MICRO-CHEMICAL INVESTIGATIONS OF VOLATILE CHEMICALS OF SOME MYRMICINE ANTS

BY

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"*Anything man can do ants can do better*"

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ABSTRACT

The volatile chemicals originating from the poison glands, Dufour glands, mandibular glands and postpharyngeal glands of several species of myrmicine ants have been investigated. A number of novel micro-methods were developed and several advances made in existing methods for handling and identification of small quantities of natural products, including GC trapping, ozonolysis, epoxidation and cleavage of epoxides, borohydride reduction, and formation of chiral derivatives.

The trail pheromone of workers of the ant *Tetramorium caespitum* L. is a 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine with an average total amount of 3.9 ng per worker or 0.03% of the volume of the poison gland (2.7 ± 0.4 ng of 2,5-dimethylpyrazine and 1.15 ± 0.25 ng of 3-ethyl-2,5-dimethylpyrazine). A 70:30 mixture of the respective synthetic pyrazines evoked the highest activity in artificial trail following tests.

The postpharyngeal gland of *Solenopsis geminata* contains about 10 µg of hydrocarbons per ant. Three components, heneicosane, (Z)-9-tricosene and tricosane represent 85% of the volatile components of the gland. The poison vesicle contains about 19 µg of alkaloids per ant and

the major components are *cis*- and *trans*-2-methyl-6-undecylpiperidines. The Dufour gland is filled with a variety of hydrocarbons of C_{15}-C_{23} range. The Dufour gland also contains its trail pheromone which appears to be a sesquiterpenoid hydrocarbon derivative.

The Dufour glands of *Myrmica sulcinodis*, *M. rugulosa* and *M. schencki* contain species-specific mixtures of linear alkanes and alkenes of C_{13} to C_{19} range and terpenoid hydrocarbons, namely (Z-E)-α-farnesene, homofarnesene and bishomofarnesene. The structures of the two latter compounds were confirmed as 7-ethyl-3,11-dimethyldodeca-1,3,6,10-tetraene and 7-ethyl-3,11-dimethyltrideca-1,3,6,10-tetraene by micro-degradation. The Dufour and mandibular gland contents of a further species, here called *M. albuferensis*, was found to be different from all the other eight, chemically examined species. By its glandular substances, it most closely resembles *M. scabrinodis*.

The 3-octanol from the mandibular glands of *Myrmica* ants consists essentially of the (R)-enantiomer and in at least one species where this substance is a pheromone, this enantiomer is ethologically active, while the antipode is inactive.

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INTRODUCTION

1.1 Pheromones

Odours, sight, sound, tastes and touch enable individual animals to communicate with the environment for their sustenance and survival. The insects rely on the sense of smell as the major channel for exchange of information more than any other group of animals. As early as 18th century, Rene de Reaumur[1] observed that virgin females of the silkworm moth *Bombyx mori* could lure males of that species from a distance of one kilometre. In 1879, the great naturalist Jean Henri Fabre[2] demonstrated that male emperor moths are attracted to a caged virgin female moth, and even to an empty cage that had contained a female. Although olfactory communication seems obvious in the above examples, no significant progress had been made in its study until the late 1950s. In 1959, pioneer work of Butenandt *et al.*[3], led to the isolation and identification of a substance called Bombykol [1] from female silkworm moths (*B. mori*), that elicited the sexual excitation response from male moths.

bombykol [1]

In fact, even before Butenandt, an American group had worked nearly 30 years to identify such an attractant from *Porthetria dispar* but unfortunately the structure published in 1960 was incorrect[4]. The correct structure appeared in 1970[5].

The term "pheromone" (from the Greek *pherein*, to transfer, and *horman*, to excite or stimulate) was coined by Karlson, Luscher and Butenandt[6,7] to represent this class of substances. With incorporation of some minor modification as proposed by Kalmus[8], pheromones can be defined as "substances secreted to the exterior by an organism which cause one or more specific reactions, such as, a definite behaviour or developmental process in a receiving organism of the same species." The pheromones are employed only for intraspecific transmission of information, however olfactory communication is much broader than that.

According to the most widely accepted terminology, chemicals which convey information between organisms are termed *semiochemicals*[9]. Those semiochemicals used for interspecific communication are called *allelochemics*[10]. Three main types of allelochemics are recognized, *kairomones*[11], *allomones*[11] and *synomones*[12]. An allomone is a chemical emitted from an insect, which gives adaptive advantage to the odour-releasing individual (*e.g.* defensive and repellent secretions). In contrast, a kairomone gives the adaptive advantage to the receiver (*e.g.* substances that enable the predators to locate its prey. The trails of the army ant, *Neivamyrmex nigrescens* are picked up by predators like the blind snake *Leptotyphlops dulcis*[13] and the beetle *Hellumorphoides texanus*[14], which feed mainly on the brood of the ants). A synomone benefits both the producer and recipient (*e.g.* floral scents that attract pollinating insects). Pheromones are only one type of semiochemical and used to transmit information only between members of the same species.

Pheromones can be classified according to the responses they elicit on the recipient. Chemical stimuli that trigger an immediate,

reversible and specific change in the behaviour of the recipient are called *releasers*. Those pheromones that induce long term, irreversible, morphological and physiological changes are referred to as *primers*. Perhaps the most familiar and most dramatic releaser pheromones are the sex attractants, trail pheromones and aggregation pheromones. However, no less dramatic are the primer pheromones that bring about remarkable physiological and morphological changes *e.g.* transformation of solitary to gregarious phases of locusts or inhibition of ovary development in worker honeybees by the "Queen substance"[15].

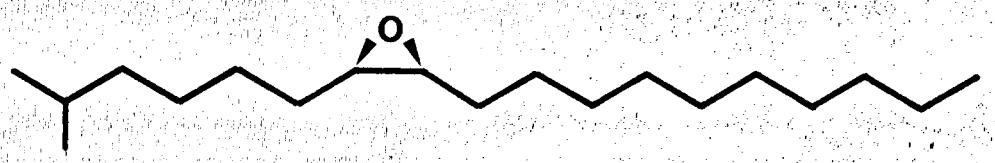
The major categories of semiochemicals are not mutually exclusive. A chemical or a mixture may act simultaneously as a pheromone, kairomone and allomone. A good example for such a pheromone is exhibited by the bark beetle *Ips paraconfusus*[16]. Its sex pheromones emitted by an actively boring male to attract conspecifics, also serve either as a kairomone to attract predators that prey on *Ips* or as an allomone to repel competitors from utilizing the same resource.

1.2 Characteristics of Pheromones

Higher animals rely mostly on hearing and sight, for communication while lower animals, including insects, use pheromones. Although chemical communication is the most primitive (it probably allowed the most primitive single-celled organisms to locate each other for the exchange of genetic material), it is very widespread and occurs througout the animal kingdom. At least for the three-quarters of a million or so species of insects it is the major method of communication. The major characteristics of pheromonal communication are listed below.

i. Pheromones are effective in darkness and they circumvent obstacles.

ii. Only a very minute quantity is required to initiate a biological response. For instance, fewer than a hundred sex pheromone molecules on the antenna of a male moth may be sufficient to stimulate a behavioural response. The mean rate of release of disparlure [**2**] the sex attractant of female gypsy moth (*Lymantria dispar*), is about 7-8 ng/h[17]. The lowest concentration of its trail pheromone detected by the fire ant *Solenopsis richteri* is reported to be as low as 10 fg/cm of the trail[18].



disparlure [2]

iii. The effective distance of activity of the pheromone may usually be 50-100 m for moths. But this depends on many factors such as the rate of pheromone release, properties of substratum etc.

iv. Pheromones demonstrate a high species specificity, thus most species may have their own chemical signals. This is realised because, usually pheromones are mixture of several components, precise to the blends of correct geometrical and optical isomers[19]. Table 1, shows an example, where two compounds in different ratios, provide a species-specific sex pheromone for three species of Lepidoptera of the same genus[20].

v. The pheromones show a very strong structure-activity relationship. A small change in structure may eliminate the activity and may even cause inhibition. For instance the activity of disparlure [2] decreases more than 1000 times when the position of the methyl group or oxirane ring is changed[21].

Table 1. Variation of Mixture Composition of the Same Components in Sex Pheromones of Three Species of *Pectinophora*

Species	Components	
	(Z,Z)-7,11-C_{16}OAc	(Z,E)-7,11-C_{16}OAc
Pectinophora gossypiella	1	1
P. endema	1	0.5
P. scutigera	1	0.1

vi. For some species, their mere survival depends on sex pheromones as their populations are so low the males and females can scarcely meet without the aid of sex pheromones.

vii. The substances employed in pheromonal communication may be either biosynthetic products derived from acetate, fatty acids, terpenes, amino acids etc, or compounds derived from the food or surroundings.

1.3 Stereobiology of Insect Pheromones

1.3.1 Multicomponent pheromones

The pheromonal transmission of information in insects is now accepted to be through multicomponent pheromones, consisting of several stimulus compounds[19]. Since the first isolation of bombykol by Butenandt *et al.*[3] and throughout the 1960s, each pheromone was considered to consist only of a single component with the exception of beetle (Coleoptera) pheromones, which were considered more complex. In 1964 Wright[22] suggested that a multicomponent pheromone could convey more information since evolutionary selectivity would favour an organism that had the better communication system, however his proposal was generally ignored till 1970s. In 1971, Silverstein[23] influenced by findings of multicomponent pheromonesin several beetle species and by the repeated failures of field tests based on single compounds, recognized the importance of Wright's suggestion.

