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Review

Pheromone analysis using capillary gas chromatographic techniques ☆

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Abstract

The application of capillary gas chromatography to the analysis of pheromones is reviewed together with associated sampling and detection techniques. Solventless injection and solid-phase micro-extraction are highlighted and examples of the utility of micro-chemical derivatisations to assist structure determination are given. The use of a variety of column phases to increase resolution, confirm structure and estimate vapour pressure is discussed along with the separation of chiral pheromones on chiral phases and the use of dual-column GC systems. In addition to traditional detection systems, including mass spectrometry and Fourier transform infrared spectroscopy the specialist techniques of electroantennography and single cell recording are described.

Keywords: Reviews; Electroantennography; Enantiomer; Extraction methods; Derivatization, GC; Detection, GC; Single-cell recording; Pheromones

Contents

☆Dedicated to Professor E. David Morgan on the occasion of his retirement.

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1. Introduction

Many pheromones are multicomponent mixtures, present in nanogram or picogram amounts. Therefore, their analysis and structure elucidation requires a separation technique capable of high resolution and sensitivity. Capillary gas chromatography, perhaps more than any other method, fulfils these criteria. This, together with the fact that the vast majority of pheromones are suitable for GC analysis (i.e. they are thermally stable and volatilise below 300°C), means the technique has become central to the study of pheromone chemistry. In this review we aim to highlight the major methods used in the analysis of pheromones by capillary GC; with sections on derivatisation and microreactions, sampling and injection techniques, columns and separation, and detection. Because of the large subject area, this article is confined to current methods, with an emphasis on the recent literature. Examples include the use of GC in pheromone separation and identification, quantification, and biosynthesis.

2. Sampling and injection techniques

Solvent extraction and volatile trapping are the two established techniques for sampling pheromones. A general outline of their use is given here, together with some recent examples. The remainder of this section describes the application of solventless injection methods and the few studies that have been carried out using the new technique of solid-phase microextraction.

2.1. Solvent extraction

The lipid nature of many pheromones makes them readily soluble in hydrocarbon solvents such as pentane or hexane [1]. Dichloromethane can be used if a more polar solvent is required. However, repeated injection of pheromone mixtures in methanol, or other protic solvents, is not recommended as these solvents have a detrimental effect on most GC capillary column phases.

It is sometimes necessary to extract small amounts of pheromones, from glands or whole insects, in relatively large volumes of solvent. When preparing the sample for GC, by concentrating the mixture under a stream of nitrogen, care must be taken that none of the volatiles are lost. It is also not uncommon to purify crude extracts on short silica or florisil columns to remove free fatty acids [2,3], which can interfere with the chromatography.

In some instances pheromone mixtures have been fractionated, before GC analysis, using an additional chromatographic technique. Reversed-phase HPLC fractionation of extracts of female screwworm flies *Ochliomyia hominivorax*, followed by bioassay, was used to identify the bioactive fraction that elicits mating behaviour in male flies. The structures of compounds in this fraction were then elucidated

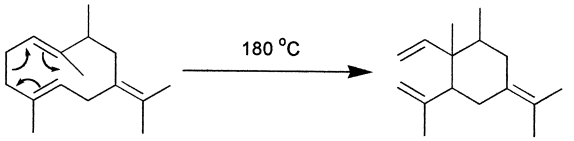


Fig. 1. The thermal Cope rearrangement of 9-methylgermacrene-B.

using microreactions and GC–MS (see Section 3.2) [4]. TLC or HPLC, using silica impregnated with silver nitrate, has routinely been used to separate unsaturated compounds from a pheromone mixture (see Section 3.5 for a number of relevant examples).

Injections of extracts containing thermally labile compounds can be problematic, an example being 9-methyl-germacrene-B, isolated from males of the sandfly *Lutzomyia longipalpis* from Lapinha, Brazil [2]. With the injector port of the GC set at 180°C, a number of additional peaks were found in the chromatogram, resulting from a thermal Cope rearrangement of 9-methyl-germacrene-B (Fig. 1). This rearrangement could be prevented by cold on-column injection, or by keeping the injector port at 110°C.

More vigorous conditions are sometimes required to extract polar compounds with solvents. In an investigation into the pheromones of the cashmere goat *Capra hicus laniger*, samples of fleece from does, gonadectomised bucks and bucks were Soxhlet extracted with a methanol chloroform azeotrope, and the extracts analysed by GC–MS [5,6]. A number of ethyl branched hexanoic, octanoic, decanoic dodecanoic and tetradecanoic acids were found to be specific to the bucks and the amount of these acids increased during the breeding season. Bioassay showed that the pheromone mixture alone could induce ovulation in does. In another example of a mammalian pheromone, the anal sac secretion of the ferret *Mustela furo* [7], was extracted by agitation with diethyl ether in a vortex mixer. GC–MS analysis of the diethyl ether phase revealed the presence of a number of highly odorous compounds, *cis*- and *trans*-2,3-dimethylthietane, 2-propylthietane, 2-pentylthietane, *cis*- and *trans*-3,4-dimethyl-1,2-ditholane and indole, the ratios of which varied between individuals.

2.2. Volatile trapping

2.2.1. Dynamic headspace analysis

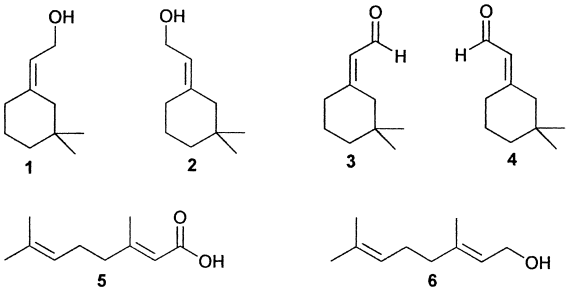
Dynamic headspace analysis has been widely used for collecting air-borne pheromones from live organisms and substrates [1]. The apparatus consists of an entrainment chamber with an inlet and outlet through which purified air can be drawn using a vacuum pump [8]. Volatile pheromones are collected either by sorbent trapping on an adsorbent, commonly activated charcoal or a porous polymeric phase such as Porapak-Q, Haysep-Q, Super-Q or Tenax-TA, or by cryogenic trapping.

The air flow-rate and quantity of adsorbent required is dependent upon the total volume of the system and the length of the sampling period. Having two traps in tandem is a simple method of ensuring that there is no breakthrough of the volatiles from the first trap [9]. All adsorbents must be cleaned and activated immediately prior to use to ensure trapping effectiveness and to minimise artifacts in the chromatograms [10].

Pheromones can be removed from the adsorbent by simple solvent washing or Soxhlet extraction [11], followed by injection of the solution onto the GC system. Thermal desorption is an alternative method that can be employed to release the pheromone from the adsorbent phase. Cryogenic retrapping of the volatiles desorbed by heating the adsorbent in the GC injector port concentrates the volatiles on the GC column before chromatography [12]. Desorption can also be carried out using commercial instruments such as a purge and trap concentrator connected to a GC [13] or a programmed temperature vaporisation GC injector port [14].

This collection technique has proved invaluable in monitoring the atmospheric ratio of compounds within pheromone mixtures. In an investigation into the aggregation pheromone of the pepper weevil *Anthonomus eugenii*, dynamic headspace analysis was used to identify the air-borne male-specific compounds [15]. Volatiles were collected from both males and females on Tenax TA or Super Q and extracted using either hexane or dichloromethane. A known amount of α-terpineol was added as an internal standard to quantify the volatiles and calculate pheromone release rates. Comparison of the

chromatograms showed six male-specific compounds (**1**–**6**) in the relative abundance of 48:32:3:2:13:2. In field tests, this mixture was found to attract large numbers of pepper weevils with an average of 72% being female.



Volatile trapping has also been important when studying the release rates of pheromones from individual insects. A method using a 60-cm×0.9-mm I.D. capillary as the volatile trap has been described that allows the pheromones of some insects to be sampled whilst they are observed to be emitting pheromone [16]. By applying this method, the mean release rate of (*Z*)-8-dodecenyl acetate from the oriental fruit moth *Grapholita molesta* was measured to be 8.48±7.26 ng/h.

Dynamic headspace analysis has also been used to sample pheromones indirectly from a variety substrates upon which pheromones are deposited. Volatiles from the froth plug placed by the desert locust *Schistocerca gregaria* over newly laid eggs were collected by dynamic headspace analysis using nitrogen gas and adsorption onto charcoal [17]. GC–MS and GC–electroantennographic detection (EAD) analysis of a dichloromethane wash of the charcoal, coupled with bioassay identified acetophenone and veratrole (see Section 5.2) as the locust oviposition pheromone.

Headspace analysis of the hair of male white tail deer, *Odocoileus virginianus*, has been carried out by passing purified helium gas over the hair at 70°C. Excess water was condensed out in a water-cooled condenser and the volatiles were collected on Tenax [18]. Thermal desorption followed by cryogenic retrapping was used to focus the volatiles at the start of the GC column. GC–MS analysis showed that hair from the forehead of males contains a number of pheromone substances, including terpenes and phenols, and that the relative amounts of these compounds varied between subordinate and dominant male deer. The same headspace analysis technique has been used to sample the volatile compounds from cotton wool swabs of the interdigital glands of the same species [19].

2.2.2. Closed loop stripping

Closed loop stripping is an alternative approach to dynamic headspace analysis. An adsorbent removes the pheromones from the air which is constantly circulated through an entrainment chamber containing the organism [20,21].

During an experiment to monitor the diel periodicity of sex pheromone release in three species of Lepidoptera, the recovery of three acetates, (8*E*)-8-dodecenyl, (9*Z*)-9-tetradecenyl and (11*Z*)-11-hexadecenyl from the system was monitored using synthetic compounds [21]. It was found that only 50–70% of the acetates were present in the charcoal trap, the losses rising with increasing molecular mass. It was suggested that this was due to adsorption of the compounds by the glass apparatus, the recovery from the charcoal using CS_2 as the solvent being near quantitative. In all three species analysed, *Mamestra brassicae*, *Cryptophlebia leucotreidae* and *Spodoptera sunia*, it was found that pheromone release occurs during the scotophase.

Closed-loop stripping has been adapted to sample pheromones from water using an apparatus described by Grob and Zurcher [22]. The water-borne pheromones from a number of marine polychaete have been extracted and identified using closed-loop stripping sampling followed by GC–MS [23,24]. Seawater containing freshly laid eggs of *Platynereis dumerilli*, and stabilised with sodium azide and acetyl iodide, was stripped of its volatile organics using a charcoal filter. The charcoal was washed with acetone and the major female specific volatiles identified as (5*R*)-5-methyl-3-heptanone and the (3*E*,5*E*) and (3*E*,5*Z*) isomers of 3,5-octadien-2-one. These compounds were shown to induce the release of eggs in females, but did not cause males to release sperm [24].

2.3. Solventless injection

A number of solventless injection techniques have been developed which allow the direct insertion of biological material into the injector port of a GC

system. However, apart from the original application there are often very few reports of studies that have used these techniques [25–29]. The simplest solventless injection method reported holds the sample of secretion or tissue in the end of a glass capillary, which is then pushed through the septum into a hot injector port operating in the splitless mode [30,31].

The most successful solventless injection technique has been developed by Morgan and Wadhams [32,33] and has been applied to the analysis of numerous insect pheromones. The sample is sealed inside a soft glass capillary and the volatiles are released when it is crushed inside the hot injection port of a GC system. This requires the use of a special crushing device known as a Keele Injector. The injection port is operated in the splitless mode and a small plug of deactivated glass wool [34] is placed in the liner to prevent glass particles from blocking the capillary column. These glass particles can be reactive towards some polar compounds such as fatty acids and, hence, it is advised that a clean pre-conditioned liner is used for each injection [34].

An advantage of this technique is that samples can be collected in the field and sealed directly in capillaries. Once inside the capillary biological material is kept free from contamination and can be easily stored and returned to the laboratory for analysis. Insect body parts [35], glands [36,37], secretions [38] and nest material [39] have all been sampled and analysed using this method.

Structure elucidation of the recruitment pheromones of the ants *Aphenogaster albisetosus* and *A. cockerelli* [40] was realised using this technique. The poison glands were sealed in soft glass capillaries and injected into the GC–MS system using a Keele injector. Analysis of the chromatograms showed *A. albisetosus* to contain (*S*)- and (*R*)-4-methyl-3-heptanone in the ratio of 8:2, and *A. cockerelli* a 9:1 mixture of (*R*)-1-phenylethanol and (*S*)-4-methyl-3-heptanone. Interestingly, it was found by bioassay that *A. albisetosus* is able to follow trails made from a racemic mixture of 4-methyl-3-heptanone and the gland extract of *A. cockerelli*. (*R*)-1-Phenylethanol, however, was found to be the active pheromone that stimulated trail following in *A. cockerelli* and this species is unable to follow the trails of *A. albisetosus*.

The solid sampling method is particularly suitable for the analysis of insect cuticular hydrocarbons, which are of interest to those studying nestmate, caste and kin recognition within social insects [41]. For these studies, the hydrocarbon profiles of individual insects need to be analysed and problems of contamination from exocrine glands can arise if solvent washing techniques are employed [42].

Another application has used this technique to locate the exact site of sex pheromone production in females of the moth *Mamestra brassicae* [43]. The abdominal tip was disected and the various parts sealed into capillaries and injected onto a GC system. (11*Z*)-11-Hexadecenyl acetate, the major compound in the pheromone mixture, was only found in the intersegmental membrane and not in the tissues beneath nor in any other parts of the abdominal tip.

2.4. Solid-phase microextraction

Solid-phase microextraction (SPME) is a relatively new technique that is growing in popularity [44,45]. It consists of a fibre coated with an adsorbent that can extract organic compounds from the headspace above a liquid or solid sample, from an aqueous solution or from the surface of a biological material. The extracted compounds are desorbed upon exposure of the SPME fibre in the heated injector port of the GC system, operating in the splitless mode. A number of different fibres are available and should be chosen depending upon the polarity of the compounds to be sampled.

The first report of SPME being used to sample pheromones was in 1995 [46]. It was used to monitor the diel rhythm of emission of air-borne volatiles from the sugar cane weevil *Metamasius hemipterus*. Sampling was carried out by exposing a polydimethylsiloxane fibre for 5 min to the exit of an airflow (100 ml/min) that passed over six male weevils. These conditions were arrived at by an optimisation procedure, carried out on synthetic pheromone mixture of 2-methyl-4-heptanol, 2-methyl-4-octanol, 5-nonanol and 4-methyl-5-nonanol. The authors found that all the components of the known pheromone mixture were present in the SPME sample and that pheromones were released during the photophase.

An interesting study comparing SPME to more traditional methods of headspace analysis has recently been published [14]. Static headspace analysis was

carried out on a synthetic mixture of putative pheromones of known concentration. Sampling by direct injection of a gas sample or trapping on Tenax, Poropak Q and Hayesep Q phases, followed by either solvent washing or thermal desorption all gave, consistently, the same ratio of compounds in the pheromone mixture. However, SPME analysis using a poly(dimethylsiloxane) fibre gave significantly different ratios (Fig. 2). Possible explanations for this are that the SPME fibre did not have enough time (30 s) to equilibrate with the sample mixture, or that the SPME phase preferentially absorbs certain types of compounds. Whatever the reasons, care must be taken when using SPME for headspace analysis and the authors suggested that it should be used in conjunction with another established sampling technique.

Studies into the extraction of insect cuticular hydrocarbons have shown that headspace SPME sampling is comparable to solvent extraction and solventless injection [47]. By exposing a polydimethylsiloxane fiber to three legs of a *Vespa orientalis* queen in an enclosed 2-ml vial at 170°C for 5 min, it was possible to extract all the cuticular hydrocarbons. The chromatogram from SPME sampling was almost identical to that of a hexane extract of the other three legs of the same insect (Fig. 3).

A carbowax-divinylbenzene fibre has been shown to be suitable for the headspace sampling of polar compounds, such as long chain fatty acids, from the sternal glands of the paper wasp *Polistes dominulus* [34]. However, in another study into the chemical secretion of the metapleural glands of the leaf cutter ant, *Acromyrmex octospinosus*, no acids could be detected using SPME. It was suggested that the sampling temperature of 140°C may decompose the polar compounds in the secretion. For these samples, sealing the secretion in glass capillaries and using a Keele injector was found to give higher sensitivity.

