EAG	Minor component	13.56	11.61	14.335	14.35
GC-MS	Minor component	20.54	11.69	15.62	14.31

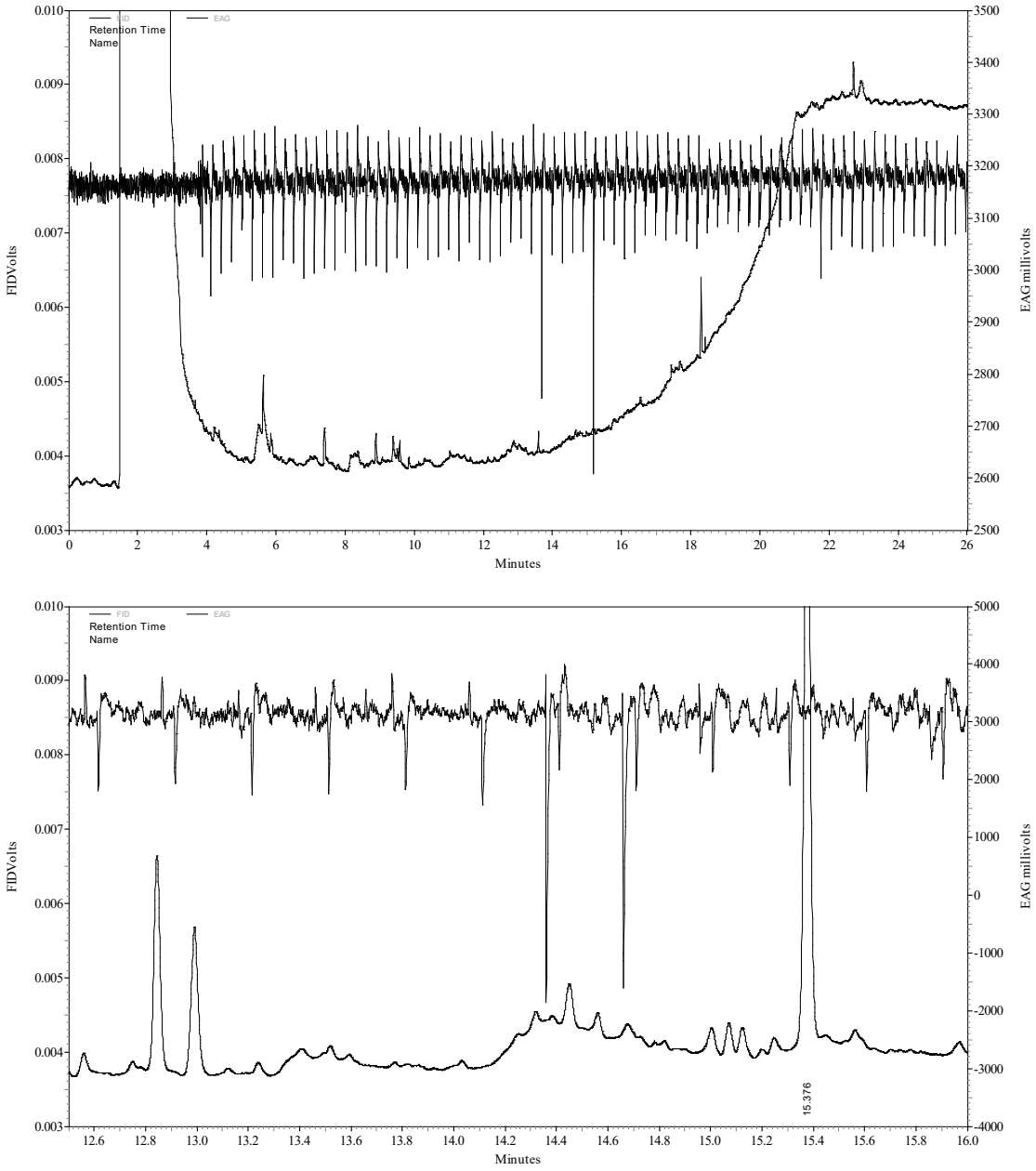

Figure 5. GC-EAG analysis of volatiles from female *C. pyrivora* on non-polar (upper) and polar (lower) GC columns.

The retention index differentials between the major components of the *D. pyri* and *C. pyrivora* pheromones suggested that carbon chain of the latter is six carbon atoms shorter than that of the *D. pyri* compound. Intercolumn differentials suggested the major component of the *C. pyrivora* pheromone has two acetoxy functionalities. The assumption that the major component is a diacetoxy undecane was strongly supported by the GC-MS fragmentations (Figure 6). Thus the peak at m/z 152 is due to the elimination of two acetoxy groups from the parent molecule with molecular weight of 272. It is suggested that the acetoxy groups are on the 2- and 7-positions of the carbon chain. Fragments m/z 95 (40%) and 113 (20%) could be

due to the elimination of butyl group (-$CH_2CH_2CH_2CH_3$) from the 11-carbon alkane chain and an acetoxy group from the parent molecule respectively. The minor component is suspected to have a structure related to that of the major component.