The word "pheromone" is now being used to indicate the mixture of compounds that causes a particular behavioural or developmental process. The active constituents are termed the pheromone components. Renwick and Vite[24] consider that the wide usage of the word "pheromone" to indicate a mixture is unfortunate, because future studies may reveal that each component is a pheromone in its own right. Each being responsible for a specific behavioural response in a complex communication system.

A multicomponent pheromone may consist of a mixture of positional isomers, functional group isomers, geometrical isomers, optical isomers or structurally similar nonisomers. In the biosynthesis of pheromones, the systems have become so finely tuned through evolution, the pheromonal components are synthesized not only to a high degree of chemical purity but also the geometrical and optical isomerism of the molecules are precisely controlled. The precise qualitative and quantitative blend of the components, ascribe the species-specificity to the pheromonal signal.

The pheromone perception is deciphered by the transformation of a molecular message, into a bioelectric response at the antennal level. This process is known as *transduction*. The pheromones are perceived via the cuticular hairs, called olfactory sensilla on the antenna, which support the sensory dendrites that ultimately transmit impulses to the central nervous system. The pheromone molecules encounter, and become bound to, the proteinaceous acceptor molecules on the membrane of the receptor dendrite, which triggers an electric impulse known as an *acceptor potential*. An acceptor molecule can be so highly tuned that it is activated by a single compound only. The rate of impulses produced depends on the number of stimulating molecules. Therefore, a certain mixture of pheromone components produces a characteristic ratio of impulses among the different receptor cell types. Only the impulse pattern induced by the species-specific pheromone complex leads to a release of a behavioural response.

The action potentials generated in a single sensillum, which travel down the neuron axon to the brain, can be monitored by means of microcapillary electrodes (*single-cell recording*)[25,26]. A recording of the summated responses of the antennal receptors to a stimulus is called an *electroantennogram* (EAG)[27]. These electrophysiological methods have clearly demonstrated the relationship between the activity and the chemical structure of a pheromone component. For instance a single-cell recording of a specialized sensillum of *Bombyx mori* shows a 1000 times less response to a "wrong" geometrical isomer than bombykol [1][17].

The pheromone molecules, after they have reacted with the acceptors, are rapidly degraded by antennal enzymes and the receptor neurons quickly regain their acceptor capabilities. For instance, an enzyme has been observed in the antenna of males and females of *Trichoplusia ni*, capable of hydrolysing their pheromone, (Z)-7-dodecenyl acetate[28].

1.3.2 Chirality in insect pheromones

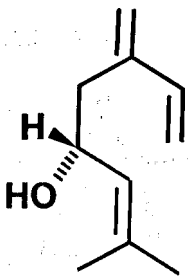
Many insect pheromones contain one or more chiral centres. A number of behavioural experiments have shown that the "odour" receptors in insects can often distinguish between optical isomers[29]. The insects often will biosynthesize, and utilize as a pheromone, only one optical isomer or a specific blend of optical isomers. Silverstein[19] has stated that a description of a racemate as a single compound would be valid only in a world devoid of chirality. Therefore a complete identification of a chiral pheromone should include a statement of enantiomeric composition and a determination of the absolute configuration of the chiral centre(s).

A number of possibilities in behavioural responses towards the optical isomers are conceivable and the main categories are given below.

i. The insect produces only a single optical isomer and it is more active than the other(s). The leaf-cutting ants, *Atta texana* and *A. cephalotes* responded to a lower concentration of the naturally occurring alarm pheromone, (S)-(+)-4-methyl-3-heptanone, than its enantiomer[29]. Another ant, *Pogonomyrmex barbatus*, which also uses 4-methyl-3-heptanone as an alarm pheromone[30], responded to the (S)-enantiomer up to 10 times more than the (R)-enantiomer[31].

ii. The insect produces only a single optical isomer and the insects response to it is inhibited by the other(s). The aggregation pheromone of the bark beetle *Ips pini* (western strain from California and Idaho) is (R)-(-)-ipsdienol [3] only, and the synthetic racemic mixture failed to elicit any positive response[32]. The beetles responded positively to (R)-(-)-ipsdienol in the laboratory, whereas (S)-(+)-ipsdienol interrupted the response to (R)-(-)-ipsdienol[33]. Under field conditions, males of gypsy moth, *Lymantria dispar*, respond

significantly to (7R,8S)-(+)-disparlure [2], only in the absence of the (-)-enantiomer[34].

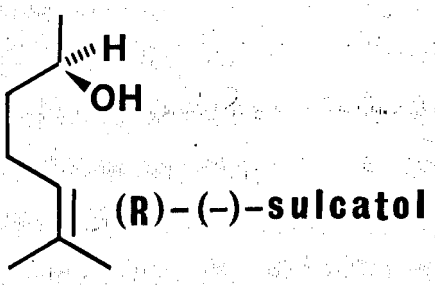


(R)-(-)-ipsdienol

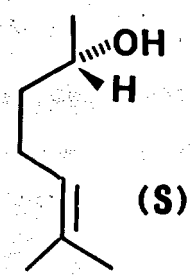
[3]

iii. The insect produces only a single optical isomer but cannot distinguish between it and the other(s). The males of nun moth *Lymantria monacha*, responded equally well to (+)-disparlure [2] as to the recemic mixture[35].

iv. The insect produces two or more optical isomers and responds optimally to the naturally produced ratio. The males of ambrosia beetle, *Gnathotricus sulcatus*, produce an aggregation pheromone, sulcatol [4] as a 65 : 35 mixture of (S)- and (R)- enantiomers. Moreover, they respond synergistically to the enantiomeric mixture but neither enantiomer by itself is attractive[36].



(R)-(-)-sulcatol

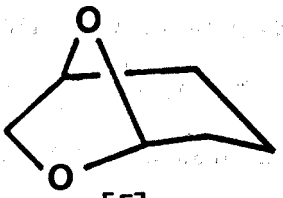


(S)-(+)-sulcatol

[4]

v. The insect produces two or more optical isomers, but responds preferentially to one than to the other(s). The female southern pine beetles, *Dendroctonus frontalis*, emit frontalin [5] as a mixture of 85 : 15 (-)-and (+)- enantiomers. In the laboratory the males showed a higher response to (-)- frontalin than to the (+)- enantiomer, but the latter caused no inhibition[37].

vi. The insect produces two or more optical isomers and responds equally to each optical isomer and to mixtures in all ratios. No examples are yet reported in this category.



frontalin [5]

The examples given above, are only an important few, from a number of chiral pheromones described in recent literature, but they exemplify the need for determining the optical isomeric composition and absolute configuration of chiral centre(s) of chiral pheromones.

1.3.3 Pest control with pheromones

The chemical communication system of pheromones is comparable with any other communication system which uses different symbols or signs for the transmission of information. The combination of chemicals to make complex statements, allows pheromonal communication to be comparable to a language. An understanding of such a language may not only help in taxonomy and chemosystematics, but also show a way how to disrupt the communication code which will eventually be useful in integrated pest control.

1.4 Types of Insect Pheromones

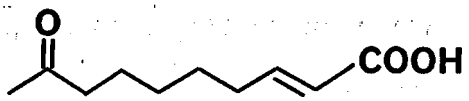
Insect pheromones can be classified according to the behavioural responses they evoke on the recipient. One of the weaknesses of this classification is that the insects use pheromones to mediate a wide variety of behaviour. The main pheromone-mediated functions in insects are orientation of the individuals towards or away from a location, signaling of danger and exchange of information for social interactions. The major categories of insect pheromones are discussed below with special emphasis on examples from the Formicidae family of Hymenoptera.

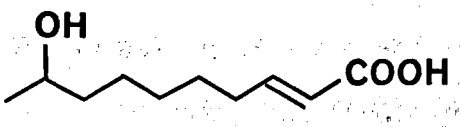
1.4.1 Sex pheromones

The primary function of sex pheromones is to bring the sexes together for the purpose of mating. A variety of acts that may take place before copulation are mediated by a number of pheromones. The chemicals involved in long range mate location are called *Sex attractants* and those which stimulate courtship are termed *courtship pheromones* ("aphrodisiacs"[38]). Some sex pheromones cause the synchronization of the time of sexual activity for the two sexes. Most of the pheromone research over the last 20 years has concentrated on the sex attractants of Lepidoptera. Moths have received a special attention mainly because they, together with beetles, comprise the majority of agricultural pests. In 1965, only three Lepidopteran sex pheromones were known. By 1970, 20 were known and by 1975 over 40 had been recognized. The number increased to 100 in 1978 and at present several hundred compounds are known and very comprehensive reviews with long lists of compounds have been published[39-42]. The female Lepidopteran sex attractants form a separate homogeneous group and consist mainly of even-numbered and linear, mono- or poly-alkenyl acetates, alcohols or aldehydes. Other insects have pheromones similar to sex pheromones in basic properties and characteristics, but not necessarily in chemical structure.