Lepidopteran sex pheromones have been sampled

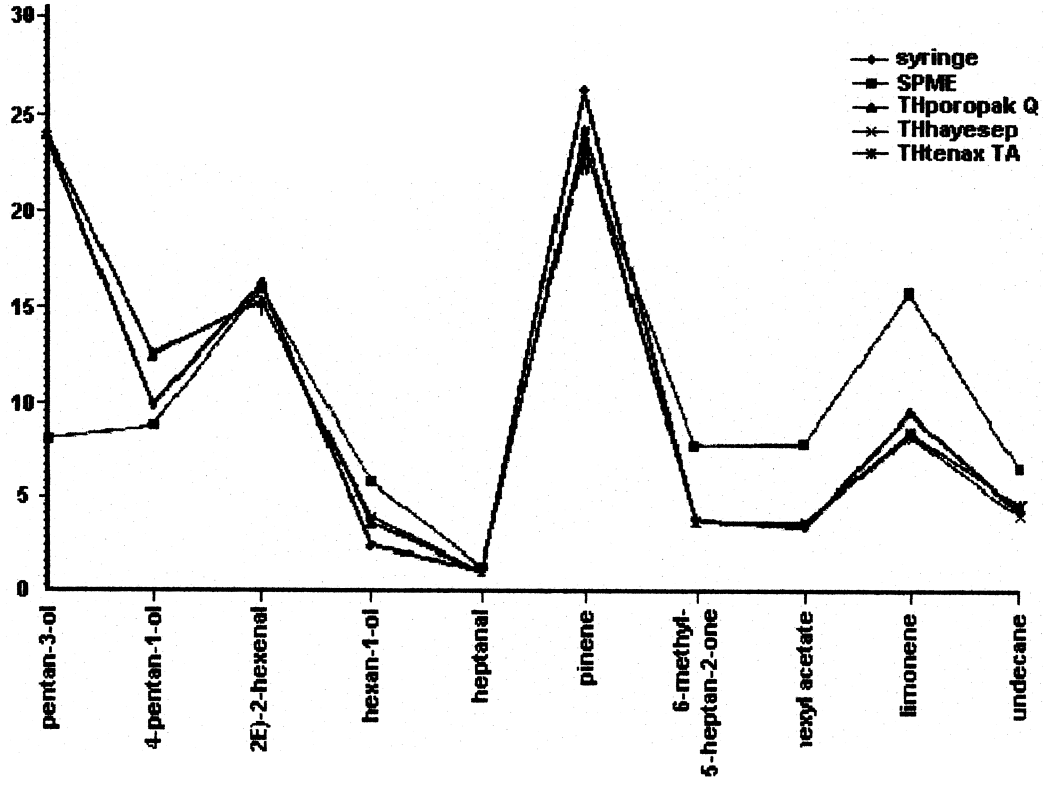


Fig. 2. Comparison of headspace sampling by SPME, syringe and porous polymers (TH, thermal desorption). From Ref. [14] with permission; ©Plenum Press, 1998.

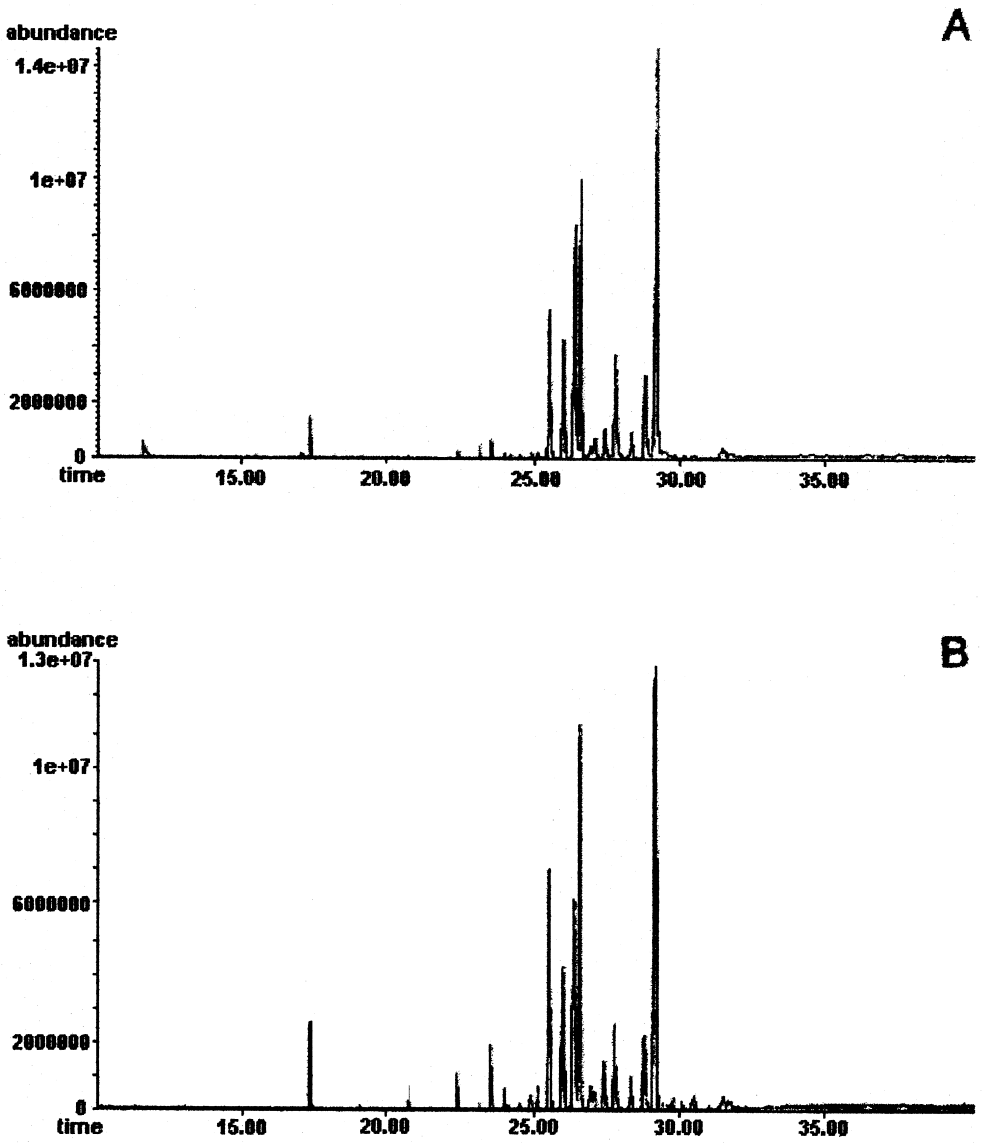


Fig. 3. Total ion chromatograms of cuticular hydrocarbons of the three legs from each side of the body of the same *Vespa orientalis* queen, sampled by SPME (A) and hexane extraction (B). From Ref. [47] with permission; ©Wiley, 1997.

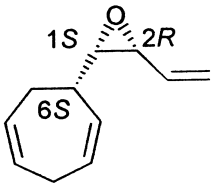
by simply rubbing a polydimethylsiloxane SPME fibre over the exposed sex pheromone gland of a female for 5 min [48]. Termed 'contact SPME' the technique was used to sample the sex pheromone of the French strain of the moth *Sesamia nopnagrioides*. This was found to be a blend of three components, (11*Z*)-11-hexadecen-1-ol, (11*Z*)-11-hexadecenyl acetate and hexadecanyl acetate. Comparison of the SPME results with those from washing the gland in ether showed that SPME extracted greater quantities of (11*Z*)-11-hexadecenyl acetate.

Monnin et al. [49] used contact SPME with a polydimethylsiloxane fibre to extract cuticular hydrocarbons directly from the cuticle of the Ponerine ant *Dinoponera quadriceps*. In this species of ant there is no queen but an alpha worker who mates and reproduces. The alpha worker exerts her dominance on the other workers in the colony by a specific

behaviour that involves biting the antenna of a subordinate worker and rubbing it against the intersegmental membranes between her abdominal tergites V and VII. SPME sampling and GC–MS analysis showed the cuticular hydrocarbon profile to contain 81 hydrocarbons. The cuticular hydrocarbon profile of the alpha female, however, was found to contain significantly higher amounts of (9Z)-9-hentriacontene.

For insect studies, the great advantage of contact SPME is that its non-destructive nature allows repeated sampling from the same individual insect, something previously not possible.

Finally, in aqueous solution, SPME has been used to extract the spermatozoid releasing pheromone of the brown algae *Laminaria digitata* [50]. The pheromone was extracted by exposure of a polydimethylsiloxane SPME fibre to a saturated sodium chloride solution of seawater medium containing newly released eggs. By chiral column GC, and with reference to synthetic samples, it was possible to identify that the algae produced the pheromone, Lamoxirene, as a mixture of two diastereoisomers (**7**) and (**8**) in the ratio of 29:71.



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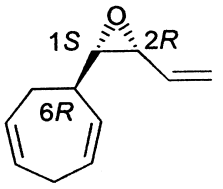

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3. Microreactions

Before the widespread availability of GC–MS, microscale reactions played a crucial role in the determination of the structure of insect pheromones [51,52]. Nowadays microreactions have three main uses; derivatisation to aid chromatographic properties, functional group modification to help with MS structure determination, and assignment of absolute configuration to chiral centres. All of these require highly chemoselective reactions that leave other functionality within the molecule untouched. This section is structured by functional group, a selected number of examples serving to illustrate the scope and limitations of each method.

3.1. Carboxylic acids

Direct GC analysis of carboxylic acids is possible using polar stationary phases (see Section 4). It is more common practice, however, to methylate carboxylic acids to give the corresponding methyl esters, which are much more amenable to analysis. Despite its explosive and toxic nature, diazomethane is regularly used, the reactions being clean and highly selective. This technique was recently employed to aid in identification of dodecanoic acid as the oviposition pheromone of the sandfly *Lutzomyia longipalpis* [53].

The position of methyl branching in fatty acids found within the fat body of two insects, the housefly *Musca domestica* [54] and the german cockroach *Blattella germanica* [55], has been correlated with the methyl branching of hydrocarbons found on the insects' cuticle. This study employed a whole series of microreactions to convert the branched fatty acids to their corresponding branched alkanes to aid GC–MS analysis (Fig. 4).

Saponification of the fat body, followed by extraction and separation of the non-polar lipids by TLC, gave the free fatty acids, which were esterified using 14% boron trifluoride in methanol. The methyl esters were reduced to alcohols and treated with triphenylphosphine dibromide [56] followed by sodium borohydride, or sodium borodeuteride, to give the corresponding hydrocarbons, which in the latter case were mono-deuterated on the original carboxyl carbon. The unsaturated and saturated hydrocarbons were then separated by silver nitrate chromatography, and the linear alkanes removed by inclusion in 5A molecular sieves, from a solution of the hydrocarbons in isooctane. Thus, the remaining methyl branched alkanes, corresponding to the methyl branched fatty acids, were isolated and analysed by GC–MS.

For each branched hydrocarbon two fragment ions, resulting from α-cleavage to the methyl group, were observed in the mass spectra. Those reduced by sodium borodeuteride exhibited one fragment ion with an even mass, identifying it as the fragment

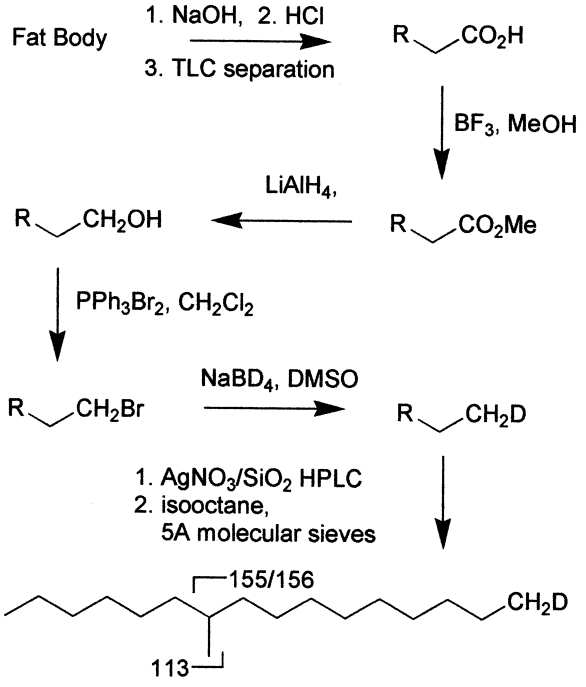


Fig. 4. The conversion of insect fatty acids to hydrocarbons and separation of the methyl branched alkanes.

containing the deuterium marking the original carboxyl carbon. This allowed precise location of the methyl group, relative to the carboxyl carbon in the original fatty acid. In both species it was found that there was a close correlation between the methyl branching found in the fatty acids and the methyl branching found in the cuticular hydrocarbons.

3.2. Carboxylic esters

Microsaponification of pheromones containing ester groups results in the production of the corresponding acid and alcohol. An interesting series of microreactions were used to identify the position of the acetate and methyl groups in the pheromone mixture of the female screwworm fly *Cochliomyia hominivorax* [4].

Saponification or reduction of the pheromone mixture using lithium aluminium hydride gave a mixture of alcohols. Microscale oxidation using chromium trioxide on celite produced the corresponding ketones which, when subjected to GC–MS, gave characteristic McLafferty ions, locating the original position of the acetate along the carbon chain (Fig. 5).

Some of the pheromones contained methyl branches, remote from the acetate group, the positions of which were established using two microchemical techniques. Reaction of the corresponding alcohols with thiocarbonyldiimidazole, followed by microreduction with hydrogen and palladium on carbon (Pd/C) catalyst produced methyl branched alkanes. Alternatively, Grignard addition of ^{13}C-methyl magnesium bromide, followed again by complete reduction of the alcohol, gave dimethyl branched alkanes in which the ^{13}C-methyl group marked the original position of the acetate. GC–MS analysis of both these products allowed identification of all the

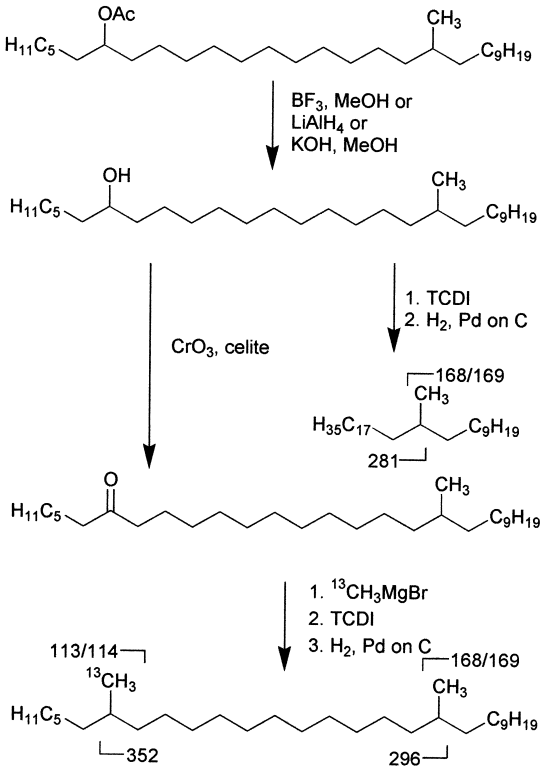


Fig. 5. Method of locating the relative position of remote acetate and methyl groups in the pheromones of the female screwworm fly *Cochliomyia hominivorax*.

fragment ions, thus confirming the relative position of the methyl and acetate groups (Fig. 5).

3.3. Alcohols

In general it is not necessary to derivatize simple alcohols for routine GC analysis. However, to aid the separation of enantiomers on chiral columns the trifluoroacetates of alcohols are sometimes prepared by reaction with trifluoroacetic anhydride [57,58] (see Section 4.2).

A number of investigations into mammalian steroidal pheromones by GC–MS have required the conversion of hydroxyls on the steroid to their corresponding trimethylsilyl and *tert*.-butyldimethylsilyl ethers. This greatly enhanced their chromatographic properties and, by the use of GC–MS, allowed the characterisation and quantification of a number of androst-16-enes, putative pheromones in the testis of the macaque *Macaca fascicularis* [59] and in human semen [60].

3.4. Chiral derivatisation

Microreaction of a chiral pheromone with a homochiral derivatising reagent produces diastereoisomers, which are separable by GC on an achiral stationary phase. This method can be a useful alternative when GC of chiral compounds on a chiral stationary phase (see Section 4.2) fails to give satisfactory separation. Homochiral derivatisation has not only been used for pheromone analysis, and a number of reviews on the subject have been published [61,62].

Methods have been described for the homochiral derivatisation of chiral alcohols, lactones and hydroxy acids using (2*S*)-2-acetoxypropionyl chloride [63]. This reagent was used to assign the absolute configuration of (10*E*)-10-tridecen-2-yl acetate, the female sex pheromone of the hessian fly *Mayetiola destructor* [64] (Fig. 6). An extract of 200 female abdominal tips was hydrolysed using potassium hydroxide in methanol and then derivatised with an excess of (2*S*)-2-acetoxypropionyl chloride in pyridine, and analysed by GC–MS using a DB-5 column. Comparison of the natural derivative with the derivatives of a synthetic racemic sample and a

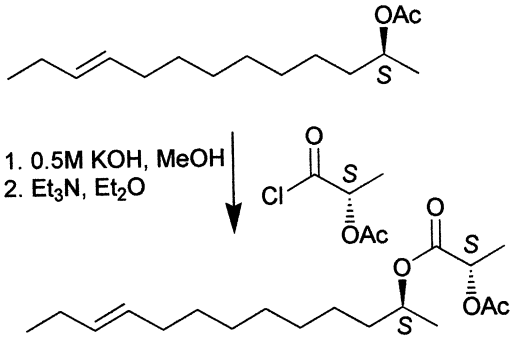


Fig. 6. Homochiral derivatisation of (2*S*),(10*E*)-10-tridecen-2-yl acetate using (2*S*)-2-acetoxypropionyl chloride.

sample of known chirality allowed the configuration at the 2 position to be determined as *S*.

(*R*)-*trans*-Chyrsanthemyl chloride has been used to prepare diastereomeric esters of 3-octanol to assign the configuration of (3*S*)-3-octanol a component of the alarm pheromone in the ants *Crematogaster castanea* and *Cremanodater liengmei* [65]. Kruse et al. [66] introduced the use of *N*-trifluoroacetyl-L-alanine for the homochiral derivatisation of chiral alcohols in the presence of dicyclohexacarbimide and dimethylaminopyridine. Using this they were able analyse an extract of the males of *Ips amitinus* by GC–MS and identify (*R*)-ipsdienol as the aggregation pheromone.