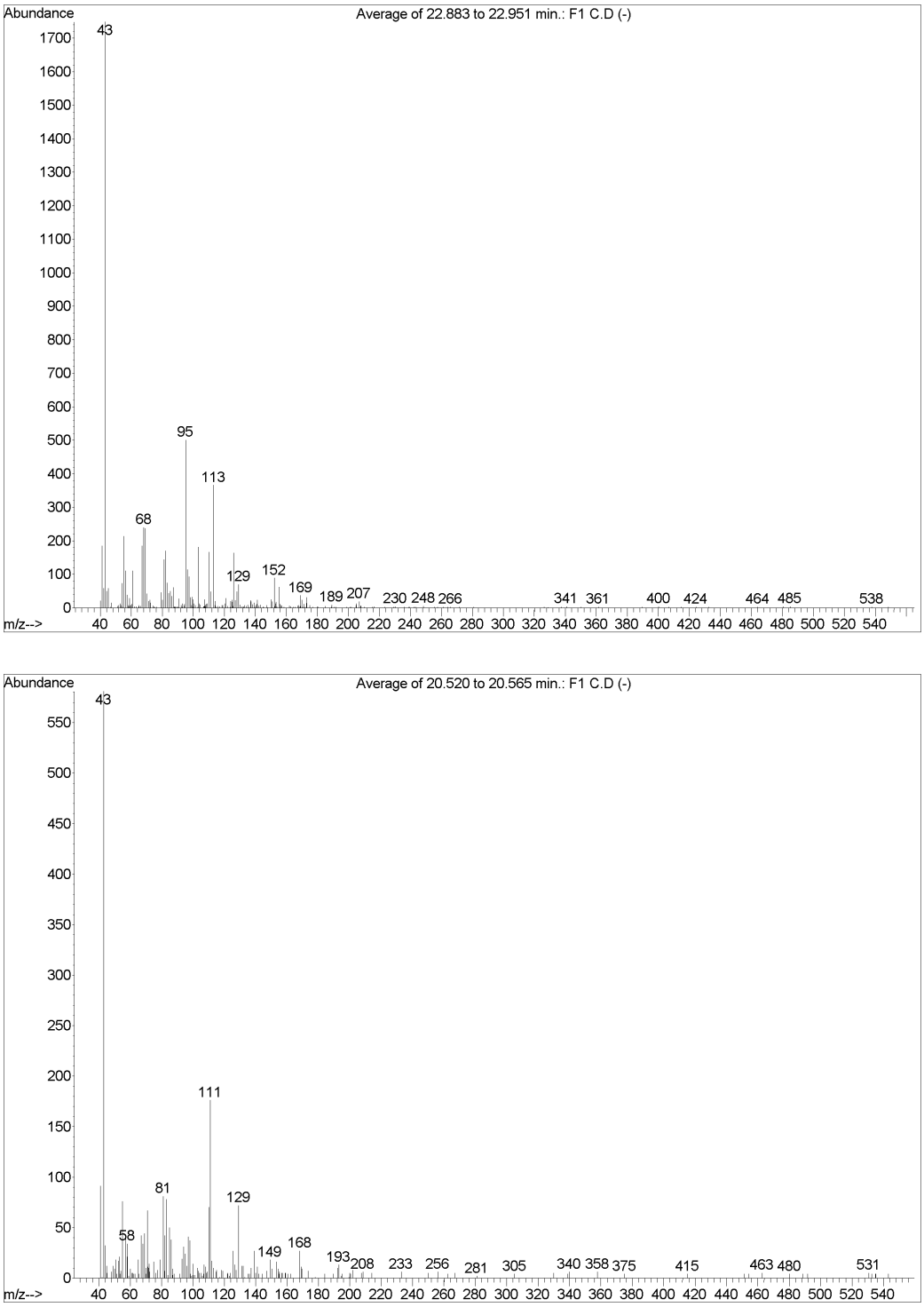

Figure 6. Mass spectra of proposed major (upper) and minor (lower) pheromone components of *C. pyrivora* (from GC-MS analysis on non-polar column).

Conclusions

Pear leaf midge

The major component of the female sex pheromone of *D. pyri* is thought to be (*Z*)-2,13-diacetoxy-8-heptadecene which is chemically related to the pheromone of the apple leaf midge, *D. mali*, (*Z*)-13-acetoxy-8-heptadecen-2-one (Cross and Hall, 2006). Further work is required to determine which of the four possible stereoisomers is produced by the female midge, and to identify the second "minor" pheromone component.

In the coming year further collections of insects will be made to make possible collection of more pheromone. It is hoped to be able to obtain a mass spectrum of the minor pheromone component and to identify its chemical structure. Stereoisomers of the major component will be prepared by new techniques of chiral high performance liquid chromatography and/or enzymatic resolution. An attractive lure for use in the field will then be developed.

Pear midge

Two active components produced by females have been found. Tentative structures have been proposed for both components and full identification and synthesis is in progress.

It will not be possible to test synthetic pheromone in the field in the UK until spring 2008 as the pear midge has only one generation per annum. The possibility of carrying out field testing is New Zealand during September-October 2007 is being investigated.

Technology transfer

Investigations on the sex pheromone of pear leaf midge, *Dasineura pyri* (Bouché), and other gall midge pests of fruit crops. Lakmali Amarawardana, David Hall and Jerry Cross. Poster and Proceedings of IOBC Workshop on Arthropod Pest Problems in Pome Fruit, Lleida, Spain, 4-6 September 2006.

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References

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Cork, A., Beevor, P.S., Gough, J.E. and Hall D.R. 1990. Gas chromatography liked to electroantennography: a versatile technique for identifying insect semiochemicals. In: Chromatography and isolation of insect Hormones and Pheromones. Ed. A.R. McCaffery and I.D. Wilson, Plenum Press, London, pp 271-279.