Very little work has been done on the sex pheromones of Hymenoptera. In the carpenter ant, *Camponotus herculeans* the males release a pheromone before the nuptial flight to initiate the virgin queens to take flight also [43]. Infact Holldobler and Maschwitz were able to induce the females to swarm, with a mandibular gland secretion of the males, provided the temperature and time of the day were also favourable [43]. Falke[44], chemically investigated the mandibular glands of the carpenter ants and found five different substances, of which he identified four. However, no behavioural effects could be attributed to them. A female sex attractant produced in the poison gland, which not only attracts the males but also stimulates them sexually, has been demonstrated in the myrmicine ant *Xenomyrmex floridanus*[45]. A similar female sex attractant, but produced in the Dufour gland has been described for *Monomorium pharaonis*[46]. This sex pheromone is absent in the workers and even in the older females who have already laid eggs [46,47]. Buschinger[48] demonstrated that *Harpagoxenus sublevius* males fly to females as well as isolated poison glands from a distance of 3-4 m[49]. None of the sex attractants of the ants have been chemically identified yet.

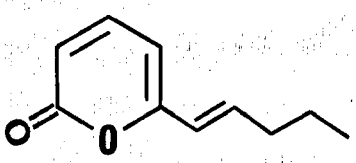
In a number of species of ants not only the presence of the queens but also larvae can suppress the fertility of workers[46]. The influence of the queens on the fertility of workers has been extensively studied in honey bees (*Apis mellifera*). The "queen substance" produced in the mandibular gland, which inhibits ovarian development of the workers is distributed through trophyllaxis in the colony. The queen pheromone consists of at least two components[50], the major being 9-oxo-*trans*-2-decenoic acid [6] and the minor is 9-hydroxy-*trans*-2-decenoic acid [7].

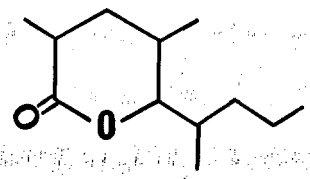


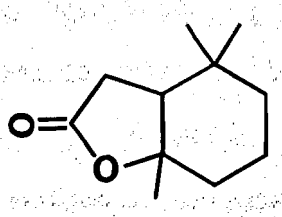


[6] [7]

The existence of a similar primer pheromone (or pheromones) in the ant, *Solenopsis invicta* is reported but no definite chemical structure has been assigned. This substance produced by the mated queen is relatively nonvolatile and it inhibits the virgin females and workers becoming functional egg layers[51]. Recently Rocca *et al.*[52,53] described three compounds [8-10] responsible for "Queen recognition" in *S. invicta*.







[8] [9] [10]

1.4.2 Maturation pheromones

The primer pheromones, which maximize the developmental synchrony especially in insects living in large populations are termed *maturation pheromones*. The reproductive potential of the population is enhanced by the simultaneous availability of large number of sexually mature adults as a result of synchronization of maturation. One well documented example, found in the desert locust *Schistocerca gregaria*, is the acceleration of development of immatures of both sexes by the presence of mature males[54].

1.4.3 Brood pheromones

The substances released by the brood to advertise their identity to the conspecific workers, are named *brood pheromones*. The recognition of

the brood is aided by the brood pheromones, when they are being fed, maintained, defended and moved by the workers. The sexual brood pheromone of *Solenopsis invicta* has been recognized as triolein[55]. However, the structure assignment based solely on TLC R_f values has been strongly criticized by Vander Meer[56].

1.4.4 Aggregation pheromones

An *aggregation pheromone* causes the members of the same species to aggregate in a particular area, leading to an increase in their density[57]. The aggregation serves a variety of functions including bringing the conspecifics to a source of food or a suitable habitat, overcoming the natural resistance of a selected host, and defence against predators. The aggregation pheromones of bark beetles (Family Scolytidae) have been extensively studied[58] and they are also being found in some cockroaches, bees and wasps.

In the workers of the ant *Camponotus pennsylvanicus* an oriented reaction occurs and aggregations lasting up to 12 h are caused by undecane a compound from its Dufour gland[59]. The aggregative behaviour of this species is governed by an exocrine product that also functions as a key orienting agent when alarm behaviour is released.

1.4.5 Dispersal or spacing pheromones

Pheromones of this nature causes increased spacing between the conspecifics. One adaptive advantage of possessing such pheromones is to reduce intraspecific competition. An interesting example is found in the apple maggot fly (*Rhagoletis pomonella*), when the female oviposits in a fruit, she marks the fruit with a pheromone that deters other females from ovipositing in the same fruit[60]. Similar oviposition deterrents have been reported for the European corn borer, *Ostrina nubilalis*[61] and other insects[62]. When the western pine beetles (*Dendroctonus brevicomis*) colonise

a pine tree they release initially *exo*-brevicomin and frontalin to attract more females and males, but once they mate they release verbenone and *trans*-verbenol, which deter further arrivals [58].

1.4.6 Alarm pheromones

The *alarm pheromones* are used as signals to alert conspecifics that a threatening situation is present. Some respond by vigorously attacking the intruder whereas others may escape by retreating into the interior of the nest. The ants and termites open their mandibles in an aggressive response when they perceive high concentration of their species' alarm pheromone. The aphid *Myzus persicae* produces *trans*-β-farnesene when disturbed, which induces other aphids to stop feeding, disperse or drop from the plant and escape[63].

In the species among the Formicidae, the alarm pheromones are generally synthesized in their mandibular glands. In certain formicine species the alarm signals can be generated with secretions from the poison and Dufour gland in addition to the mandibular glands[64]. Dolichoderine ants produce alarm pheromones in the supra-anal glands[65]. The ants of *Myrmeciinae* subfamily produce the alarm signals from their rectal glands[66].

Ants employ a large variety of compounds which release alarm behaviour, but aliphatic ketones appear to be the major group. Many comprehensive reviews are available with lists of alarm substances identified from ants[67-70].

1.4.7 Territorial pheromones

The territory of an animal is the area within which it normally confines its day to day activities. The *territorial pheromones* serves the purpose of conditioning the area with a familiar odour. The work of Cammaerts on the ants of the genus *Myrmica*, demonstrates the use of their Dufour gland contents as a territorial marking pheromone[71]. The glands

contain a mixture of volatile, low molecular weight oxygenated compounds and a comparatively less-volatile, linear and branched hydrocarbons. The volatile oxygenated compounds act as short range non-species specific attractants. The less volatile hydrocarbon mixture is highly species-specific and used by the workers on a new foraging territory to encourage other workers to search for food[72]. The ants move rapidly on a territory already marked with their own Dufour gland secretions. They move less rapidly in a new territory and even more slowly in a territory marked with an alien secretion, until they overmark it with their own secretion. The existence of a colony-specific territorial marking pheromone in the ant *Oecophylla longinoda* has also been recognized[73]. The workers mark newly acquired home range with randomly placed drops of rectal vesicle fluid[74].

1.4.8 Trail pheromones

The social insects (*e.g.* bees, wasps, ants and termites) utilize an array of pheromones to maintain the high level of organization in their colonies. Many of these insects, especially the ants and termites because they are essentially wingless, lay terrestrial odour trails leading to food sources or nesting sites. Short range trail pheromones, laid with foot prints in the vicinity of the hive or nest are also known for bees and wasps[75,76]. Trail pheromones can also facilitate migration of the colony to a new site. In the termites, a gland on the ventral surface of the abdomen serve as the source of the trail pheromones but in ants they can arise from a number of glandular sources including the hind gut, rectal gland, poison gland, tarsal gland, Dufour gland, Pavan gland, tibial gland and anal (pygidial *?*) gland. The location of the general exocrine glands of a typical ant is shown in Figure 1. The trail is deposited on the ground either by the sting, anus, abdominal sternum or tarsi on the hind legs, as the ant moves along. Table 2 shows the reported glandular sources of the trail pheromones of some genera of Hymenoptera.