For chiral alcohols, probably the most common homochiral derivatisation is to prepare the Moshers ester – the (*R*) or (*S*)-methoxy(trifluoromethyl)-phenylacetate [67]. The absolute configurations of 3-hydroxy-2-hexanone and 3-hydroxy-2-octanone, male sex pheromone components of the longhorn beetle *Anaglyptus subfasciatus*, were assigned by derivatisation with (*R*)-methoxy(trifluoromethyl)-phenylacetyl chloride in pyridine [68] (Fig. 7). Interestingly, whereas complete resolution on a CP-Sil 5 CB capillary column was achieved for both pairs of Moshers esters, on a Chilraldex GTA capillary column the two enantiomers of 3-hydroxy-2-hexanone failed to give baseline separation. Comparison of the chromatograms of Moshers esters produced from the natural hydroxy ketones with those of synthetic samples allowed the absolute configuration at the 3 position to be assigned as *R* for both compounds.

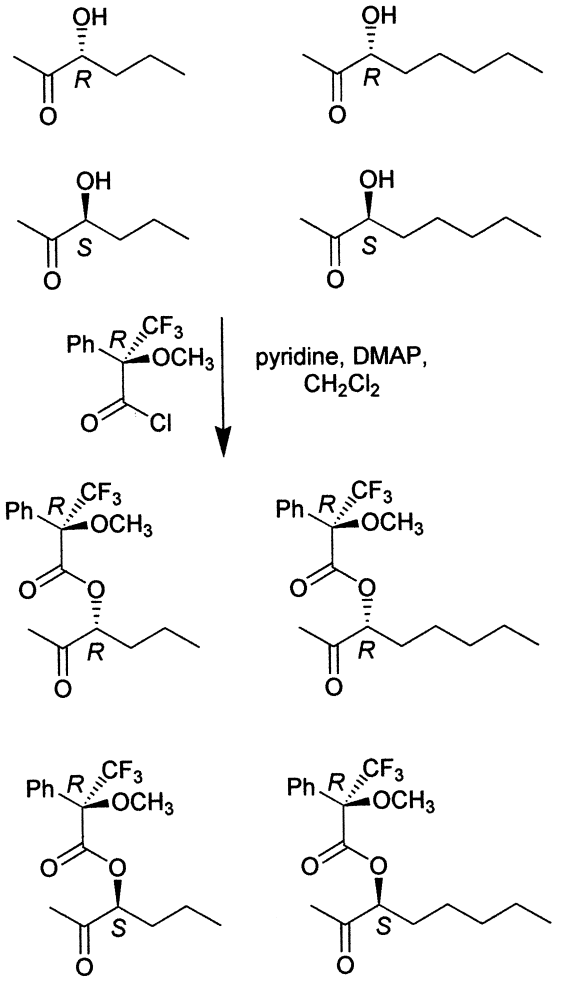


Fig. 7. Homochiral derivatisation of 3-hydroxyhexanone and 3-hydroxy-2-octanone using (*R*)-methoxy(trifluoromethyl)phenylacetyl chloride.

3.5. Double bonds

3.5.1. Reduction

Micro-reduction coupled with GC–MS is commonly used to determine the degree of unsaturation within a pheromone. For example, micro-reduction with hydrogen over a palladium on charcoal catalyst [69] was used to help identify (3*Z*,6*Z*,9*Z*)-1,3,6,9-heneicosatetraene as the sex pheromone of *Epirrita autumnata* [70]. An alternative to catalytic hydrogenation is diimide reduction, which was used to aid the identification of (9*Z*)-16-methyl-9-heptadecenyl isobutyrate as the sex attractant pheromone of the major female tussock moth *Euproctis taiwana* [71].

Micro-reduction is particularly useful for indicating the degree of unsaturation in terpenoid compounds, which are often cyclic or bicyclic. Catalytic reduction of an extract of males of *Luzomyia longipalpis*, from Jacobina Brazil, using palladium black gave two main diastereoisomer peaks in GC–MS, each having an M+ of 222, four mass units higher than the major pheromone component (Fig. 8). This indicated that the molecule contained two double bonds and, hence, from the molecular formulae of $C_{16}H_{26}$, it was deduced that the molecule it must be bicyclic. Together with other analytical and spectral data the male sex pheromone was identified as the homosesquiterpene 3-methyl-α-himachalene [3].

3.5.2. Ozonolysis

For acyclic unsaturated compounds a number of microreactions have been developed in order to help determine the position of double bonds within unsaturated pheromones. Micro-ozonolysis was the first method to be developed and, unlike other techniques for determining double bond position, can be carried out using a GC and does not require GC–MS. Bubbling low concentrations of ozone in oxygen through a solution of the pheromone at low temperature (−50 to −78°C), followed by quenching the ozonide with either dimethylsulphide or triphenylphosphine results in the double bond being cleaved to give two aldehydes. Immediate injection of the mixture onto the GC system is necessary to minimise over-oxidation. In an early study, Jackson and Bartelt carried out an extensive investigation into the changes in cuticular hydrocarbons with age and sex of the fly *Drosophila virilis* and used silver nitrate/silica chromatography and ozonolysis to determine double bond position in a complex mixture of alkanes, alkenes and alkadienes [72].

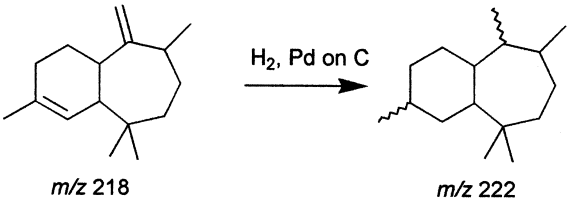


Fig. 8. Reduction of 3-methyl-α-himachalene.

Thermal decomposition of an ozonide to aldehydes, in the GC injector port, is an alternative to chemical quenching. This was recently used to identify (5*Z*)-5-dodecenyl acetate the major component of the female sex pheromone of the rice looper, *Plusia festucae* [73], the aldeyhdes being identified by reference against standards (Fig. 9).

An alternative solventless methodology has been described in a study directed towards identifying the female sex pheromone of the carob moth *Ectomyelois ceratonaie* [74]. First, the pheromone was collected from the end of a capillary GC column in a melting point tube cooled with dry ice. The tube was then placed inside a flask, containing oxygen and ozone, for 2 s and then immediately washed out with a solution of CS_2 and injected onto a GC system (Fig. 10). Using this methodology the authors identified the major pheromone component (9*Z*,11*E*)-9,11,13-tetradecatrienal and two minor components (9*Z*)-9-tetradecenal and (9*Z*,11*E*)-9,11-tetradecadienal.

3.5.3. Epoxidation

Epoxidation using *m*-chloroperoxybenzoic acid (*m*-CPBA), followed by GC–MS analysis, is an alternative to ozonolysis. Unfortunately, *m*-chlorobenzoic acid, the by-product from the reaction, can sometimes interfere with the chromatography. To overcome this, dimethyldioxirane [75] is recommended as an alternative cleaner reagent that is simply reduced to acetone [2,3].

An interesting example of the use of epoxidation is in the analysis of multi-unsaturated compounds such as (3*Z*,6*Z*,9*Z*,11*E*)-3,6,9,11-nonadecatetraene the sex pheromone of the fall cankerworm *Alsophila pomentaria* [76]. Mono-epoxidation with *m*-CPBA

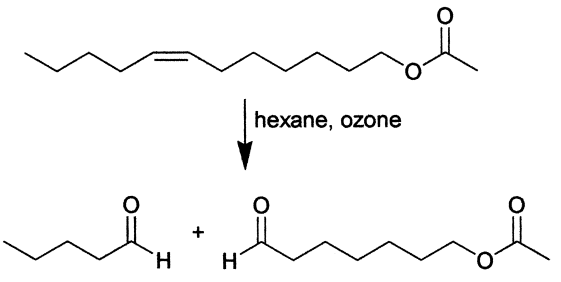


Fig. 9. Ozonloysis of (5*Z*)-5-dodecenylacetate the sex pheromone of the rice looper *Plusia festucae*.

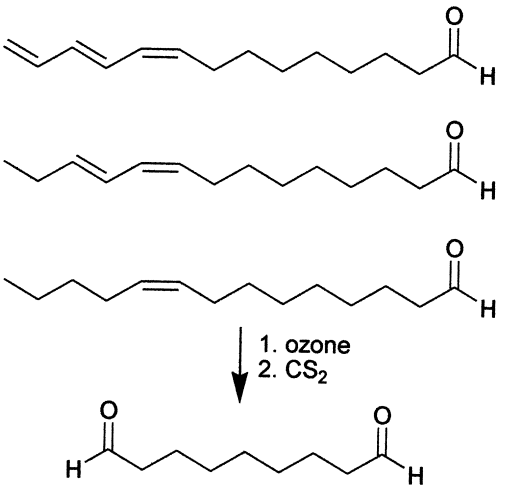


Fig. 10. Ozonolysis of (9*Z*,11*E*)-9,11,13-tetradecatrienal, (9*Z*,11*E*)-9,11-tetradecadienal and (9*Z*)-9-tetradecenal the sex pheromones of the female carob moth *Ectomyelois ceratonaie*.

followed by catalytic hydrogenation using H_2 and 5% Pt on neutral alumina produced an isomeric mixture of four epoxides by GC–MS, the diagnostic ions of which are shown (Fig. 11).

3.5.4. Methoxy-mercuration-demercuration

Methoxy-mercuration-demercuration has also been used for the determination of double bond position in pheromones [77–79], an example being in the analysis of the sex pheromone of the female pine beauty moth *Panolis flammea* [80]. An advantage of this method is that during the mercuration step only unsaturated compounds in the pheromone mixture react with mercury acetate and dissolve in the methanol solvent. The remaining saturated compounds are imiscible with methanol and can easily be removed with a syringe. The mixture is then reduced using sodium borohydride to give approximately equal amounts of both isomers of the methoxylated compounds (Fig. 12). These isomers are not resolved by GC, the mass spectrum showing characteristic fragmentation adjacent to the carbon bearing the methoxy groups. Using this methodology it was possible to identify the unsaturated pheromone components as 9-tetradecenyl acetate, 11-tetradecenyl acetate, 11-hexadecenyl acetate, methyl 9-hexadecenoate and methyl 9-octadecenoate. Bioassay showed that a mixture of the first three alkenes in a

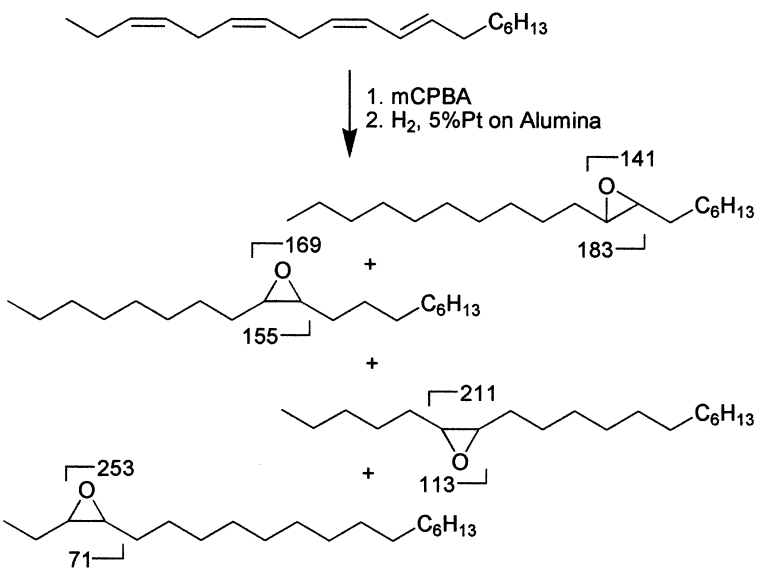


Fig. 11. GC–MS determination of double bond position in (3*Z*,6*Z*,9*Z*,11*E*)-3,6,9,11-nonadecatetraene the sex pheromone of the fall cankerworm *Alsophila pomentaria* by a monoepoxidation and reduction sequence.

100:1:5 ratio was able to elicit full sexual behaviour in the male moth.

3.5.5. Dimethyldisulphide derivatisation

By far the most common method currently used for double bond position determination in pheromones is derivatisation with dimethyldisulphide (DMDS) followed by GC–MS [81]. The reaction is catalysed by iodine and being radical in nature is quenched in the presence of oxygen. Therefore, in

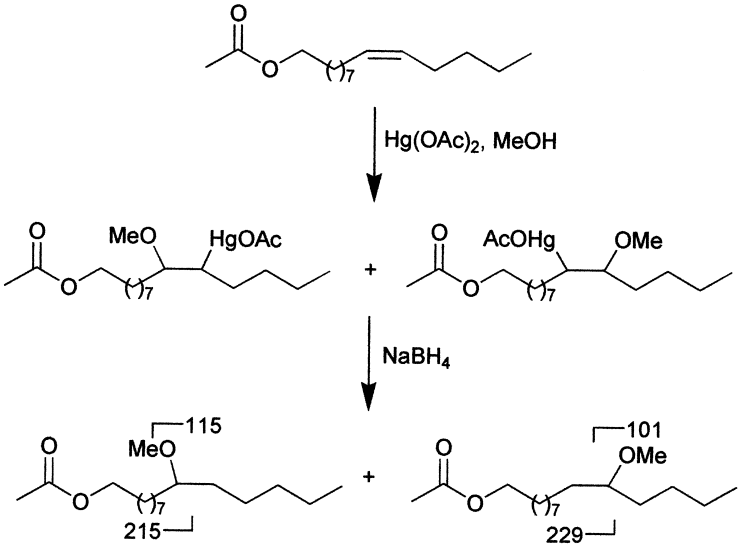


Fig. 12. Methoxy-mercuration-demercuration double bond position determination in the sex pheromone of the female pine beauty moth *Panolis flammea*.

order to achieve quantitative yields it is recommended that the reactions are performed under an inert atmosphere such as nitrogen [36]. Fragmentation in the mass spectrum of the DMDS derivatives occurs across the carbon carbon bond between the methylsulphide groups, thus locating the original position of the double bond.

A comprehensive study into the reaction of DMDS with a range of long-chain alkenes, alkadienes and alkatrienes was undertaken by Carlson et al. [82]. They used this in a chemotaxonomic study of three races of bee, the European honeybee *Apis mellifera mellifera*, *Apis mellifera scutellata* from Southeast Africa termed the African honey bee and the Africanised honey bee commonly found in South and Central America. In order to simplify the chromatograms, the alkenes and alkadienes were separated from saturated hydrocarbons using silver nitrate chromatography.

The alkene DMDS adducts were amenable to GC and gave interpretable mass spectra. For example, the double bond position in 9-hentriacontene was located by the characteristic fragment ions with *m*/*z* 173 and 355 (Fig. 13). The bees contained a series of alkenes from C_{23} to C_{35} with the position of unsaturation changing from positions 7 and 9 in lower chain lengths to 8, 10 and 12 in longer chain lengths. The ratio of the different positional isomers was taken from the abundance of the diagnostic fragment ions in an averaged spectrum across the chromatographic peak. The major difference between the races was that in the African and Africanised bees about 37% of the C_{31} alkene was the 9 isomer, whereas the European bee contained less than 2%.

It should be noted that it is sometimes possible to resolve the DMDS adducts of different positional isomers of alkenes on GC. The DMDS adducts of (9*Z*)-9- and (11*Z*)-11-tetradecenyl acetate the female sex pheromone of the summer fruit moth *Adoxophyes orana* [83] were separable by GC on a 50-m Silar 10c capillary column.

Problems were encountered with higher-molecular-mass C_{37} alkadiene bis-DMDS derivatives because of their very long retention times on GC, sometimes greater than 80 min [82]. However, the mass spectra of these adducts were again easily interpretable, e.g. (9*Z*,19*Z*)-9,19-heptatriacontadiene gave two diagnostic fragment ions at *m*/*z* 173 and 299 together with an $(M)^+$ at *m*/*z* 704 (Fig. 14). Interestingly, the two other fragment ions, at *m*/*z* 311 and 437, result from α-cleavage and concurrent loss of DMDS (M-94). All the dienes identified in the bees were in the range of C_{29} to C_{35} with the double bonds being most commonly positioned at C_9 and C_{23}, separated by 12 methylene units. Compared with the alkenes, differences between the alkadienes found in the races of bees was much less significant. This methodology has also been used to identify (5*Z*,27*Z*)-5,27-tritriacontadiene as the major sex pheromone from the female fruit fly *Drosophila pallidosa* [84].

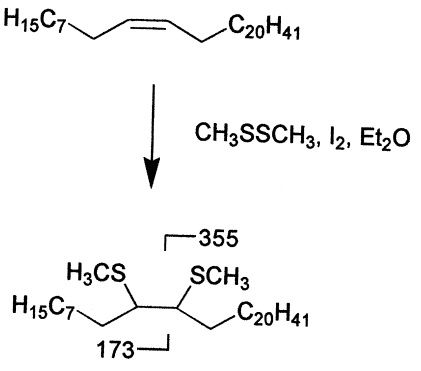


Fig. 13. DMDS derivatisation of (9*Z*)-9-hentriacontene.

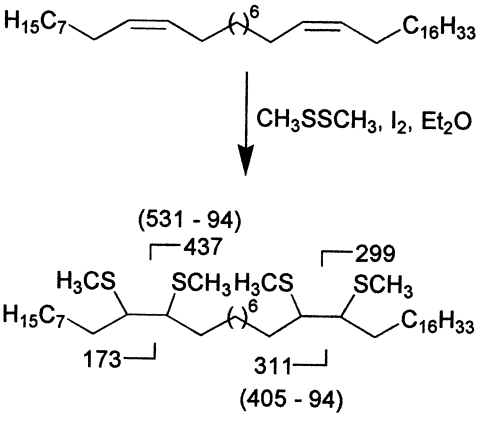


Fig. 14. DMDS derivatisation of (9*Z*,19*Z*)-9,19-heptatriacontadiene.