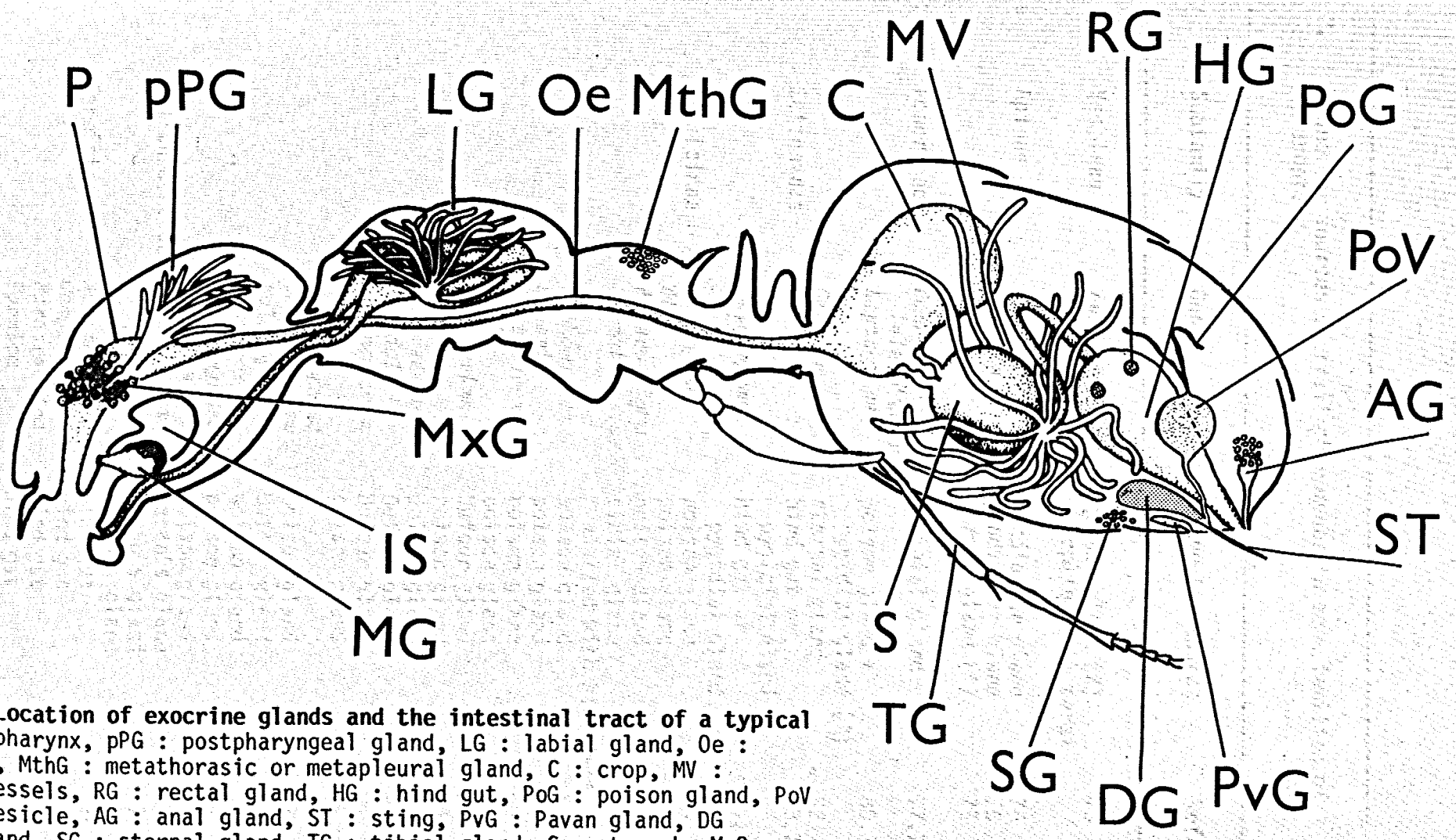
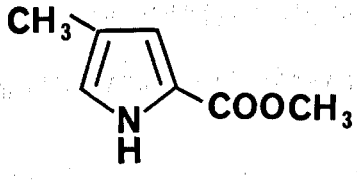


FIGURE 1. Location of exocrine glands and the intestinal tract of a typical ant. P : pharynx, pPG : postpharyngeal gland, LG : labial gland, Oe : oesophagus, MthG : metathorasic or metapleural gland, C : crop, MV : malpighi vessels, RG : rectal gland, HG : hind gut, PoG : poison gland, PoV : poison vesicle, AG : anal gland, ST : sting, PvG : Pavan gland, DG :Dufour gland, SG : sternal gland, TG : tibial gland, S : stomach, MxG : maxilliary gland, IS : infra buccal sac, MG : mandibular gland.

Table 2. Glandular sources of trail pheromones in some families of Hymenoptera.

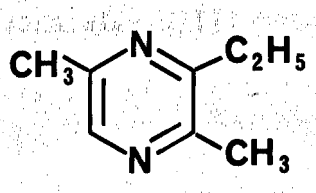
Family	Subfamily	Genus	Glandular source	References
Vespidae	Vespinae	*Vespula*	dermal gland (?) applied with tarsi	75
Apidae	Apinae	*Melipona*	mandibular gland	76
		Trigona	mandibular gland	76
		Apis	dermal gland (?)	75
Formicidae	Ponerinae	*Pachycondyla* (=Termitopone)	Pygidial gland	77,78,79
		Leptogenys	poison gland + pygidial gland (?)	80,81
		Onychomyrmex	sternal gland	82
	Ecitoninae	*Eciton*	hind gut or pygidial gland	83,78
		Neivamyrmex	hind gut or pygidial gland	84,78
	Myrmicinae	*Acromyrmex*	poison gland	85
		Atta	poison gland	85
		Apterostigma	poison gland	86
		Crematogaster	tibial gland applied with hind legs	87
		Cyphomyrmex	poison gland	85
		Huberia	poison gland	77
		Leptothorax	poison gland	88
		Manica	poison gland	89
		Monomorium	Dufour gland	90
		Myrmica	poison gland	89,91
		Novomessor	poison gland	92
		Pheidole	Dufour gland	93
		Pogonomyrmex	Dufour gland	94,95
			poison gland	89,96
		Pristomyrmex	?	97
		Sericomyrmex	poison gland	138
		Solenopsis	Dufour gland	98
		Tetramorium	poison gland	99
		Trachymyrmex	poison gland	86
		Veromessor	poison gland	89
	Dolichoderinae	*Iridomyrmex*	Pavan's gland	65
		Monacis	Pavan's gland	65
		Tapinoma	Pavan's gland	100
	Formicinae	*Acanthomyops*	hind gut	101
		Camponotus	hind gut	102,103
		Paratrechina	hind gut	104
		Formica	hind gut	105
		Lasius	hind gut	122,123,124
		Myrmelachista	hind gut	104
		Oecophylla	rectal gland	74

As early as 1779, Bonnet observed that some ants use trails to recruit workers of the same species to a food source. However, relatively little information is available yet, on the chemistry of the trail pheromones. Methyl 4-methylpyrrole-2-carboxylate [11], a poison gland substance of the myrmicine ant *Atta texana* was the first ant trail pheromone to be identified[106]. The compound has a very high behavioural efficiency and the detection threshold is as small as 0.08 pg/cm. The same compound was subsequently



[11]

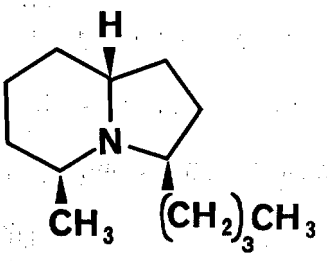
demonstrated to be the trail pheromone of *A. cephalotes*[107] and *Acromyrmex octospinosus*[86]. It is apparent that the pyrrole is only one component in the trail pheromone because Tumlinson *et al.*[106] isolated at least four other active fractions but the nature of the other constituents remains unknown. *Atta sexdens*, a species related to *A. texana* and *A. cephalotes* did not follow trails made of synthetic methyl 4-methylpyrrole-2-carboxylate. Subsequently another compound, 3-ethyl-2,5-dimethylpyrazine [12]



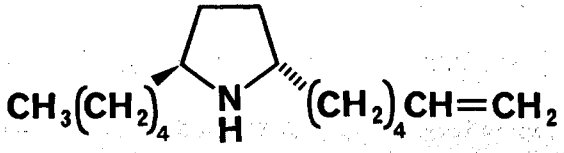
[12]

was identified as the major component of the trail pheromone of *A. sexdens rubropilosa*[108]. Recently the same compound has been isolated from the poison glands of eight species of *Myrmica* and identified as the single component of their trail pheromone[109,110]. Among the poison gland constituents of the Pharaoh's ant are two substances, 5-methyl-3-butyloctahydroindolizine

(Monomorine I) [13], and 2-(5'-hexenyl)-5-pentylpyrrolidine (Monomorine III) [14], able to attract worker ants and show some activity in trail following tests[111,112].

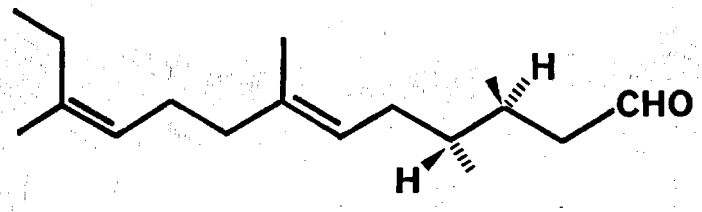


[13]



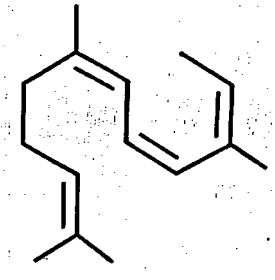
[14]

However the much more active true trail pheromone of the pharaoh's ant is found in the Dufour gland in trace quantities. It was later identified as (+)-(3S,4R)-3,4,7,11-tetramethyltrideca-6E,10Z-dienal (Faranal)[133] [15].



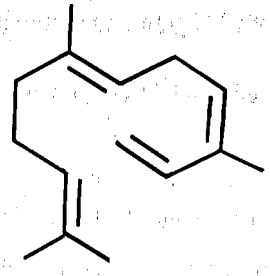
[15]

The stereochemistry and geometry of faranal have been confirmed by stereospecific synthesis by several groups[113-117]. The trail pheromone of the red imported fire ant, *Solenopsis invicta* is certainly multi-component but the composition is controversial. Williams *et al.*[118,119] report it to be (2Z,4Z,6Z)-3,7,11-trimethyl-2,4,6,10-dodecatetraene (Z,Z,Z-allofarnesene) [16].