In a parallel study of the hydrocarbons of *Drosophila melanogaster*, C_{25}, C_{27} and C_{29} alkadienes, in which the double bonds were separated by only two methylene units, were treated with DMDS [82]. These gave characteristic cyclic adducts containing a tetrahydrothiophene ring, which were separable from the mono DMDS adducts by GC. Fragmentation of the cyclic adducts preferentially occurred adjacent to the ring. A general observation is that dienes with four methylene units, or less, separating the double bonds form cyclic adducts [85,86] (Fig. 15). It has also been reported that conjugated dienes are amenable to derivatisation [87].

Finally, Carlson et al. prepared the DMDS derivatives of a homologous series of alkatrienes from the stable fly *Stomoxyz calcitrans*. For the major alkatriene 1,7,13-pentacosatriene, both the triadduct and diadducts could be observed, the diadducts being unresolved by GC [82]. Although the fragmentation was complex, in both spectra all the expected fragment ions were found and the double bond positions could be assigned.

The DMDS reaction conditions are highly chemoselective, tolerating a wide variety of other functional groups such as acetates [83,88,89], esters [90], alcohols [85,88] and aldehydes [85,91].

When analysing unsaturated aldehydes care has to be taken in assigning the position of the double bond relative to the aldehyde group. A good example is the identification of octadecenal, (11*E*)-11-octadecenal, (14*E*)-14-octadecenal and (11*E*,14*E*)-11,14-octadecadienal as the sex pheromones of the female tea cluster caterpillar *Andraca bipunctata* [85] (Fig. 15). The fragment ions from α-cleavage of the DMDS adducts of (11*E*,14*E*)-11,14-octadecadienal had fragment ions of 103/287 and 175/215, all of which could include the aldehyde functional group, thus locating the double bonds at either C_{11} and C_{14} or C_3 and C_6. To clarify the exact position of the double bond the pheromone secretion was reduced using lithium aluminium hydride. The mass spectrum of the DMDS adduct of the resulting alcohol had fragment ions at 103/289 and 175/217, thus locating the double bond at C_{11} and C_{14}.

As can be seen, DMDS derivatisation is highly versatile and has been used to determine double bond position in a wide variety of substrates. Its main disadvantage is an increase in the molecular weight

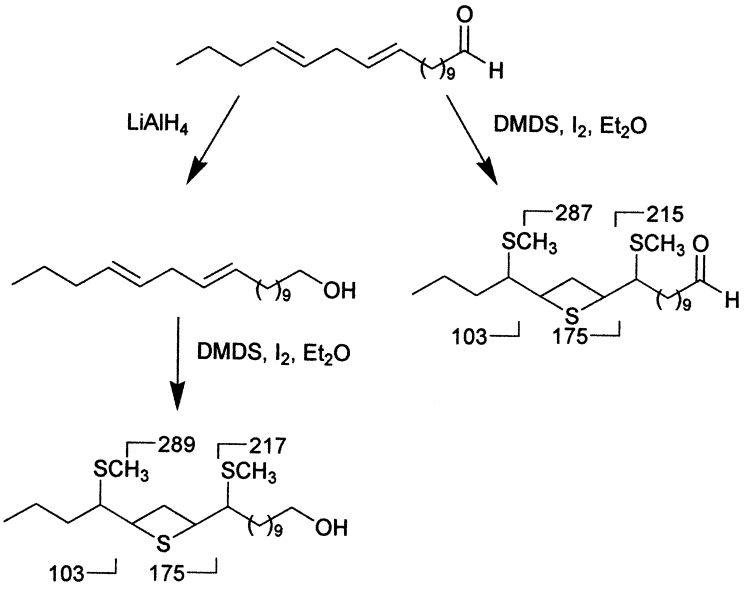


Fig. 15. DMDS derivatisation of (11*E*,14*E*)-11,14-octadecadienal a component of the sex pheromone of the female tea cluster caterpillar *Andraca bipunctata*.

by 94 mass units per double bond, which can lead to difficulties with chromatography. This makes chemical ionisation-MS techniques an attractive alternative, as the double bond 'derivatisation' takes place after the chromatography, inside the mass spectrometer (see Section 5.2).

4. Columns and separation

4.1. Achiral phases

Most pheromones (or pheromone components) possess relatively small ($<C_{30}$) and simple chemical structures of low polarity, making them amenable to analysis on a number of GC phases. In general, the more nonpolar polysiloxane derived phases are preferred, because of their broad range, thermal stability and long lifetimes. A 100% polydimethylsiloxane or a 5% polyphenylmethylsiloxane phase is the most common starting point for pheromone analysis. However, more polar phases are often employed if the above fail to give adequate separation, or if it necessary to confirm identification by comparison of retention times/indices on different phases. For example, identification of the sex pheromone of the bristly cutworm, *Lacinipolia renigera*, was achieved using three capillary columns coated with phases of differing polarities [92]. The two components of the pheromone were determined to be (9*Z*)-9-tetradecenyl acetate and (9*Z*,12*E*)-tetradecadienyl acetate by comparison of their retention times with those of synthetic standards on DB-5 (5% polyphenylmethylsiloxane), HP-17 (50% polyphenylmethylsiloxane) and Carbowax 20M (polyethylene glycol (PEG)). The latter also permitted unambiguous identification of stereochemistry, since both monene and all four diene isomers were resolved.

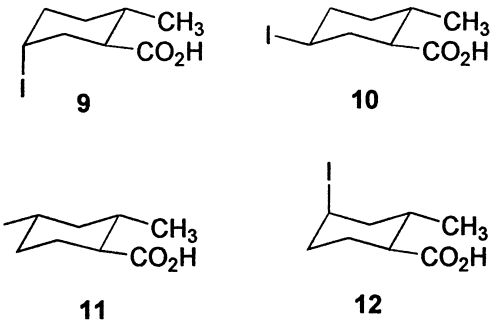
A similar approach was employed to identify the sex pheromone of the nettle caterpillar, *Setothosea asigna*, which is composed of a mixture mono- and diunsaturated C_{12} aldehydes and alcohols [93]. Here, DB-5, DB-210 (50% polytrifluoropropylmethylsiloxane) and DB-23 (50% polycyanopropylmethylsiloxane) phases were used. The high polarity of the DB-23 column gave excellent separation of aldehydes from the alcohols (for (9*E*)-9-dodecenal, retention index $I=1822$; (9*E*)-9-dodecenol, $I=1971$), and of (*E*)- and (*Z*)-isomers (for (9*Z*)-9-dodecenal, $I=1834$).

A DB-210 column was also used to aid structure elucidation of the sex pheromone of the eastern blackheaded budworm, *Acleris variana*. Retention index (*I*) calculations suggested the presence of a structurally related C_{14} alcohol ($I=1959$), aldehyde ($I=2038$) and acetate ($I=2165$) [94]. The compounds eluted too late to be monoenes, but too early to be conjugated internal dienes. Thus, they were identified as conjugated terminal dienes, which possess lower retention indices. Subsequent analysis of synthetic standards and use of GC–EAD confirmed the structures as (11*E*)-11,13-tetradecadienol, (11*E*)-11,13-tetradecadienal and (11*E*)-11,13-tetradecadienyl acetate.

Gas chromatographic behaviour of the sex pheromone of the alfalfa blotch leafminer, *Agromyza frontella*, proved useful in its identification [95]. The retention indices on DB-1 (100% polydimethylsiloxane) and Carbowax columns were 2013 and 1994, respectively, suggesting a C_{21} branched alkane. GC–MS analysis confirmed this result, and the pheromone was found to be 3,7-dimethylnonadecane.

More polar pheromones, such as carboxylic acids, are best analysed by PEG columns, although long chain carboxylic acids can be chromatographed on less polar phases. For example, dodecanoic acid, the ovipositon pheromone of the sandfly *Lutzomyia longipalpis* was identified by GC–MS analyses using both HP-5MS (5% polyphenylmethylsiloxane) and DB-Wax phases [53].

A study into odour binding proteins used a capillary GC column with a Stabilwax (PEG) phase to analyse (*E*)-3-methyl-2-hexanoic acid, the major volatile component of human axillary (under arm) odour [96]. Proteins were collected from the axillary region of healthy male volunteers and separated by electrophoresis. Each protein band was subjected to base hydrolysis, followed by acidification and extraction in chloroform. The chloroform solution was then analysed by GC–MS to detect the presence of the liberated odour. This allowed the identification of two odour binding proteins, which transport the acid to the surface of the skin where it is released by the action of cutaneous microorganisms.



Isomer separation often requires careful column choice. This is highlighted by attempts to analyse the four *trans* isomers of ceralure (**9**–**12**), a commercial synthetic attractant of the Mediterranean fruit fly, *Ceratitis capitata* [97]. Non-polar columns are unable to separate the isomers, and ceralure decomposed on polar columns (both PEG and polydicyanopropylsiloxane types). Medium-polarity phases, however, were found to give excellent resolution without decomposition. SPB-35 (35% polydiphenylsiloxane) and DB-17 (50% polydiphenylsiloxane) gave near baseline separation, but optimum results were obtained using a specially prepared 60-m SPB-608 column.

Separation of the geometric isomers of tetradecenyl acetate, common components of Lepidopteran pheromones, has been investigated using liquid crystal stationary phases [98]. The authors employed a range of *para*-substituted derivatives of cholesteryl cinnamate that form liquid crystals suitable for coating capillary columns. The ability of such columns to separate (*E*)- and (*Z*)-tetradecenyl acetates was found to be dependent on the position of the double bond in the chain. When the double bond was located more than four carbon atoms from the terminal methyl group the resolving power of the phases was *p*-Cl>*p*-Me>*p*-H>*p*-OMe>*p*-NO_2. However, when the unsaturation occurred four carbons or less from the terminal methyl group, the separation efficiency of the series was reversed. Overall, the cholesteryl *p*-chlorocinnamate phase was found to give the best results.

In addition to allowing the high-resolution separation of multicomponent pheromone mixtures, capillary GC also enables the determination of pheromone vapour pressures. These are among the most important physico-chemical properties of pheromones, since the majority are transmitted through the vapour phase, and knowledge of vapour pressure values is important in determining the efficacy and longevity of pheromone-baited traps in the field. The vapour pressures (P) of a sample (s) and reference compound (r) are related by

$$\ln P_s = \Delta H_s/\Delta H_r \ln P_r + c \quad (1)$$

where ΔH is the latent heat of vaporisation and c a constant [99]. These pressures are also related to GC retention times (t) by

$$\ln P_s = \ln P_r - \ln(t_s/t_r) \quad (2)$$

Combination of (1) and (2) yields

$$\ln(t_s/t_r) = (1 - \Delta H_s/\Delta H_r) \ln P_r - c \quad (3)$$

Thus, plotting $\ln(t_s/t_r)$ against $\ln P_r$ yields a straight line of gradient $(1-\Delta H_s/\Delta H_r)$ and intercept c. Eq. (1) can then be used to calculate the vapour pressure P_s of a sample at a given temperature.

Using this approach, Koutek and co-workers have determined the vapour pressures of a range of pheromones, and pheromone-like saturated and unsaturated alcohols [100] and acetates [101,102]. By employing short (2–4-m) columns with relatively thick (0.52-μm) polydimethylsiloxane coatings, the authors were able to achieve reasonable resolution without prohibitively long retention times. The methodology was validated by comparison of the vapour pressures of a series of saturated primary alcohols, measured by GC, with those given in the literature. The results were correlated using a logarithmic plot of literature vapour pressure values (P_L) against those determined by GC (P_{GC}) (Fig. 16). The regression line is almost identical to that for $x=y$, confirming the suitability of this method for vapour pressure determination.

Application of the technique to monounsaturated alcohols revealed a series of subtle trends. For example, those alkenols with the double bond closer to the centre of the chain tend to have higher P values [100]. Whilst analysis of over 100 (un)saturated long-chain acetates allowed the development of empirical equations for correlating P and ΔH with

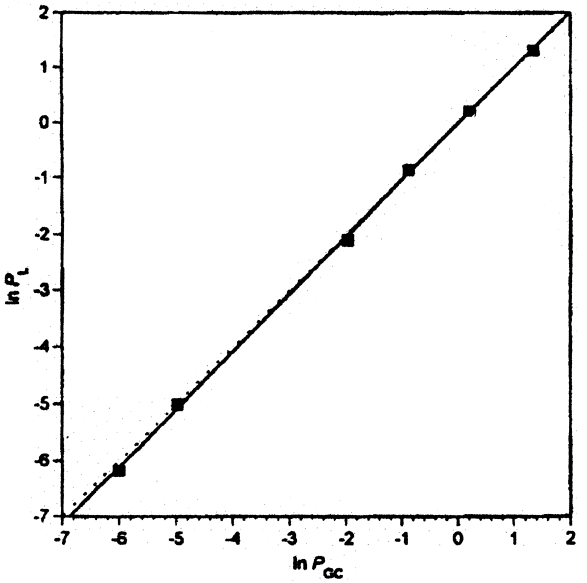


Fig. 16. Logarithmic plot of the literature vapour pressure values P_L of a series of saturated fatty alcohols (at 25°C) against the vapour pressures determined by gas chromatography P_{GC}. The dotted line corresponds to $y=x$. From Ref. [100] with permission; ©Elsevier 1994.

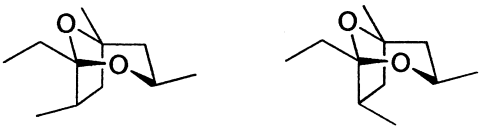
the number of carbon atoms and double bond position [102].

Liquid crystal stationary phases have also been used to determine pheromone vapour pressures. The length-to-breadth ratio (distortion of linearity) dependence of elution order on liquid crystal phases [103], such as cholesteryl-*p*-chlorcinnamate, is also related to vapour pressure and, thus, retention time data from these columns can be used to generate vapour pressure values. In a study of monounsaturated C_{10} acetates, Heath and Tumlinson demonstrated a linear relationship between ln *P* and retention times of the acetates (in equivalent chain length units (ECLU)) with a coefficient of determination of 0.999 [104]. The relationship was used to calculate the vapour pressures of related (un)saturated acetates and alcohols and these values correlated against reported half-lives of the compounds. The curve fit of 0.995 provided a direct link between retention time on liquid crystal columns and half-life of the material. This relationship could then be used to predict the half-lives of pheromone components and blends used in field traps and, therefore, provide a method for estimating the useful lifetime of a trap [104].

4.2. Chiral phases

The introduction of chiral GC phases has allowed determination of the absolute configuration of many chiral pheromones. This is of particular importance, since biological activity is often associated with only one enantiomer, or a specific ratio of enantiomers. In many cases the antipode of the natural compound is inactive and occasionally it is even repulsive [62]. Whilst chiral derivatising agents permit the separation of enantiomers on achiral phases (see Section 3.4), their use is restricted to compounds containing suitable functional groups. Chiral columns, in contrast, provide the possibility to resolve a wide range of compound classes. Most commercially available chiral phases are based on modified cyclodextrins, normally bearing methyl, trifluoroacetyl, propionyl, butyryl or *tert.*-butyldimethylsilyl substituents [105].

The male-produced aggregation pheromone of Australian populations of the banana weevil, *Cosmopolites sordidus*, was identified as a mixture of (1*S*, 3*R*, 5*R*, 7*S*)-1-ethyl-3, 5, 7-trimethyl-2, 9-dioxabicyclo[3.2.1]octane (sordinin, **13**) and its (7*R*)-epimer (**14**) (>81% enantiomeric excess (ee) and >85% ee, respectively) using a permethylated β-cyclodextrin GC column, together with enantiospecific synthesis [106]. This assignment was in agreement with an earlier study on African populations [107,108], although much lower levels of pheromone were emitted by the Australian insects.



13 **14**

Determination of the isomer ratio of ipsenol and ipsdienol in the male-produced aggregation pheromone of the bark beetle *Pityokteines elegans* was performed on a modified β-cyclodextrin phase. The enantiomeric excess of (*S*)-ipsenol was greater than 99% However, ipsdienol was found to consist of a mixture of (*R*)- and (*S*)-enantiomers in the ratio 2:1

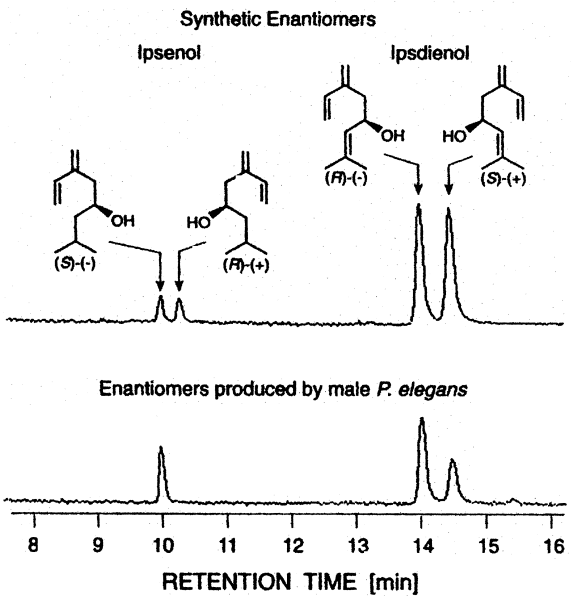


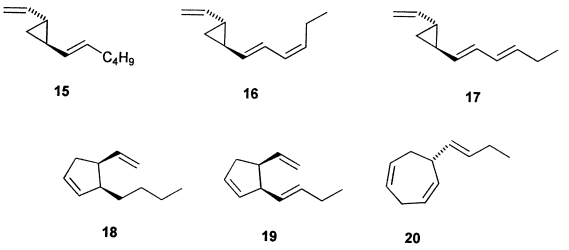
Fig. 17. Chiral separation of the synthetic enantiomers of ipsenol and ipsdienol, and of the enantiomers produced by males of *P. elegans*, on a β-cyclodextrin phase. From Ref. [109] with permission; ©Plenum Press, 1997.