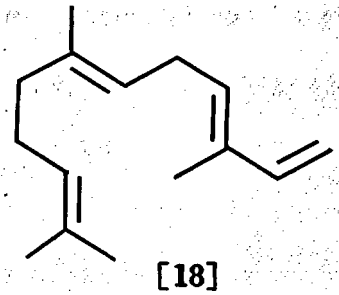


[16]

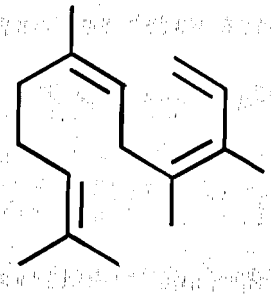
According to Vander Meer *et al.*[120], *Solenopsis invicta* trail pheromone is multicomponent and four of them are identified as (3Z,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene (Z,E-α-farnesene) [17], (3E,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene (E,E-α-farnesene) [18], (3Z,6Z)-3,4,7,11-tetramethyldodeca-1,3,6,10-tetraene (Z,Z-homofarnesene) [19] and (3Z,6E)-3,4,7,11-tetramethyldodeca-1,3,6,10-tetraene (Z,E-homofarnesene) [20].



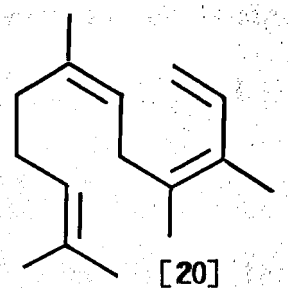
[17]



[18]



[19]



[20]

A preliminary study had been done on the trail pheromones of few other species of *Solenopsis*. Barlin *et al.*[18] reported the main trail pheromone of *S. richteri* to possess a M.W. of 218 and an empirical formula of $C_{16}H_{26}$. They assume the trail pheromone of *S. xyloni* and *S. geminata* are of a similar chemical type and suggests the empirical formula to be $C_{17}H_{28}$.

Hexanoic, heptanoic, octanoic, nonanoic, decanoic and dodecanoic acids are reported by Huwyler *et al.* as components of the trail pheromone isolated from the hind gut of the formicine ant, *Lasius fuliginosus*[121]. It is also reported that the active material is composed of an acidic and a non-acidic fraction. The former appears to account for the greater part of the total activity. Although the absence of pentanoic, undecanoic and tridecanoic acids have been tested, it is unknown whether any other lower and

higher homologues of the fatty acid series are present in the rectal fluid. In fact, Hangartner[122] had observed that the activity of an aqueous trail pheromone extract disappeared to a large extent, upon the addition of alkali and reappeared at the original level when it was re-acidified. Commercial samples of the aforementioned six fatty acids were all found to elicit trail-following behaviour in *L. fuliginosus* workers, when tested individually for activity, but the activity towards an appropriate mixture of the acids has not been examined. It is interesting to note that the trail pheromone of *L. niger* isolated from the rectal fluid, is non-acidic and can be recovered from the GC effluent, although no corresponding peak can be observed[121].

A mixture of nine fatty acids, similar to those found in *L. fuliginosus*, have been reported as the components of the trail pheromone of the myrmicine ant *Pristomyrmex pungens*[97]. This mixture of saturated and unsaturated fatty acids of C_{14} to C_{20} range, falls out of line, when compared with the chemical structures described as the trail pheromone components of the other myrmicine ants (Table 3). Nevertheless the query whether they are in fact the true trail pheromone components remains, because the activity of the synthetic analogues has not been reported. Furthermore, the glandular origin of the pheromone remains unknown.

In all reported dolichoderine ants the trail pheromones are derived exclusively from the Pavan's gland[65]. In *Iridomyrmex humilis* (Z)-9-hexadecenal, a Pavan's gland constituent, is indicated as a trail pheromone component by behavioural evidence[125,126]. Although high concentrations of (Z)-9-hexadecenal alone elicit intense trail following by recruited workers, the true trail pheromone is considered to be multi-component. In fact gaster extract trails containing 100 times less (Z)-9-hexadecenal were comparable in activity to the synthetic trails.

Table 3 summarizes all the chemicals identified so far in trail pheromones of termites and ants. In the present study, the trail pheromone of *Tetramorium caespitum* and *Solenopsis geminata* are investigated.

Table 3. Substances identified in the trail pheromones of termites (Isoptera) and ants (Formicidae).

Compound	Insect	Species	References
12-isopropenyl-1,5,9-trimethyl-cyclotetradeca-1,5,9-triene (neocembrene A)	termite	*Nasutitermes sp.*	127,128
Z-3,Z-6,E-8-dodecatriene-1-ol	termite	*Reticulitermes flavipes*	129
		R. hesperus	129
		R. santonensis	130
		R. tibialis	129
		R. virginicus	129
Z-3-hexadecene-1-ol	termite	*Calotermes flavicollis*	131
		Microcerotermes edentatus	131
4-phenyl-Z-3-buten-1-ol[a]	termite	*Coptotermes formosanus*	129
		Reticulitermes speratus	129
Z-3,Z-6-dodecadien-1-ol[a]	termite	*Coptotermes formosanus*	129
		Reticulitermes speratus	129
3,7,11-trimethyl-E-2,E-6,10-dodecatriene-1-ol (farnesol)	termite	*Zooteromopsis nevadensis*	132
hexanoic acid	termite	*Z. nevadensis*	132,133
methyl 4-methylpyrrole-2 carboxylate (attalure)	ant	*Atta cephalotes*	107
		Atta texana	106,134
		Acromyrmex octospinosus	86
methyl 4-chloropyrrole-2-carboxylate[a]	ant	*Atta texana*	135
methyl 4-bromopyrrole-2-carboxylate[a]	ant	*A. texana*	135
hexanoic acid	ant	*Lasius fuliginosus*	121,136
heptanoic acid	ant	*L. fuliginosus*	121,136
nonanoic acid	ant	*L. fuliginosus*	121,136
decanoic acid	ant	*L. fuliginosus*	121,136
3-ethyl-2,5-dimethylpyrazine	ant	*Atta sexdens rubropilosa*	108
		Atta sexdens sexdens	108
3-ethyl-2,5-dimethylpyrazine	ant	*Myrmica sp.*	109,110
(+)-(3S,4R)-3,4,7,11-tetramethyltrideca-6E,10Z-dienal (Faranal)[b]	ant	*Monomorium pharaonis*	90

Table 3. (continued)

Compound	Insect	Species	References
5-methyl-3-butyloctohydroindoli -zine (Monomorine I)	ant	*M. pharaonis*	111,112
trans-2-pentyl-5-(5'-hexenyl) pyrrolidine (Monomorine III)	ant	*M. pharaonis*	111,112
(3Z,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene (Z,E-α-farnesene)	ant	*Solenopsis invicta*	120
(3E,6E)-3,7,11-trimethyldodeca-1,3,6,10-tetraene (E,E-α-farnesene)	ant	*S. invicta*	120
(3Z,6Z)-3,4,7,11-tetramethyl-dodeca-1,3,6,10-tetraene (Z,Z-homofarnesene)	ant	*S. invicta*	120
(3Z,6E)-3,4,7,11-tetramethyl-dodeca-1,3,6,10-tetraene (Z,E-homofarnesene)	ant	*S. invicta*	120
(2Z,4Z,6Z)-3,7,11-trimethyl-dodeca-2,4,6,10-tetraene (Z,Z,Z-allofarnesene)	ant	*S. invicta*	118,119
tetradecanoic acid[c]	ant	*Pristomyrmex pungens*	97
hexadecanoic acid[c]	ant	*P. pungens*	97
hexadecenoic acid[c]	ant	*P. pungens*	97
octadecanoic acid[c]	ant	*P. pungens*	97
octadecenoic acid[c]	ant	*P. pungens*	97
octadecadienoic acid[c]	ant	*P. pungens*	97
eicosatetraenoic acid[c]	ant	*P. pungens*	97
eicosapentenoic acid[c]	ant	*P. pungens*	97
(Z)-9-hexadecenal	ant	*Iridomyrmex humilis*	125

[a] compound not isolated from the insect, but the synthetic sample is active

[b] Faranal is the true trail pheromone, Monomorine I and III may act as attractants.

[c] compound isolated from insect but activity response towards a synthetic sample not reported

1.5 Source and Specificity of Ant Trail Pheromones

An examination of the behaviour response of one ant species, to an artificial trail made from the trail substances of another species may help in the identification of the trail pheromones and also in the understanding of their phylogeny. Such cross-examinations are called transposition studies. Initially, the trail pheromones were considered to be highly species-specific and the concept appeared incidentally to provide a simple method, to distinguish one species from another within a given genus[65,137]. However this expectation was short lived because the existence of a wide variability in specificity in the trail-following behaviour has become apparent recently. In fact, it is not surprising, as ants do not use a single specialized gland for the synthesis of the trail pheromones but a number of glands, which also have other functions (Table 2). It seems plausible that the chemical recruitment mechanism was derived from a more primitive tandem calling behaviour (similar to that used by *Leptothorax*[88]) by the conversion of a gland normally used for other functions, into a social organ. Such an evolutionary process may be common to almost all subfamilies of ants, except perhaps the dolichoderine ants, which utilize Pavan's gland, a gland with no other known function. Gabba and Pavan[138] have reviewed the results from transposition studies up to 1970.

1.5.1 Dolichoderine ants

The trail pheromones of species of four dolichoderine genera, namely *Iridomyrmex*, *Tapinoma*, *Liometopum* and *Monacis* were demonstrated by transposition studies, to be highly species-specific[65]. The trail pheromones of the dolichoderine ants are produced in the Pavan's gland and secreted via the posterior border of the sixth sternite. According to Blum[69] such a great specificity of dolichoderine trail pheromones may be possible because Pavan's gland, a unique organ for the synthesis and dispensing of trail pheromones,

has been selected for the production of particular metabolic end products which serve as trail pheromones. Presumably, highly species specific trail pheromones, similar to those found in the dolichoderine ants, would be much more difficult to achieve in subfamilies, which utilize trace constituents of the poison gland, the Dufour gland or the hind gut as trail pheromones.

1.5.2 Myrmicine ants

Three different glands, the poison gland, Dufour gland or tibial gland may serve as the source of the trail pheromones of myrmicine ants. The tibial glands and the elaborate trail laying mechanism appear to be unique only to the genus *Crematogaster*[87]. Given that the trail following behaviour has arisen several times separately and the myrmicine ants are the largest and the most diverse group of ants, it is not surprising to find that they utilize a number of different glands for the production of their respective trail substances.

In contrast to dolichoderine ants, the trail substance of myrmicines investigated so far show a marked absences of species-specificity in transposition studies conducted in the laboratory. However, field observations indicated that the natural trails are considerably more specific. The wide interest on leaf-cutting ants has provided considerable amount of information about the variability of specificity in their trail following behaviour. The information available from transposition studies is summarized in Table 4. The trail following behaviour of some leaf-cutting ants towards two synthetic trail substances, methyl 4-methylpyrrole-2-carboxylate [11] and 3-ethyl-2,5-dimethylpyrazine [12], is summarized in Table 5. The workers of *Atta sexdens* followed synthetic trails of the pyrazine [12] only, not the pyrrole [11], and they do not follow trails made of the poison gland of *Acromyrmex*, therefore it can be expected that the pyrazine [12] is absent in the poison gland of *Acromyrmex*. The recent chemical investigation of the poison gland of *Acromyrmex octospinosus*

Table 4. Responses of Some Myrmicine Ants to Artificial Trails Laid from Their Poison Glands.

Source species	Test species						
	A.texana	*A.cephalotes*	*A.sexdens*	*A.octospinosus*	*T.septentrionalis*	*S.urichi*	*D.armigerum*
Atta texana	+++[b,c]	+++[a]	+++[a]	+++[a]	+++[b,c]	0[c]	
Atta cephalotes	+++[a]	+++[a]	+++[a]	+++[a]			
Atta sexdens	+++[a]	+++[a]	+++[a]	+++[a]			
Acromyrmex octospinosus	+++[a]	+++[a]	0[a]	+++[a]			
Trachymyrmex septentrionalis	+++[b,c]				+++[b,c]	0[c]	
Sericomyrmex urichi	0?[c]				0?[c]	+++[c]	
Daceton armigerum	+++[c]	+++[c]	+++[c]	+++[c]	+++[c]	0[c]	0[c]

[a] Robinson *et al.*[86]

[b] Blum and Ross[96]

[c] Blum and Portocarrero[140]

0 :no trail following activity

+++ :high trail following activity

Table 5. Responses of Some Leaf-cutting Ants to Artificial Trails Laid with Two Synthetic Substances.

Test species	Test substance		References
	Pyrrole	Pyrazine	
Atta texana	+++		86
Atta cephalotes	+++	++	86,108
Atta colombica	+++		86
Atta laevigata	++		86
Acromyrmex octospinosus	+++	++	86,108
Acromyrmex versicolor	+++		86
Atta sexdens sexdens	0	+++	86,108
Atta sexdens rubropilosa	0	+++	86,108
Trachymyrmex septentrionalis	+++		86
Trachymyrmex urichi	+++		86
Cyphomyrmex rimosus	+++		86
Apterostigma collare	+++		86

0 :no trail following activity

\+ :detected but not followed convincingly

++ :trail following by most ants, but few with hesitation

+++ :natural following

Pyrrole:methyl 4-methylpyrrole-2-carboxylate [11]

Pyrazine:3-ethyl-2,5-dimethylpyrazine [12]

by Evershed and Morgan[139], proved the absence of the pyrazine [12]. The poison glands of *Atta cephalotes*, *A. sexdens sexdens* and *A. sexdens rubropilosa* contain both the pyrrole [11] and pyrazine [12][139]. Hence it becomes clear why *Atta sexdens* would follow the poison gland trails of *Atta cephalotes*, which also contains the pyrazine [12], but not a synthetic trail of the pyrrole [1] only. A number of non-attine ants showed no response to artificial trails of pyrrole [11][86].

Sericomyrmex apparently differs from the genera representing the mainstream of attine ants, as it does not follow trails generated from the poison glands of *Atta texana* or *Trachymyrmex septentrionalis* (Table 4). Furthermore, the poison gland contents of *Sericomyrmex urichi* induces feeble or no trail-following behaviour on *Atta texana* or *Trachymyrmex septentrionalis* workers[140]. Hence the pheromone which releases the trail following behaviour in *Sericomyrmex urichi* is species-specific and expected to be different from pyrrole [11] or pyrazine [12]. An interesting discovery, which indicates how the poison gland substances have evolved to assume the secondary function of trail releasing, was reported from a non-trail laying myrmicine, *Daceton armigerum*[140]. A trail laid with the poison gland of *Daceton armigerum* was not followed by the workers of *Daceton armigerum* itself, but surprisingly, it was strongly followed by *Trachymyrmex septentrionalis*, *Atta texana*, *A. cephalotes* and *Acromyrmex coronatus*. These results demonstrate though the trail-following behaviour is not evolved yet in the primitive *Daceton* but its venom contains compounds (most probably the pyrrole [11] and the pyrazine [12]) utilized by some other more advanced ant species as pheromone components. Furthermore, *Sericomyrmex urichi* did not follow a trail made of *Daceton* venom as one might expect[140].

Further evidence on the variability of specificity in the trail following behaviour of myrmicine ants, as summarized in Table 6, was found by Blum[99] by the study on *Tetramorium*. The source of odour trail pheromone of *Tetramorium* is the poison gland and they do not follow the artificial trails

Table 6. Responses of Some Myrmicine Ants to Artificial Trails Laid from the Glandular Sources of Their Odour Trail Pheromones[a].

Source species	Test species: *Tetramorium guineense*	*Tetramorium caespitum*	*Atta texana*	*Trachymyrmex Septentrionalis*	*Solenopsis saevissima*
Tetramorium guineense	+++	0	+++	++	0
Tetramorium caespitum	0	+++	0	0	0
Atta texana	+++	0	+++	+++	0
Trachymyrmex septentrionalis	+++	0	+++	+++	0
Solenopsis saevissima	0	0	0	0	+++

[a] After Blum and Ross[99]

0 :no trail following activity

\+ :detected but not followed convincingly

++ :trail following by most ants, but few with hesitation

+++ :natural following

laid with either the hind gut or the Dufour gland[99]. The trails are completely species-specific between *Tetramorium guineense* and *T. caespitum* (Table 6) but in contrast, *T. guineense* trails are well followed by two not so closely related species, *Atta texana* and *Trachymyrmex septentrionalis* and vice versa. Furthermore, *Tetramorium caespitum* trails are not followed by the two attine species.

The least species-specificity in the trail following behaviour is demonstrated by the genus Myrmica. With the exception of *Myrmica monticola*, thirteen other *Myrmica* species almost equally follow trails made of each other's poison glands[89,110]. It is interesting to note that *Veromessor pergandei*, *Pogonomyrmex badius* and three species of *Manica* also follow each other's trails and those of *Myrmica*[89]. The poison gland extracts of *Aphaenogaster fulva*, *Novomessor cockerelli* and *Veromessor pergandei* do not evoke any positive trail following behaviour in *Myrmica* or *Manica* workers.

According to Holldobler[141], the trail pheromone of *Monomorium* originates from the Dufour gland, although Blum[77] had previously reported the source as the poison gland. The trail of *M. floricola*, *M. minimum* and *M. striate* are strictly species-specific, although the trail pheromone of *M. pharaonis* produced trail following responses in workers of *M. minimum* as well as workers of its own[141].

In a similar study on two species of *Novomessor*, Holldobler *et al.*[92] found that *N. albisetosus* also followed artificial trails laid with the poison gland extracts of *N. cockerelli*, but *N. cockerelli* did not follow the poison gland extracts of *N. albisetosus*. According to Barlin *et al.*[18], among the fire ants, *Solenopsis richteri* and *S. invicta* follow each other's artificial trails, laid separately from their Dufour gland extracts. But their Dufour gland contents were found to be different from each other on gas chromatographic examination. On the other hand *S. geminata* and *S. xyloni* appear to have a common trail pheromone as they follow each other's trails and the Dufour gland contents are also similar to each other. The Dufour

gland contents of *S. geminata* produce virtually no response on the workers of *S. invicta* and *S. richteri*. The results are summarized in Table 7. These results are somewhat different to those reported previously by Wilson[98] on fire ant trails but Jouvenaz *et al.*[142] have obtained similar results using purified whole ant extracts. Furthermore the Dufour glands contents of dolichoderine ant *Monacis bispinosa* can release strong trail following activity on *Solenopsis invicta (saevissima)* workers. However, *M. bispinosa* produces its own trail pheromone in the Pavan's gland[98]. This indicates that the trail releasing by the constituents of the Dufour gland has evolved as a secondary function.