(Fig. 17) [109]. Field experiments demonstrated that neither (*S*)-ipsenol or (±)-ipsdienol were active alone, but together they attracted both sexes of *P. elegans*. Interestingly, both enantiomers of ipsdienol were required for biological activity.

An investigation of the enantiomeric composition of (3*Z*,9*Z*)-*cis*-6,7-epoxynonadecadiene, a sex pheromone component of *Colotois penaria* and *Erannis defoliaria*, two species of winter-flying moths, revealed a chirality based species discrimination mechanism [110]. Using a 1:1 mixture of heptakis-(2,6-di-*O*-methyl-*O*-pentyl)-β-cyclodextrin and OV-1701 as a stationary phase, it was shown that *C. penaria* employs the (6*R*,7*S*)-enantiomer, while *E. defoliaria* uses the (6*S*,7*R*)-form. Trap-catch experiments demonstrated that each species is only attracted to its own enantiomeric form of the epoxide, thus confirming species separation based on chirality.

A β-cyclodextrin column was also used in the identification of *N*-2′-methylbutanoyl-2-methylbutylamine, the sex pheromone of the longhorn beetle, *Migdolus fryanus*. However, although the authors were able to assign (*S*)-absolute configuration to the 2′ centre, they were unable to determine the stereochemistry at position 2, due to poor GC separation [111].

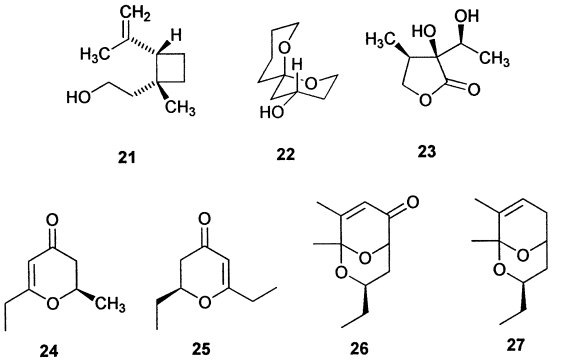
Pheromones of many marine brown alga species are composed of a mixture of cyclic hydrocarbons (**15**–**20**). The absolute configurations of the cyclopropyl components were determined to be (1*R*,2*R*)- and the cyclopentenyl constituents to be (3*S*,4*S*)-, using a heptakis-(3-*O*-2,6-di-*O*-pentyl)-β-cyclodextrin, while the cycloheptadiene enantiomers were best resolved on an octakis(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin phase [112]. Baseline separation was achieved in each case and enantiomeric excesses were found to range from 97% ee for **18** to 26% ee for **20**.



In order to obtain enantiomeric resolution of alcohols it is sometimes necessary to form the trifluoroacetate derivatives. For example, the enantiomers of grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol, **21**), a component of the aggregation pheromone of the pine weevils *Pissodes strobi* and *P. nemorensis* [113] are not separable on cyclodextrin columns. However, near baseline separation of their trifluoroacetates can be achieved easily. Using this approach, the ee of (1*R*,2*S*)-grandisol produced by *P. strobi* was found to be 98%, while that released by *P. nemorensis* was in excess of 99% [57]. Trifluoroacylation was also required to separate the enantiomers of 1,7-dioxaspiro[5.5]undecan-4-ol (**22**), from males of the fruit fly *Bactocerca cacuminatus* [58]. The (4*S*,6*S*)- isomer was found to be the dominant form, with an ee of approximately 80%.

The dihydroxy-lactone, 2-hydroxy-2-(1-hydroxyethyl)-3-methyl-γ-butyrolactone (**23**), is a scent gland constituent of male ithomiine butterflies [114]. Its stereochemistry was determined to be (1′*S*,2*S*,3*R*)- by analysis of the dioxolane derivative on a 3-*O*-acetyl-2,6,-di-*O*-pentyl-γ-cyclodextrin

phase. Unlike the free diols, excellent isomer separation was achieved with the dioxolanes, permitting unambiguous assignment of absolute configuration [115].



In addition to cyclodextrin-based capillary columns, a number of other chiral phases have been used in pheromone studies. For example, a XE-60–L-valine-(*S*)-α-phenylethylamide (PEA) phase was employed to determine the absolute configuration of two 2,6-dialkyl-2,3-dihydro-4H-pyran-4-ones (**24** and **25**) and 3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-en-9-one (**26**), components of the male produced pheromone of the swift moth, *Hepialus hecta* [116]. In the same study, a Ni(II)-bis(heptafluorobutanoyl-(1*R*,5*S*)-pinan-4-onate) column was used to separate the enantiomers of 3-ethyl-1,8-dimethyl-2,9-dioxabicyclo[3.3.1]non-7-ene (**27**).

A PEA column was also used to study the stereospecificity of an epoxide hydrase enzyme present in various tissues of the gypsy moth, *Lymantria dispar*, where it is believed to deactivate the pheromone after perception by the insect [117]. The enzyme was shown to open the epoxide ring of disparlure to yield the corresponding *threo*-(*R*,*R*)-diol with high specificity [118] (Fig. 18). The diol product was analysed as its bis(trifluoroacetate) derivative, and gave near baseline separation on the PEA phase.

Finally, although it is clear that the development of chiral columns has had a tremendous impact on pheromone analysis, it should be pointed out that there are limits to the types of compounds that can be resolved. At present, there are no phases capable of separating enantiomers of methyl and dimethyl branched hydrocarbons. This prevented direct determination of the stereochemistry of 3,13-dimethylheptadecane, the sex pheromone of the western false hemlock looper, *Nepytia freemani* [119]. In field studies, it was established that the (3*S*,13*R*)-isomer was the only active form when tested individually, but that a combination of all four stereoisomers was 3.6 times more attractive. However, as a result of the limitations of chiral phases, it was not possible to determine the exact blend of isomers released by the moth.

4.3. Dual-column GC

There are often considerable advantages in analysing compounds on a number of different phases. Comparison of retention index data on two or more columns does not only serve to confirm an identification, when used with a synthetic standard, but can also give structural information about an unknown (see Section 4.1). The different resolving powers of polar and non-polar phases may also be used to permit separation of complex mixtures, where each column is incapable of resolving all the individual components alone. Dual column chromatography exploits these advantages by simultaneously, or sequentially, analysing material on two or more columns. Simultaneous separation is achieved by connecting columns in parallel, and splitting the sample between them, whilst sequential analysis uses two, or more, columns connected in series.

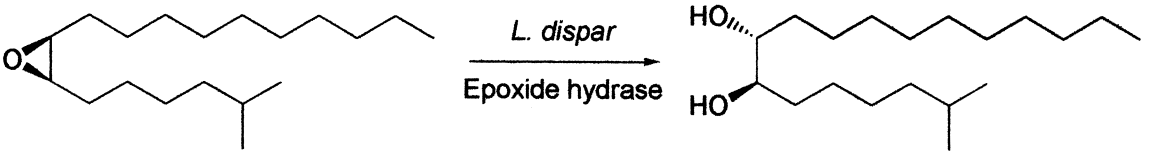
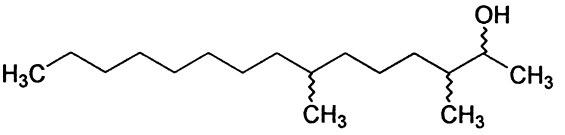


Fig. 18. Stereospecificity of disparlure hydration in *L. dispar*, as demonstrated by GC analysis of the bis-TFA diol derivative on a XE-60–l-valine-(*S*)-α-phenylethylamide (PEA) phase.

Parallel dual-column GC was employed in an identification of the sex pheromone of the South American potato tuber moth, *Symetrischema tanolias* [120]. A DB-1 and a DB-Wax (PEG) column of equal length, I.D. and film thickness were connected to a single split/splitless injector and to two, separate, flame ionisation detection (FID) systems. Thus, the sample was split equally between the two columns and the effluents detected independently. Comparison of retention indices of the active components on the two columns with standard values from the IPO pheromone data bank (Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands) [121] permitted their rapid identification as a tetradecen-1-ol isomer and a non-conjugated tetradecadien-1-ol. Subsequent DMDS derivatisation and GC–MS analysis confirmed the structures as (3*E*)-3-tetradecen-1-ol and (3*E*,7*Z*)-3,7-tetradecadien-1-ol.

GC–MS analysis of pheromones has also been performed using a parallel dual-column system. Here, both columns (a DB-1 and DB-210) were connected to a single mass spectrometer, rather than two separate detectors [122]. The large difference in retention times of most compounds on polar and non-polar phases, together with the additional information provided by a mass spectrometer, removes the need for two detectors. Although, interpretation of data from complex mixtures is difficult. This approach proved useful in identifying (7*Z*)-7-tetradecenal as the sex pheromone of *Spaelotis clandestina*, since positional isomer separation was only achieved on the DB-210 phase [122].

Sequential dual column (tandem) GC, in its simplest form, consists of two or more columns joined together, in series, by low dead-volume column connectors. Combining columns with different phases, in this way, can substantially increase the overall resolution. For example, isomers of dipropionol (**28**), and its acetate, which constitute the sex pheromones of a number of pine sawfly species could be separated with this technique [123]. *Threo*- and *erythro*-**28** were resolved using a 25-m Carbowax column coupled to a 25-m CP-Sil-88 (50% polycyanopropylmethylsiloxane) column. However, analysis of the mixture by chiral chromatography on a PEA phase failed to separate all eight isomers (analysed as isopropyl carbamate derivatives). This problem was overcome by combination of a CP-Sil-88 (25 m), a DB-Wax (25 m) and a 50-m PEA column. The achiral phases served to separate the *threo*- and *erythro*- forms, and the chiral phase was then capable of resolving the remaining enantiomers and diastereoisomers.



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The more sophisticated tandem GC technique of heart cutting has been employed to identify synergists of the bark beetle (*Pityogenes chalcographus*) aggregation pheromone [124]. Here, the effluent from a non-polar SE-33 (polydimethylsiloxane) column was trapped, using a specially designed splitter system [125], to form three fractions. Each fraction in turn was passed onto a second, polar, column (OV-351 (modified PEG)) by rapid vaporisation, in order to achieve a sharp injection. Combination of this approach with novel bioassay methods allowed identification of (2*E*,4*Z*)-methyl-(2,4)-decadienoate as a potent synergist of the bark beetle aggregation pheromone, from an extremely complex mixture of insect and plant volatiles.

5. Detection, quantification and structure elucidation

5.1. Conventional detection

The most common conventional GC detection method used in pheromone analysis is FID. Its high sensitivity, linear response over a wide concentration range and near universal detection capability [126] make it ideal for studying the diversity of organic compounds encountered in pheromone research. However, in cases where higher sensitivity/selectivity is required, nitrogen–phosphorous detection (NPD) has been employed. For example, methyl 4-methylpyrrole-2-carboxylate, the trail pheromone of a number of leaf cutting ant species, has been quantified using NPD [127].

A number of studies have used radio-gas chromatography to examine the biosynthesis of pheromones. In a particularly elegant example, Blomquist et al. monitored the incorporation of radio-labelled acetate into (*Z*)-9-tricosene, the female sex pheromone of the housefly *Musca domestica* [128], and used this assay to investigate the function of insect hormones in pheromone production. (*Z*)-9-Tricosene biosynthesis was found to be under the control of ecdysteroids, and independent of juvenile hormone. Radio-detection has also been exploited for the investigation of pheromone catabolism. Tritiated analogues of (11*Z*)- and (11*E*)-11-tetradecenyl acetate, the female sex pheromone of the European corn borer, were applied to the insect antennae in picogram amounts, and their degradation products analysed by radio-GC [129]. Both sexes were shown to convert the acetates to 11-tetradecenoic acid.

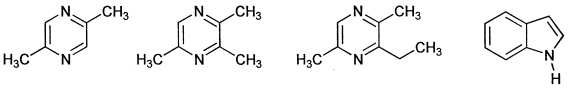
Other common GC detection methods, such as thermal conductivity detection (TCD) and electron capture detection (ECD), are seldom used for pheromone analysis, because of the lower sensitivity of TCD and the requirement for electronegative functional groups in compounds detected by ECD.

5.2. Detection by mass spectrometry

Without doubt the introduction of hyphenated techniques, particularly gas chromatography linked to mass spectrometry (GC–MS), has revolutionised the analysis of pheromones. The mass spectrometer functions as a sensitive detector (typically nanogram to picogram sensitivity, or higher) and, crucially, provides extremely valuable structural information. Thus, combination of the resolving power of capillary gas chromatography with the ability of mass spectrometry to identify pure substances provides a powerful technique for the analysis of mixtures [130]. This is particularly useful for the study of pheromones, which often occur as multi-component blends in complex matrices, such as biological tissue. In addition to providing a spectrum, capable of interpretation, detection by mass spectrometry also gives a useful 'fingerprint' of a compound which can be used in computer-based library searches and for comparison with standard samples. Thus, combined GC–MS can provide a much more reliable identification of a pheromone than either GC or MS alone.

Gas chromatographs can be coupled to most types of mass spectrometers directly, with the capillary column passing through a heated transfer line into the ion source (normally electron impact (EI) or combined electron impact/chemical ionisation (EI/CI)) of the MS. However, because of their relatively low cost, and compact design, quadrupole [131,132] and ion-trap [133,134] mass spectrometers have been most widely used in pheromone analysis. Quadrupole analysers have the advantage of giving highly reproducible 'classical' mass spectra, while ion-trap instruments display higher sensitivity. The use of GC–MS has become so widespread in the detection and structure elucidation of pheromones that it is impossible to list all the studies here. Instead, we have chosen a number of representative examples to highlight the utility of this technique.

Identification of the trail pheromone of the ant *Tetramorium meridionale* illustrates the point clearly. The pheromone, stored in the poison gland, is composed of a mixture of four components: 2,5-dimethylpyrazine (**29**), trimethylpyrazine (**30**), 3-ethyl-2,5-dimethylpyrazine (**31**) and indole (**32**) (Fig. 19) [135]. Although the pyrazines were present in sub-nanogram amounts (10–100 pg per insect), they were unambiguously identified from their mass spectra and GC retention times. The importance of detection and identification of these minor components was demonstrated by the biological testing of synthetic pheromone blends, which showed that all four compounds are required in order to recreate the trail following activity of the poison gland secretion.



29 **30** **31** **32**

Another example of a multi-component pheromone is the oviposition-aggregation pheromone of the desert locust *Schistocerca gregaria*. Its components were found in the foam plug of the locust egg pod and identified as acetophenone (**33**) and veratrole (1,2-dimethoxybenzene, **34**), by GC–MS [136]. In two and four choice bioassays, both components attracted females to oviposit in treated sand. However, unlike the *Tetramorium* trail pheromone, the components do not act synergistically.

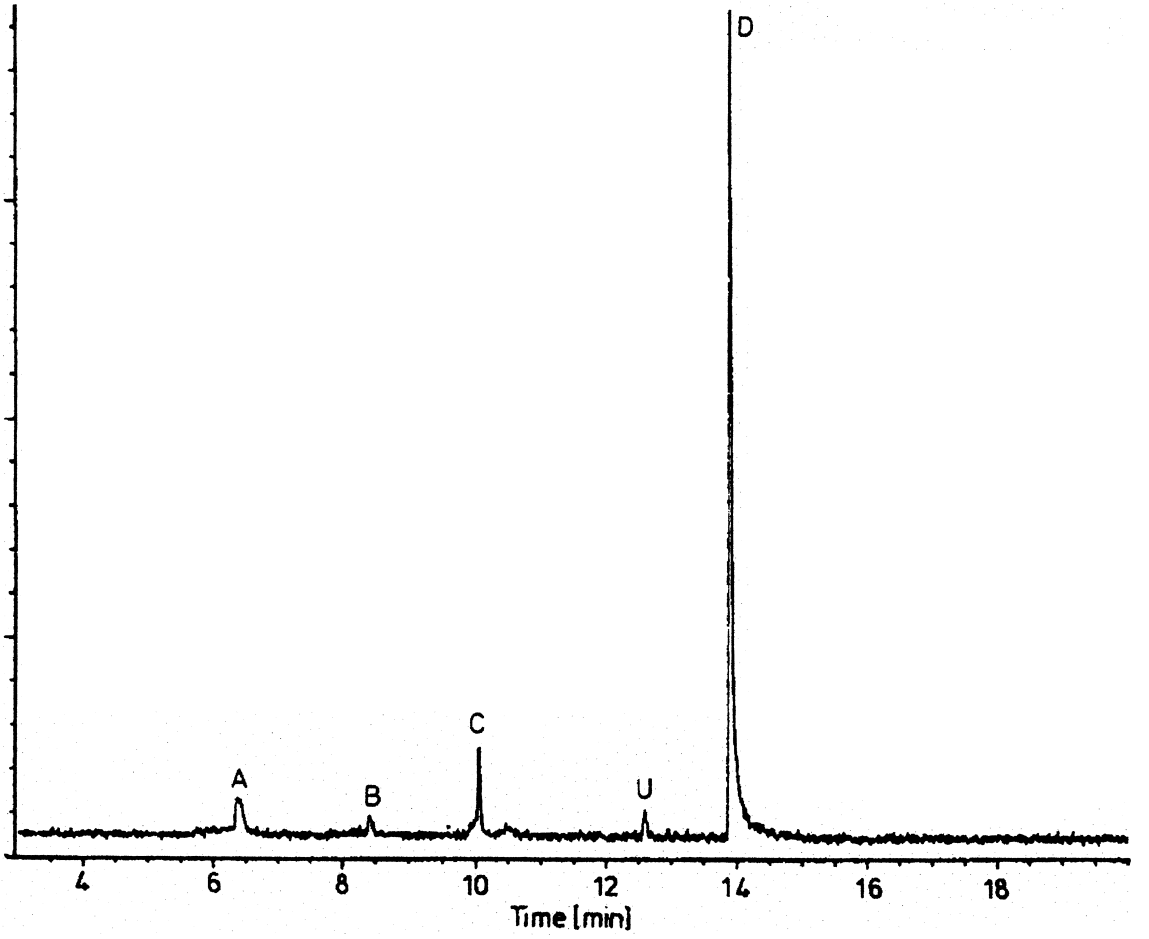
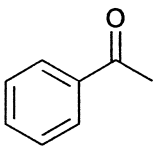


Fig. 19. Total ion chromatogram of the poison gland secretion of the ant *T. meridionale*, showing the components of the trail pheromone. (A) 2,5-Dimethylpyrazine (**29**); (B) trimethylpyrazine (**30**); (C) 3-ethyl-2,5-dimethylpyrazine (**31**); (U) unknown aldehyde; (D) indole (**32**). From Ref. [135] with permission; ©1990 Springer.