1.5.3 Formicine ants

The hind gut or the rectal gland is the source of the formicine trail pheromones. Hangartner[122,123] used artificial trails made of hind gut contents and demonstrated that the trail substance of *Lasius fuliginosus* did not evoke any response among other *Lasius* species, such as *L. emerginatus*, *L. niger* and *L. flavus*. On the contrary *L. fuliginosus* was able to follow the trails of *L. emerginatus* and *L. niger*. *L. flavus*, an underground species produces no trail substance, or only a minimally effective one. The workers of *L. fuliginosus* were also found to follow the trails of *Formica rufibarbis* and *F. rufa*[122].

Another formicine, *Camponotus pennsylvanicus* strongly followed trails prepared from the hind guts of *C. americanus*. *C. pennsylvanicus* and *C. americanus* belong to the same subgenus. It was also shown that the trail pheromones of six other species of *Camponotus* of other subgenera were particularly specific[143]. Wilson has observed that the natural trails of the dolichoderine ant *Azteca chatifex* were followed by the formicine *Camponotus beebei*, but not vice versa[144].

Table 7. Species Specificity of Trails Laid with Dufour Gland Extracts of Four Species of *Solenopsis*[b]

	Test species			
Source species	*S. richteri*	*S. invicta*	*S. geminata*	*S. xyloni*
S. richteri	+++	+++	0	+
S. invicta	+++	+++	0	+
S. geminata	+	0	+++	+++
S. xyloni	+++	0	+++	+++

[b] after Barlin *et al.*[18]

0 :no trail following activity

\+ :detected but not followed convincingly

++ :trail following by most ants, but few with hesitation

+++ :natural following

1.5.4 Ecitonine ants

This subfamily of ants includes the New World army ants, long placed within Dorylinae. The trails of army ants are long lasting and could be followed by workers weeks after they had been laid. The source of the trail substances in ecitonine ants is either the hind gut or the pygidial glands. The trails laid by four species of *Neivamyrmex* and by *Labidus coecus* were followed by all five species, but, in general, each species preferred its own trail when presented with a choice[84,145]. However, a fifth species *Neivamyrmex pilosus* would not follow any trail other than its own. Later, Torgerson and Akre[146] showed utilizing five species of *Eciton*, two of *Labidus* and a single species each of *Neivamyrmex* and *Nomamyrmex*, that all possible combinations of specificity can be encountered in this subfamily.

1.5.5 Ponerine ants

The hind gut was long considered to be the source of ponerine trails, however recent discoveries show trails substances can originate from the pygidial glands[78,79], poison gland[80] or the newly discovered sternal gland[82]. Very little information is available on transposition studies of the trails of ponerine ants. The three species of *Onychomyrmex* studied by Holldobler[82] demonstrated a partial species specificity.

1.6 Exocrine Gland Substances of Ants

A great variety of natural products are found in the exocrine glands of ants. The location of the major glands in a typical ant is illustrated in Figure 1. In the head region are the mandibular, propharyngeal (maxillary) and the postpharyngeal glands. The labial glands also open into the head region, but are actually situated in the thorax. The metapleural glands are also found in the thorax. The tibial glands described in the species of *Crematogaster* provide a unique source for their trail substances[87]. The poison and Dufour glands are the main glands found in the abdomen. The hind gut is the main source of the formicine trail substances. The supra-anal glands and Pavan's gland were thought to be restricted to dolichoderine ants only. However Holldobler and Engel have recently reviewed tergal and sternal glands, and they believe that the anal glands are analogous to the tergal pygidial glands which are found in several sub-families[78].

Definite ethological activities have been assigned to a number of compounds found the exocrine glands but many appear to have no significant behavioural activity. Closely related species are often found to produce similar substances in a particular gland, however the qualitative and quantitative compositions are usually characteristic of the species. Although it is not easy to explain the occurrence of these species-specific mixtures of compounds, nevertheless they are diagonostically useful at all taxonomic levels, especially to distinguish between morphologically similar species. Furthermore, the investigations on the composition of exocrine glands can be helpful as an indicator of the phylogeny of a particular species. The chemistry and morphology of the exocrine glands of the ants have been reviewed recently[70,147,148]. In the present study the poison, Dufour, mandibular and postpharyngeal glands of some myrmicine ants are investigated.

1.6.1 Venom apparatus

The original function of glands associated with the venom apparatus was the production of proteinaceous compounds that coated the eggs to facilitate the adhering of the eggs to a substrate. The venom and Dufour glands arose from a pocket of modified hypodermal cells and developed into a paired, saclike structure. One developed into a complex poison gland while the other (Dufour gland) remained relatively simple in structure. All the subfamilies of ants, with the exception of the formicines, contain species which use their stings to subdue their prey. However in anumber of species during phyletic development, an assortment of other functions have being assigned to the glands associated with the venom apparatus. These secondary functions include the production of communication pheromones, defensive allomones and other deterrents.

1.6.2 Poison gland substances

The characteristic chemical components found in the venoms of stinging ants are proteinaceous and alkaloidal. The formicine ants are stingless but their venoms often contain aqueous formic acid in concentrations up to 60%[149], which act as a highly effective deterrent against predators. Formic acid is the only volatile compound reported from formicine venom, however, the presence of peptides and free amino acids in the venoms of *Formica polyctena* [149] and *Camponotus pennsylvanicus*[150] have also been reported.

Proteinaceous venoms appear to be widely spread in the subfamilies, *Myrmeciinae*, *Ponerinae*, *Dorylinae*, *Pseudomyrmecinae* and *Myrmicinae*. No information is available yet about the chemistry of dolichoderine venom. The presence of constituents with a wide range of pharmacological activities has been demonstrated in the venom of two species of *Myrmecia*[151,152]. The venom of *Pogonomyrmex badius*[153] contains histamine and a series of free amino acids, enzymes of six classifications and a number

of other non-enzymic proteins. Similar constituents have also been found in *Myrmica ruginodis*[154].

In contrast to the general proteinaceous themes exhibited by the venoms of many subfamilies of *Formicidae*, many ants in the *Myrmicinae* subfamily possess the ability to biosynthesize a variety of alkaloids in the venom gland. All the alkaloids reported in myrmicine venoms are summarized in Table 8. The members of the genus *Solenopsis*, undoubtly leads the myrmicine ants, which possess the ability to produce alkaloid-rich and low protein venoms. The ants belonging to the subgenus *Solenopsis* of the genus *Solenopsis* are referred to as *fire ants* because of the potency of their venoms which exhibit pronounced necrotic[167], hemolytic[168] and antibiotic[169] activity. The venoms of *Solenopsis(Solenopsis)* species are characterized by a predominance of 2-alkyl-6-methylpiperidines [**31,32**]. Both *cis* and *trans* isomers of 2,6-disubstituted piperidines are usually present, with either *trans* isomer predominating as found in *S. invicta*[162] or *cis* isomer predominating as in *S. xyloni* or *S. geminata*[163]. These ring configurational isomers are conveniently separated by GC, with the *cis* isomer eluting first on polar phases like Carbowax 20M. The configuration of the double bonds, when present in the side chains, always appear to have a Z-configuration[163]. The absolute configurations of the chiral centres of these piperidines still remains unknown. The venom of *S. xyloni* contains, besides the usual piperidines, a 2-alkyl- 6-methyl-1-piperideine [**33**][162].

Many species of *Solenopsis* belonging to the subgenus *Diplorhoptrum* are called *thief ants* because they steal brood from the nests of other species of ants[141]. The raiding thief ants secrete offensive alkaloidal substances which repel the host ants from defending their brood. *Solenopsis (Diplorhoptrum) fugax* utilizes 2-butyl-5-heptylpyrrolidine [**25**] in this context[158].