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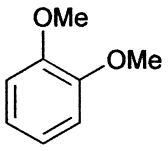

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In a study of the mandibular gland secretion of honey bee queens (*Apis mellifera carnica*), Engels et al. identified over 100 compounds by GC–MS [137], illustrating the power of this technique for examining complex mixtures. In addition to detecting (2*E*)-9-oxo-2-dodecenoic acid, the well-known queen substance, they monitored changes in amounts of structurally related compounds, during the maturation period of reproductive females.

Where higher sensitivity is required, selective ion monitoring (SIM) has been employed to detect pheromones. Here, the MS system is set to monitor only a few, selected ions, prolonging the detection time of these ions and increasing the signal-to-noise ratio. (11*E*,13*E*)-11,13-Tetradecadienal, a major component of the sex pheromone of the Western blackheaded budworm *Acleris gloverana*, was identified in extracts of the female's pheromone gland employing this technique [138].

In addition to *A. gloverana*, many other Lepidop-

teran species employ long-chain fatty acid derivatives as sex pheromones. Whilst their identification is relatively straightforward, mass spectrometry does not always provide information about the double bond position(s) in mono- and polyunsaturated compounds. Methods have been described where complex analysis of the ratios of particular fragment ions, in EI mass spectra, leads to double bond location in monounsaturated aldehydes [139], acetates [140] and alcohols [141]. However, these techniques have not been widely used in pheromone studies, most workers preferring to use chemical microreactions such as dimethylthiolation (see Section 3.5).

An alternative approach for the localisation of double bond position in long-chain alkenes is the use of ion-molecule reactions in the ionisation source of a mass spectrometer. When a mixture of nitrogen, carbon disulfide and methyl vinyl ether is used as a CI reagent gas, the latter reacts with olefins to form a pair of isomeric cyclobutane ions (Fig. 20). Subsequent dissociation leads to, among other species, a pair of substituted vinyl ether ions that are characteristic of the double bond position(s) in the olefin [142]. Recently, this technique has been employed to characterise cuticular alkenes, from a number of social insects, that are believed to form part of a nestmate recognition pheromone [143].

Acetonitrile has been found to function as an efficient CI reagent gas, in ion-trap MS, capable of revealing the position of double bonds in unsaturated hydrocarbons [144]. The reactive ion, $[C_3H_4N]^+$, adds to carbon–carbon double bonds to yield a molecular species that cleaves to give two ions which are indicative of the double bond position. A recent modification has now allowed this technique to be used with a range of functionalised monoenes [145].

Nitric oxide CI has been employed to identify double bond positions in long-chain monounsaturated alcohols, acetates and aldehydes. Compounds of the general formula $CH_3(CH_2)_xCH{=}CH(CH_2)_yR$, where R=$CH_2OH$, CH_2OAc, or CHO, react with NO^+ to give fragment ions of the type $[C_{x+2}H_{2x+3}O]^+$ [146]. NO-CI was applied to the identification of 2,13-octadecadienyl and 13-octadecenyl acetates as constituents of the sex pheromone of *Zeuzera pyrana*. However, NO was not used to determine the double bond positions directly [147]. Instead, the authors relied upon microchemical methods.

Finally, there is a report of the use of *tert.*-butyl cation CI for the direct determination of double bond position in long-chain conjugated dienes [148]. Allylic cleavage about the diene moiety results in ions characteristic of the position of the diene in the chain.

Another aspect of positional isomerism, which often arises in the identification of insect pheromones, is the location of alkyl (normally methyl)

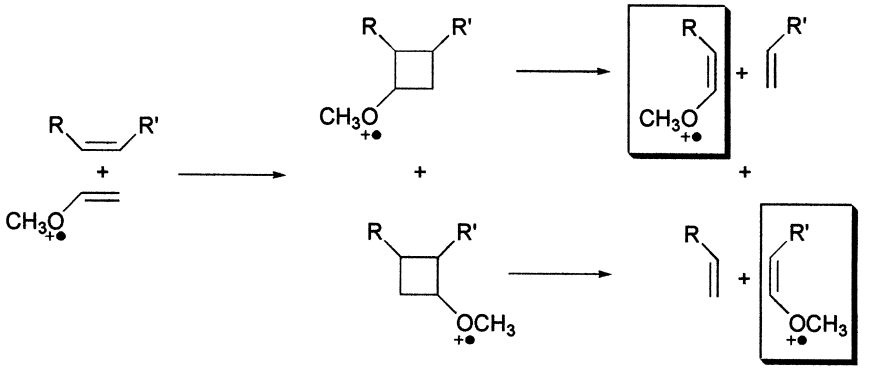
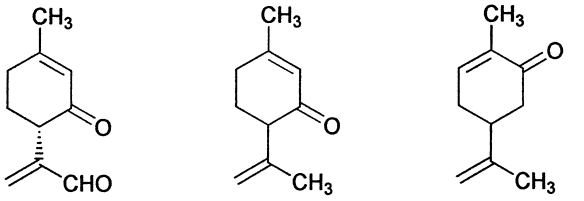


Fig. 20. Ion–molecule reaction between methyl vinyl ether and an olefin, used for the determination of double bond position by CI-MS. Characteristic ions are shown in boxes.

branches in long-chain fatty acid derivatives (normally hydrocarbons). However, here an EI mass spectrum usually provides sufficient information to allow determination of the branching position(s), due to favourable cleavage either side of the alkyl group [149]. Ion-trap mass spectrometers have been shown to be particularly appropriate for this application, since they yield intense ions resulting from cleavage about the branch [150].

Pheromones possessing novel chemical structures often require sophisticated GC–MS techniques to enable elucidation of their structure. Recent identification of the female sex pheromone of the longhorn beetle *Vesperus xatarti* illustrates this point clearly. The pheromone is a new monoterpenoid, 10-oxo-isopiperitenone (**35**), and has been given the trivial name vesperal [151]. GC–CI(NH_3)-MS of the airborne volatiles of *V. xatarti* females revealed a compound giving molecular species at m/z 165 ($[M+H]^+$) and m/z 182 ($[M+NH_4]^+$).



35 **36** **37**

Accurate mass measurement, by high-resolution GC–MS in the EI mode, gave the molecular formula as $C_{10}H_{12}O_2$ and micro-hydrogenation established two of the five double bond equivalents to be carbon–carbon double bonds. Interpretation of the low-resolution mass spectrum led to structure **35**, with the retro Diels–Alder and McLafferty ions being particularly characteristic (Fig. 21). Subsequent fragmentation of the retro Diels–Alder product, at m/z 82, by GC-collision induced dissociation-tandem mass spectrometry (GC–CID-MS–MS) gave a spectrum identical to that of the m/z 82 ion generated from isopiperitenone (**36**), but different from that arising from carvone (**37**). Thus, the relative position of the α,β-unsaturated ketone moiety was established. This structure was confirmed by synthesis, and the absolute configuration of vesperal

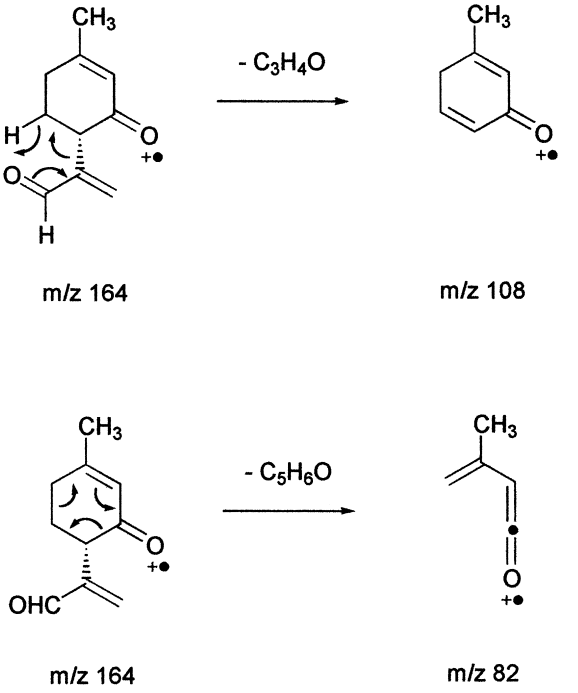
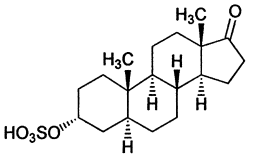


Fig. 21. The McLafferty and retro Diels–Alder rearrangements of vesperal under EI-MS conditions.

was determined to be (*S*)- by comparison of chiral HPLC retention time with that of a synthetic homochiral standard. The total identification was conducted on a crude extract of airborne volatiles containing less than 2 μg of vesperal.

GC–MS may also be used as a powerful method in applications requiring precise, time dependent, quantification of pheromone components. A study of the contents of human axillary (under arm) extracts monitored the temporal variation of three steroids, androsterone sulfate (**38**), dehydroepiandrosterone sulfate and androstenol (**39**), in men and women using GC–MS [152]. These extracts have been shown to have a primer pheromone function, causing shortening and synchronisation of the menstrual cycle [153,154]. Quantification was carried out using an individual calibration curve for each compound. Changes in instrumental conditions, over the analysis period, were corrected by use of an internal standard (androstane).



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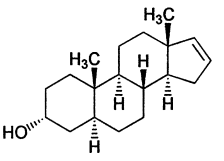

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The investigation revealed that androstenol (**39**) concentrations vary with time and that, in women, the variation is linked to menstruation, with levels of the volatile steroid at a maximum just prior to ovulation.

One further advantage of using MS detection for the quantification of pheromones is that SIM (see above) provides the possibility for selective detection of trace components in the presence of large quantities of other compounds.

In addition to its capabilities for the structure elucidation and quantification of pheromones, GC–MS has also found application in the study of pheromone biosynthesis. Mass spectrometry permits the use of stable isotope (e.g. ^{2}H, ^{13}C, ^{18}O and ^{15}N)-labelled precursors and often removes the need for hazardous radio-labelled compounds. Biosynthesis of the sex pheromone of the cabbage looper moth, *Trichplusia ni*, has been studied using such an approach [155]. The main component of the pheromone, (7*Z*)-7-dodecenyl acetate is produced from oleyl and palmityl coenzyme A (CoA) by Δ11 desaturation, chain shortening by β-oxidation, reduction and finally acetylation of the alcohol [155]. Chain shortening steps were studied using (11*Z*)-11-[13,13,14,14,15,15,16,16,16-$^{2}H_9$]hexadecenoyl CoA (and other labelled acyl CoAs) in an in vitro assay, and the products analysed (as methyl esters) by GC–MS in the selective ion monitoring (SIM) mode. The authors were able to compare pheromone biosynthesis in wild-type *T. ni* with that in a mutant strain, which produces large amounts of (9*Z*)-9-tetradecenyl acetate. They concluded that the mutation prevented more than one round of chain shortening, resulting in production of (9*Z*)-C_{14} acetate rather than (7*Z*)-C_{12} acetate [155] (Fig. 22).

Fatty acid Δ11 desaturation has been examined in

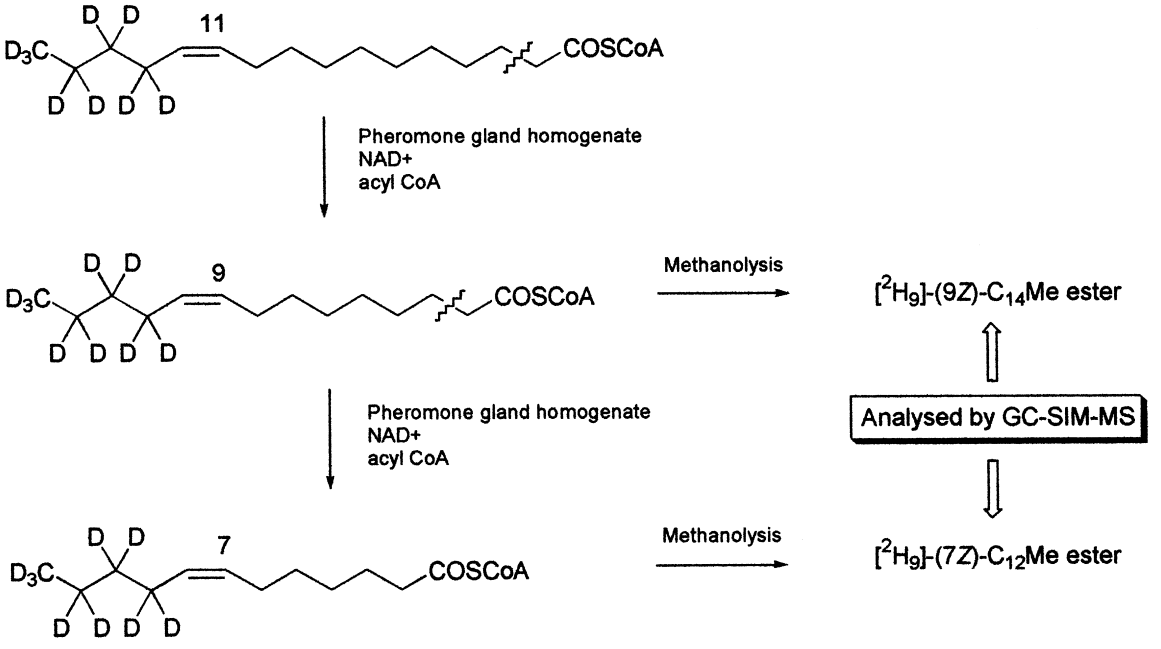


Fig. 22. β-Oxidation chain shortening of (11*Z*)-11-[13,13,14,14,15,15,16,16,16-$^{2}H_9$]hexadecenoyl CoA in normal and mutant strains of *T. ni* in vitro. Analysis of products by GC–SIM-MS revealed the mutant strain to be capable of only one round of β-oxidation.

detail in the cabbage moth, *Memestra brassicae*, using deuterium-labelled fatty acid precursors and GC–MS [156]. The major component of the female sex pheromone in this species, (11*Z*)-11-hexadecenyl acetate, is produced by desaturation of palmitic acid, reduction to (11*Z*)-11-hexadecenol and acetylation. The stereochemistry of Δ11 desaturation has been determined using (11*S*, 12*R*)- and (11*R*, 12*S*)-[2,2,3,4,5,5,6,6,7,8,9,9,11,12-$^{2}H_{14}$]palmitic acid (**40** and **41**). Application of these acids to the pheromone glands of *M. brassicae* and subsequent analysis of the pheromone blend, by GC–MS, revealed significant incorporation into (11*Z*)-11-hexadecenyl acetate. Mass spectrometry showed that desaturation of the (11*R*, 12*S*)-acid (**41**) removed two deuterium atoms, whereas desaturation of the (11*S*, 12*R*)-acid (**40**) resulted in removal of two hydrogen atoms (Fig. 23). Thus, the Δ11 desaturase in *M. brassicae* abstracts the C(11)–H_R and C(12)–H_S atoms from palmitic acid by *syn*-elimination.

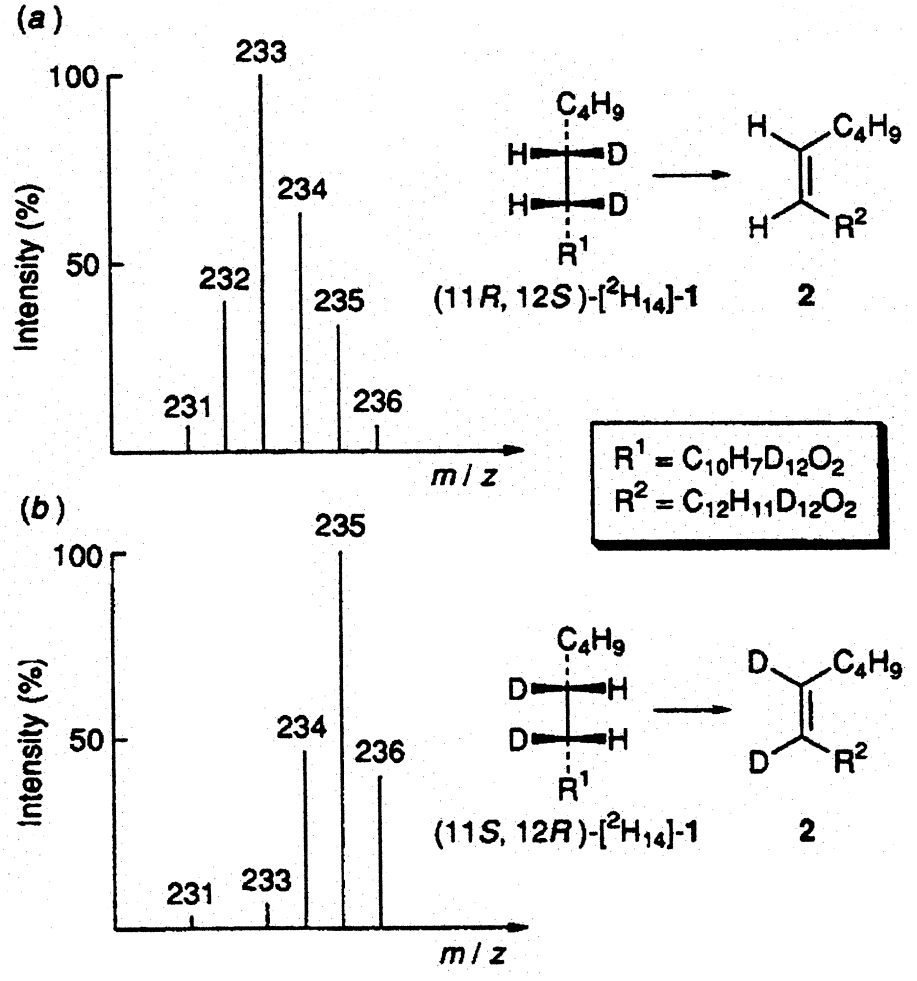
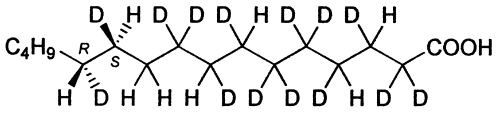


Fig. 23. Relative intensities of the fragment ions ($M^{+\cdot}$–$CH_3COO^{2}H$) in mass spectra of deuterium labelled acetate **2** after administration of (a) (11*R*, 12*S*)-[$^{2}H_{14}$]palmitic acid and (b) (11*S*, 12*R*)-[$^{2}H_{14}$]palmitic acid to pheromone glands of *M. brassicae*. GC–MS results demonstrate *syn*-elimination of C(11)-H_R and C(12)-H_S atoms by the Δ11 desaturase in *M. brassicae*. From Ref. [156] with permission; ©Royal Society of Chemistry 1993.



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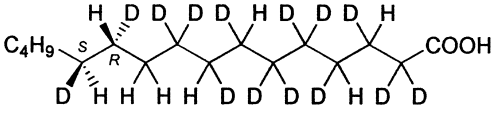

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The sex pheromone of the tobacco hornworm, *Manduca sexta*, includes a mixture of C_{16} monoene, diene and triene aldehydes [157]. Using a number of deuterium-labelled hexadecanoic, and (11*E*)- and (11*Z*)-11-hexadecenoic acids, together with GC–MS, it has been shown that (11*Z*)-11-hexadecenoic acid is the key intermediate in the production of dienes and trienes, through further desaturation and isomerisation (Fig. 24) [158].

Another fatty acid-derived insect pheromone is the queen substance of the honeybee *Apis mellifera*. The pheromone is composed of a mixture of (*2E*)-9-oxo- and (*2E*)-9-hydroxy-2-decenoic acid (**42** and **43**), produced in the mandibular gland of queens [159]. The mandibular glands of worker bees, in contrast, contain large amounts of (2*E*)-10-hydroxy-2-decenoic acid (**44**), which does not possess the pheromonal activity of **42** and **43**. Studies using deuterium-labelled fatty acids and GC–MS suggest that pathways to the 9- and 10-hydroxy acids diverge at a very early stage (stearic acid), and that there is no inter-connection between the pathways after this divergence [160]. Thus, the biochemical distinction between a queen and a worker bee occurs with their ability to metabolise stearic acid.

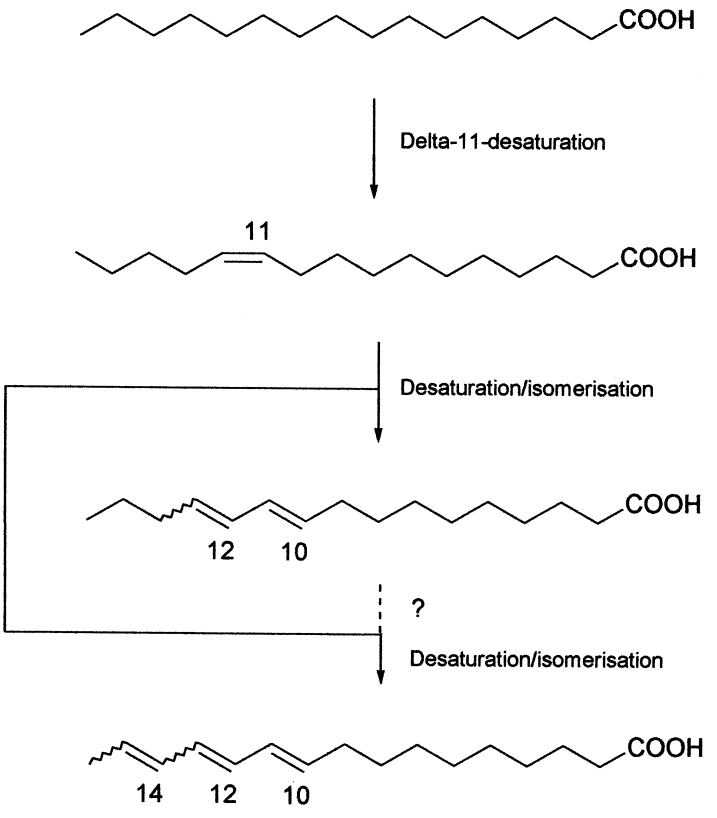
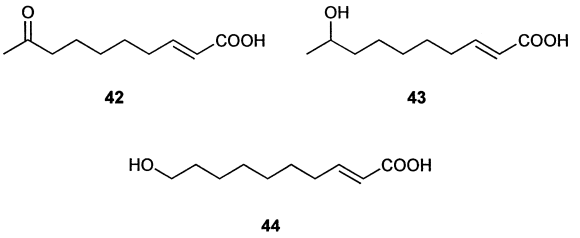


Fig. 24. The biosynthesis of mono-, di- and trienoic acid precursors of the sex pheromone of *M. sexta*, determined by GC–MS. Question mark and broken line indicates a possible conversion.



42 **43**

44

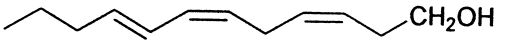
In conclusion, it can be seen from the examples presented here that the coupling of gas chromatography and mass spectrometry is an extremely powerful technique for determining the structure and biosynthetic origin of pheromones.

5.3. Detection by Fourier transform infrared spectroscopy

The combination of gas chromatography and Fourier transform infrared spectroscopy (GC–FT-IR) has, to date, found less application in pheromone analysis than GC–MS. Although, it can provide extremely valuable structural information, and is particularly useful for identifying isomeric configuration. Two main GC–FT-IR coupling techniques exist, the light-pipe [161] and the direct deposition interface [162]. In the former, 'real time' spectra of the GC eluent in the gas phase are measured, whereas in the latter the GC eluent is collected by cryogenic deposition on a moving ZnSe target, and the spectra measured later. Thus, the deposition technique has the advantage of allowing the accumulation of many more scans and is consequently 10–100 times more sensitive than the light-pipe interface. This is illustrated by the FT-IR spectra of 3-hydroxyhexanal from the beetle *Eurycotis floridana* (Fig. 25). The spectra were obtained either by using a light-pipe GC interface (Fig. 25a) or using the direct deposition technique (Fig. 25b). The amounts of hydroxyaldehyde used are 1 μg and 50 ng, respectively. In addition to higher sensitivity, direct deposition also revealed a broad absorption at 3283 cm^{-1}, characteristic of a hydroxyl group in the condensed phase.

A direct deposition interface was also used in the identification of the male sex pheromone of the longhorn beetle *Anaglyptus subfasciatus* [68]. The pheromone was shown to be a mixture of (3*R*)-3-hydroxy-2-hexanone and (3*R*)-3-hydroxy-2-octanone and, as with 3-hydroxyhexanal (see above), the condensed phase IR spectra were important in demonstrating the presence of hydroxyl groups.

An extremely useful application of GC–FT-IR is the identification of double bond geometry in alkenes. The alarm pheromone of the astigmatid mite *Tortonia* sp. was identified as a mixture of (4*Z*, 8*Z*)-4,8-heptadecadiene and (6*Z*, 9*Z*)-6,9-heptadecadiene using a combination of GC–MS, GC–FT-IR, microreactions and synthesis [163]. The main contribution of GC–FT-IR was in the assignment of double bond geometry, based on the absence of an absorption band at 970 cm^{-1} (found only in *E* double bonds).



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In the identification of the trail pheromone of *Reticulitermes speratus*, a termite species, GC–FT-IR analysis was performed on a partially hydrogenated sample of the pheromone acetate derivative to establish the geometry of the three double bonds [164]. Of the three possible diene products, two showed absorptions at 970 cm^{-1}, a result consistent with the presence of only one *E* double bond in the triene precursor. Additional absorptions at 949 and 976 cm^{-1}, in one of the dienes, showed that the *E* double bond, and one of the *Z* double bonds, were in conjugation. With the aid of microreactions and mass spectrometry, the structure of the pheromone was determined to be (3*Z*, 6*Z*, 8*E*)-3,6,8-dodecatrien-1-ol (**45**).

GC–FT-IR may also be used to identify functional groups present in pheromones. Isopropyl myristate is the most abundant compound in the mandibular gland of males of the beewolf, *Philanthus crabroniformis*, where it forms part of the territorial marking pheromone [165]. Absorptions at 1747 cm^{-1} (C=O) and 1113 cm^{-1} (C–O–C) were used to identify the ester function, and a computer search of the FT-IR library yielded a 99% match with isopropyl myristate. In addition, GC–FT-IR has been employed to show the absence of functionality. The male-produced aphrodisiac sex pheromone of *Acrolepiopsis assectella* (Lepidoptera) induces females

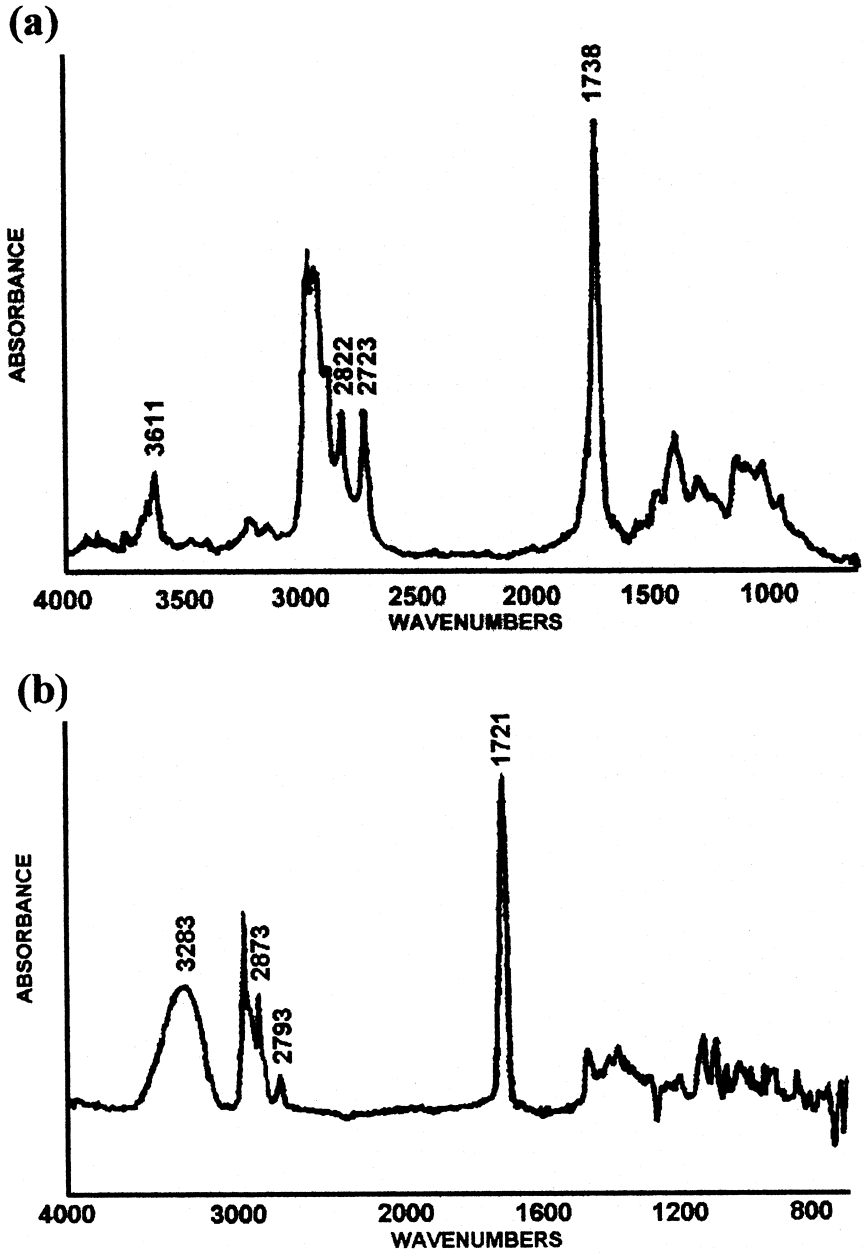


Fig. 25. Fourier transform infrared spectra of 3-hydroxyhexanal, obtained by GC–FT-IR using (a) light-pipe interface with 1 μg of hydroxyaldehyde and (b) direct deposition interface with 50 ng of hydroxyaldehyde. From Ref. [161] with permission; ©EDP Sciences 1996.

to adopt the acceptance posture, and has been identified as a mixture of homologous *n*-alkanes (C_{16}–C_{21}) by GC–MS and GC–FT-IR [166]. The IR spectra showed absorption bands in the 2900 cm^{-1} region only, corresponding to C–H stretching of the hydrocarbon chain.

Thus, the combination of GC–MS and GC–FT-IR provides a great deal of structural information for the chemical identification of pheromones.

5.4. Detection by electroantennogram recording

Electroantennography (EAG) is a neurophysiological technique that allows an insect's perception of a semiochemical to be monitored. A pair of electrodes are connected to the base and tip of an insect antenna and air, containing a pheromone, is passed over the preparation. Depolarisation across the antenna generates a potential difference in the electrodes, which may be amplified and recorded [167]. The technique was first coupled to a gas chromatograph by Moorehouse et al. [168], but it was Arn et al. [169] who developed direct coupling using capillary GC and coined the term electroantennographic detection (EAD). In the basic experimental set-up (Fig. 26), the effluent from the GC column is diluted with N_2 make-up gas and split between EAD and FID systems, providing simultaneous specific (EAD) and total (FID) detection of eluting compounds. That portion of the effluent used for EAD is transported out of the GC oven by a heated transfer line into a flow of moist air, at constant temperature, and passed over the insect antennal preparation. The amplified signal is then synchronised with the FID output and sent to a recording device [169,170].

GC–EAD–FID has been widely used in pheromone research, since it allows identification of biologically active peaks in complex chromatograms, although it does not indicate what form the activity takes (sex attractant, alarm pheromone etc.), this requires behavioural bioassays.

EAG activity of a number of male-produced volatiles was demonstrated in females of the Mediterranean fruit fly *Ceratitis capitata* using the GC–EAD–FID technique [13]. Fig. 27 shows simultaneous FID and EAD (female antenna) outputs from GC analysis of the headspace of calling males. Of the many compounds detected by FID, only five components (I–V, Fig. 27) were found to possess EAG activity. They were identified, by GC–MS, as linalool (**46**, both enantiomers being equally active), (3*E*)-3-ethyl octanoate (**47**), indole (**32**), geranyl

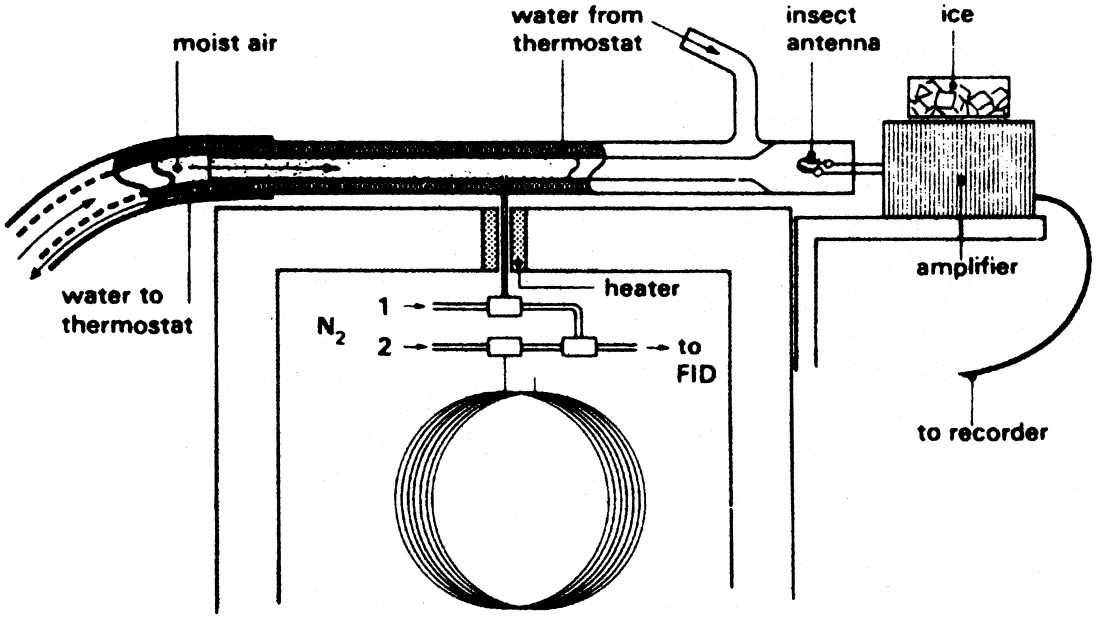


Fig. 26. Experimental set-up for GC with electroantennographic detection (EAD). The column effluent is split between the FID and EAD systems to provide simultaneous detection. From Ref. [170] with permission; ©Springer 1984.

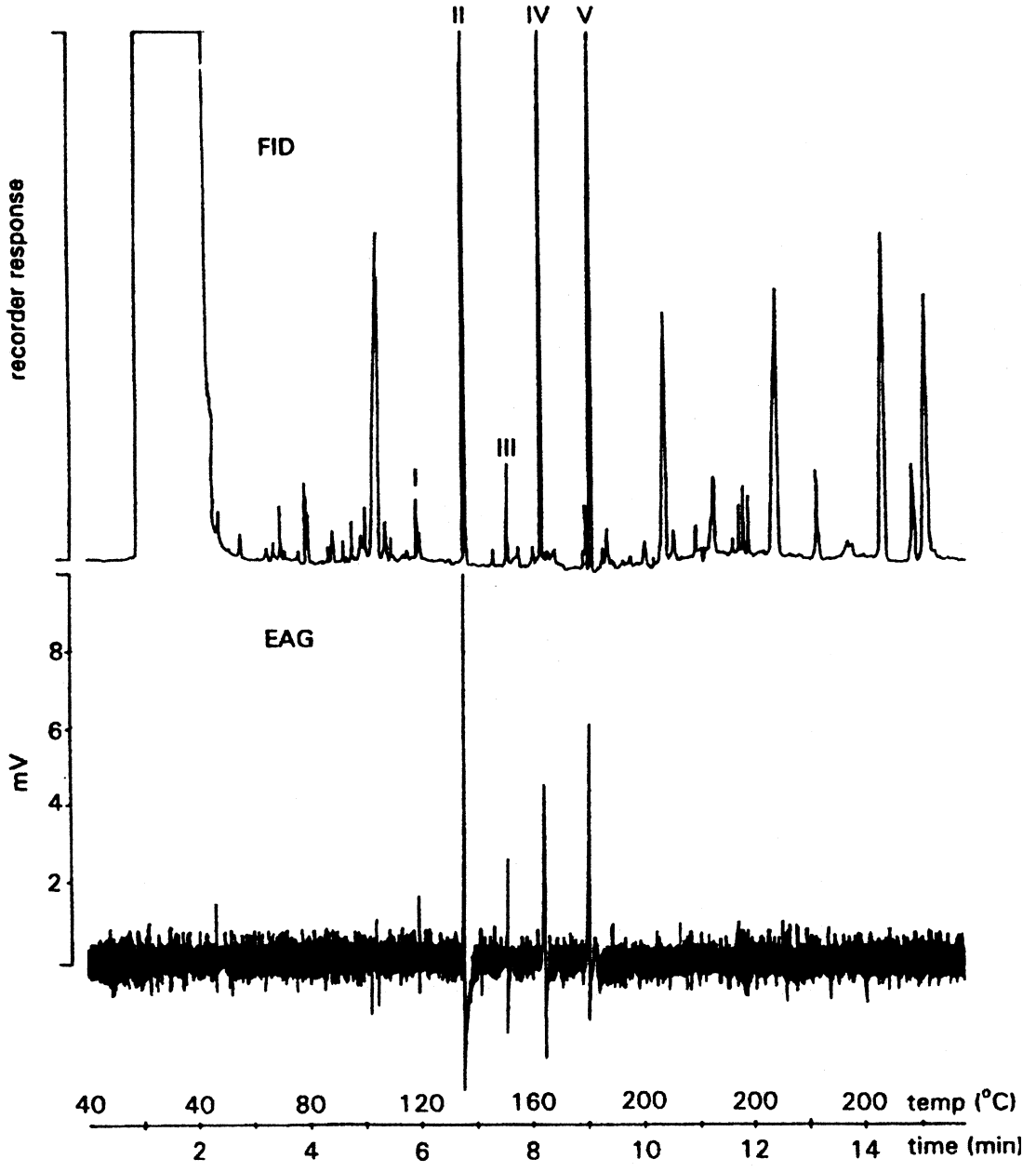
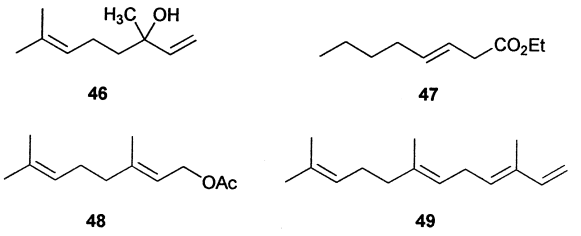


Fig. 27. Gas chromatogram of volatiles from males of *C. capitata* using simultaneous FID (upper trace) and EAD (lower trace) detection with a female antenna. Active components I–V are linalool (**46**), (3*E*)-3-ethyl octanoate (**47**), indole (**32**), geranyl acetate (**48**) and (*E*,*E*)-α-farnesene (**49**). From Ref. [13] with permission; ©Plenum Press 1995.

acetate (**48**) and (*E*,*E*)-α-farnesene (**49**), respectively. With the exception of indole, the above compounds have been tested previously and shown to attract female fruit flies in behavioural bioassays [13,171]. Thus, the use of GC–EAD identified indole as another potential pheromone component.



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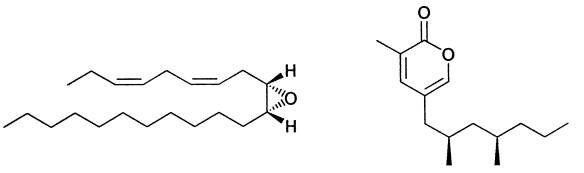
An investigation of the female sex pheromone of the evolutionary primitive moth *Eriocrania cicatricella*, using GC–EAD–FID, revealed three electrophysiologically active peaks in the chromatogram of female produced volatiles [172]. The compounds were identified as (4*Z*)-4-hepten-2-one, (2*R*)-heptan-2-ol and (2*R*,4*Z*)-4-hepten-2-ol by GC–MS and synthesis. However, in field tests, only the unsaturated alcohol proved to be attractive, and no definite behavioural activity could be attributed to the saturated alcohol or ketone. Thus, EAG activity is no guarantee of pheromonal function. In fact, in two closely related moth species, *E. semipurpurella* and *E. sangii*, that displayed EAD responses to nonan-2-one, (6*Z*)-6-nonen-2-one and (6*Z*)-6-nonen-2-ol, once more only the alcohol was attractive and (6*Z*)-6-nonen-2-one was found to totally inhibit attraction [173].

In a recent study, GC–EAD–FID has been used to demonstrate the existence of a common pheromone in two species of Arctid moths [174]. The scarlet tiger moth, *Callimorpha dominula*, and the Cinnabar moth, *Tyria jacobaeae*, have been observed taking part in interspecific mating and it was postulated that a common sex pheromone was responsible for this behaviour. GC–EAD–FID analysis of the pheromone gland extracts, from females of *C. dominula* and *T. jacobaeae*, showed a single peak that elicited a major EAG response in the antennae of males of both species. The common pheromone was subsequently identified as (3*Z*,6*Z*,9*S*,10*R*)-9,10-epoxyheneicosa-3,6-diene (**50**) by GC–MS and synthesis.

Chiral column GC–EAD–FID has been employed in a number of investigations to determine the enantiospecificity of pheromone reception. The aggregation pheromone of the palmetto weevil, *Rhynchophorus cruentatus*, was identified as (4*S*,5*S*)-5-methyloctan-4-ol using GC–MS and by comparison of the natural alcohol's retention time with those of the four synthetic isomers, on a Cyclodex-B chiral GC column [175]. GC–EAD analysis of the isomeric mixture, on the same phase, demonstrated that females of *R. cruentatus* only respond to the (4*S*,5*S*)- isomer (Fig. 28), and trap-catch experiments conducted in the field confirmed this as the only attractive isomer.



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Leal et al. examined the stereochemistry of the female sex pheromone of *Supella longipalpa*, the brownbanded cockroach, in detail using chiral GC–EAD–FID [176]. Using a modified amplifier, that

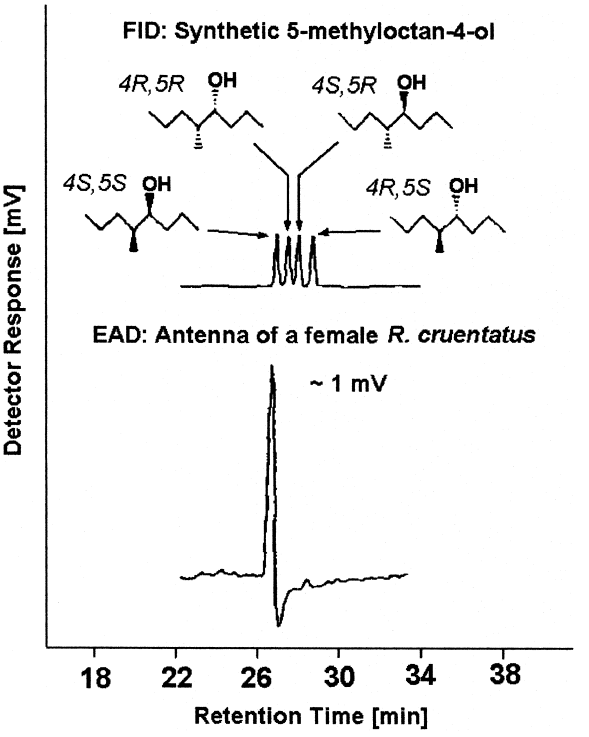


Fig. 28. Simultaneous FID and (female) EAD responses to the four isomers of 5-methyloctan-4-ol analysed by chiral GC. From Ref. [175] with permission; ©Plenum Press 1994.

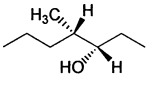
corrects for the relatively broad peaks obtained from a chiral column, they were able to identify the pheromone as 5-(2′*R*,4′*R*)-(dimethylheptanyl)-3-methylpyran-2-one (**51**).

5.5. Detection by single cell recording

Although GC–EAD is a powerful tool in pheromone research, a much more detailed understanding of the perception of odour molecules, by insects, can be obtained by recording the response of individual olfactory cells [177]. Insects possess receptor cells specialised for different pheromones, and different pheromone components. Thus, by measuring the output of single cells, upon treatment with a pheromone or pheromone component, it is possible to monitor the insect's response selectively. When coupled to gas chromatography, single cell recording provides a highly specific method for assigning neurophysiological activity to pheromone components, and is termed GC–SCR [178]. The basic experimental design is similar to that used for GC–EAD (Fig. 26). The GC column effluent is split between the SCR device and a FID system, with nitrogen as a back-up gas. The flow is then passed into a stream of moist air over the antennal preparation. However, the silver electrodes used in EAD are replaced by a pair of tungsten microelectrodes. The indifferent electrode is normally positioned in the mouthparts of the insect, and the recording electrode is placed into the base of individual antennal sensilla, using micromanipulators. The output of the cell(s) is then monitored by an oscilloscope, a chart recorder/plotter and a loudspeaker. In the absence of stimulation the cell(s) fires fairly regularly, giving rise to peaks on the oscilloscope and 'clicks' from the loudspeaker. However, when stimulated by a pheromone component, the impulse frequency of the cell increases dramatically, as detected by the oscilloscope and loudspeaker. For quantitative treatment, the cell impulses may be summed over 3-s intervals (resulting in a peak that can be integrated) and plotted against the FID response [177]. If the output from more than one cell in the sensillum is detected, the signals are often separable, since they tend to generate impulses of different amplitudes.

In an investigation of the sex pheromone of the alfalfa pest *Tephrina arenacearia*, Tòth et al. identified sensilla containing olfactory cells specific for (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, a component of the female sex pheromone of this species [179]. Fig. 29A shows a chromatogram from analysis of the female pheromone gland (peak ★ represents (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene and peak ☆ tricosane), and Fig. 29B shows the response of a male sensilla to these compounds. Initially, the trace is quiet, but as the epoxide elutes at 9.84 min (marked with ★) a cell fires rapidly, and continues to emit impulses for almost 1 min. Then, at 10.76 min (☆) another cell (with a smaller amplitude) fires in response to tricosane. Subsequent field experiments, with synthetic (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, resulted in substantial trap catches of *T. arenacearia* males, confirming the role of the epoxide in sexual attraction.

GC–SCR can also be used to determine the proportions of various olfactory cells on an insect's antenna. Sensilla are selected at random and their response to a number of pheromone components is examined. Such a study has been conducted on the elm bark beetle *Scolytus scolytus*, where 545 cells were tested with components of the aggregation pheromone [180]. Of the total number of antennal olfactory cells, 18.9% responded to (3*S*,4*S*)-4-methyl-3-heptanol (**52**), 24.2% responded to (3*R*,4*S*)-4-methyl-3-heptanol (**53**), 9.9% to (−)-α-multistriatin (**54**) and 5.3% to 4-methyl-3-heptanone. A further 20.7% showed activity towards host plant-derived volatiles, 6.1% were identified as mechanoreceptors and 14.8% were uncharacterised. Thus, over half of the antennal olfactory cells of *S. scolytus* are tuned to detect components of the aggregation pheromone.



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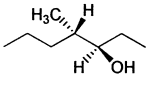

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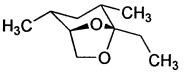

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6. Concluding remarks

In this review we have addressed the major techniques currently employed in the gas chromatographic analysis of pheromones. Since the field is such a large one, we have been limited to rather brief

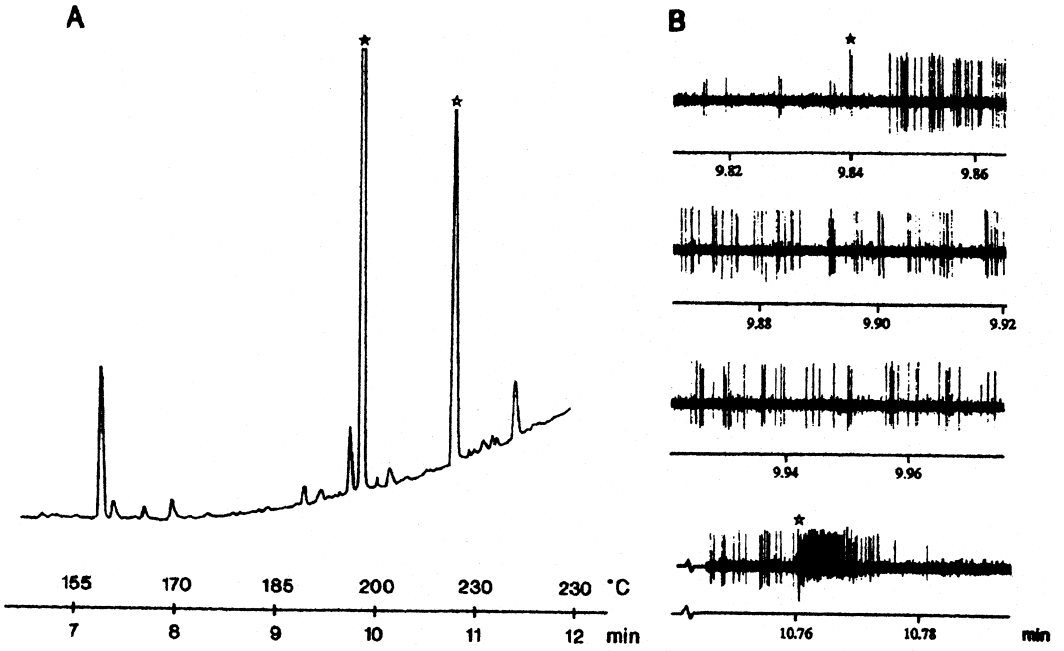

Fig. 29. (A) GC–FID chromatogram from analysis of a female *Tephrina arenacearia* pheromone gland extract (★ (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, ☆ tricosane) and (B) SCR response from a male olfactory sensillum to the two compounds (★ onset of response to (6*Z*,9*Z*)-6,9-*cis*-3,4-epoxyheptadecadiene, and ☆ onset of response to tricosane). From Ref. [179] with permission; ©Verlag der Zeitschrift für Naturforschung 1991.

descriptions of the methods employed, and have illustrated them with a relatively small number of examples. However, we hope this article demonstrates the continuing importance of capillary gas chromatography in the study of pheromone chemistry, particularly when coupled with spectroscopic techniques, such as MS, and electrophysiological methods.

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