In contrast to true fire ants(subgenus-*Solenopsis*) the thief ants are not noted for their stinging abilities. Furthermore the ants of the

Table 8. Venom Alkaloids of Myrmicine Ants

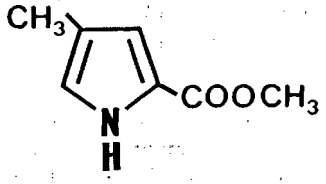
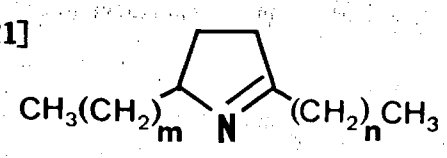
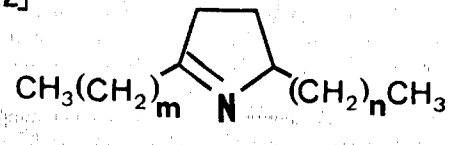
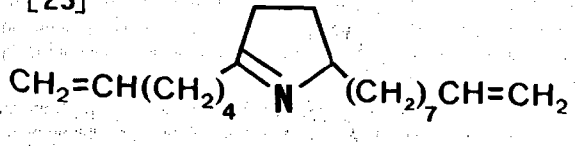
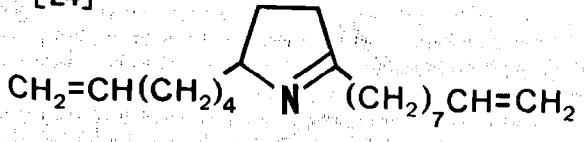
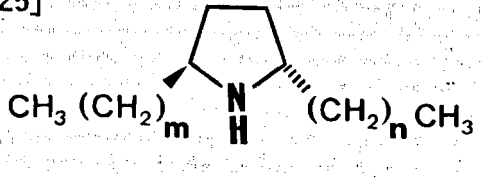
Structure		Source	References
[11]		*Atta texana* *A. cephalotes* *Acromyrmex octospinosus*	106,134 107 86
[21]		m=1, n=4 m=1, n=6 m=3, n=6 m=3, n=6	*Solenopsis punctaticeps* *S. punctaticeps* *Monomorium latinode* *M. latinode*	155 155 156 156
[22]		m=1, n=4 m=1, n=6	*S. punctaticeps* *S. punctaticeps*	155 155
[23]			*Monomorium sp.*	157
[24]			*Monomorium sp.*	157
[25]		m=1, n=6 m=3, n=4 m=3, n=4 m=3, n=6 m=3, n=6 m=3, n=6 m=4, n=5 m=4, n=5 m=5, n=8	*Solenopsis punctaticeps* *S. punctaticeps* *Monomorium sp.* *S. fugax* *S. punctaticeps* *M. latinode* *S. molesta* *S. texanas* *Monomorium sp.*	155 155 156,160 158 155 156 159 159 157

Table 8. (continued)

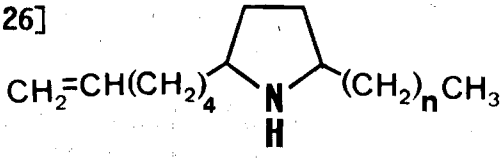
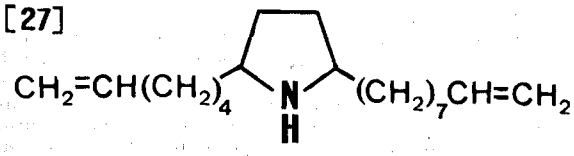
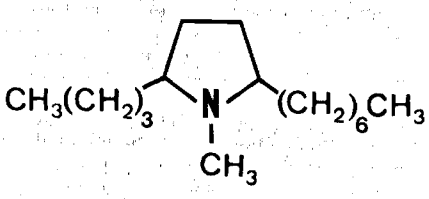
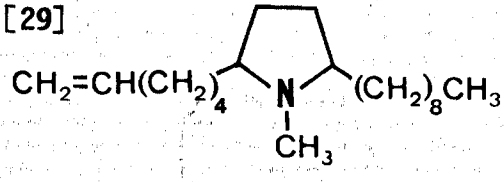
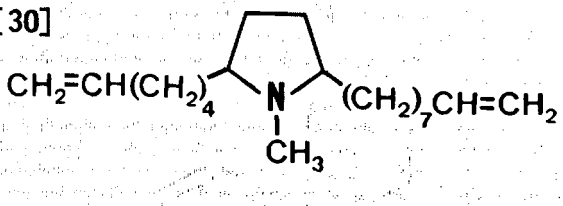
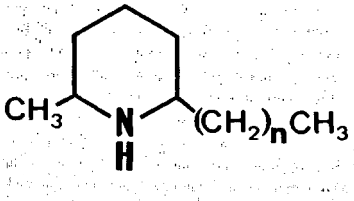
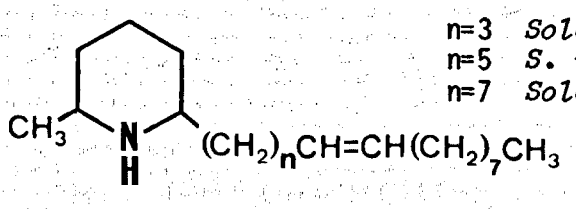
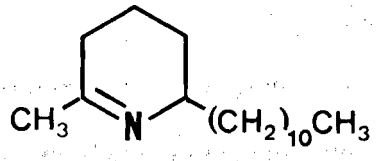
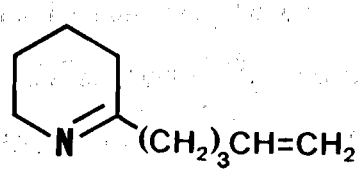
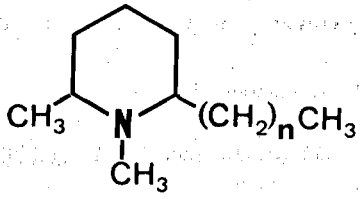
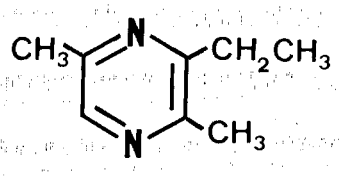
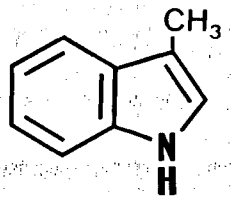
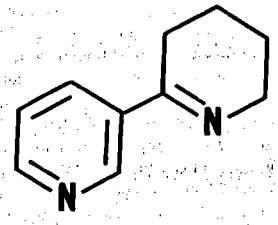
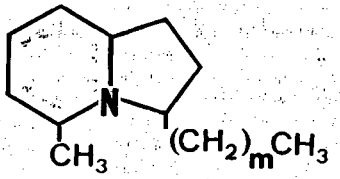
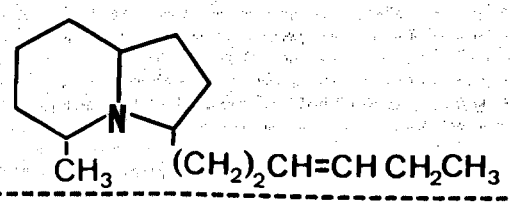
Structure		Source	References
[26] 	n=4 n=6 n=8	*Monomorium sp.*	156 157 160
[27] 		*Monomorium sp.*	157
[28] 		*M. latinode*	156
[29] 		*Monomorium sp.*	156
[30] 		*Monomorium sp.*	156
[31] 	n=6 n=8 n=8 n=8 n=10 n=10 n=12 n=14	*S. richteri* *S. carolinensis* *Solenopsis sp.* *S. richteri* *S. littoralis* *Solenopsis sp.* *S. littoralis* *S. invicta*	161 156 162 161,163 156 162 156 162,163
[32] 	n=3 n=5 n=7	*Solenopsis sp.* *S. invicta* *Solenopsis sp.*	162,163 162,163 162,163

Table 8. (Continued)

Structure		Source	References
[33]		*S. xyloni*	163
[34]		*S. (Diplorhoptrum)?*	156
[35]		n=8 *S. carolinensis* n=10 *S. pergandei*	156 156
[12]		*Atta sexdens* *Myrmica sp.*	108 109,110
[36]		*Pheidole fallax*	164
[37]		*Aphaenogaster fulva*	165
[13],[38]		m=3 *M. pharaonis* [13] m=5 *Solenopsis sp.* [38]	111,112 156,166
[39]		*M. pharaonis*	156

subgenera, *Diplorhoptrum* and *Euophthalma* produce only very small quantities of alkaloids in their venoms, compared to the true fire ants of subgenus *Solenopsis*. The venom of species of *Diplorhoptrum* and *Euophthalma* subgenera also contain 2-alkyl-6-methylpiperidines [31] but only *trans* isomers were reported in contrast to both *cis* and *trans* isomers found in the ants of *Solenopsis* subgenus[156]. Furthermore the novel N-methylpiperidines [35] reported from *S. pergandei* and *S. carolinensis* appear to be unique to thief ants of *Diplorhoptrum* subgenus[156]. A unique mono substituted piperideine [34] has also been reported from a *Solenopsis*(Diplorhoptrum) species[156].

The South African species, *Solenopsis punctaticeps* is more closely related to thief ants than fire ants. Although *S. punctaticeps* can sting, the reaction of humans to their venom is mild compared to that encountered with the stings of true fire ants. The chemical composition of the venom of *S. punctaticeps* showed a marked difference to the fire ant venom by the absence of dialkylpiperidines, but instead it is fortified with a number of 2,5-dialkylpyrrolines and -pyrrolidines [25-21][156].

The alkaloidal venoms are not restricted only to the genus *Solenopsis*, many species of *Monomorium* also have an array of alkaloids in their venoms. Jones *et al.*[157] examined the venoms of number of species of *Monomorium* and found all the species, produce mixtures of different proportions of *trans*-2,5-dialkylpyrrolidines [25], *trans*-2-alkyl-5-alkenylpyrrolidines [26], *trans*-2,5-dialkenylpyrrolidines [27], *trans*-2,5-dialkyl-N- methylpyrrolidines [28], *trans*-2-alkyl-5-alkenyl-N-methylpyrrolidines [30], 2,5-dialkyl-1-pyrrolines [21] and 2,5-dialkenyl-1-pyrrolines [23-24][156,157]. Although the purpose of these species-specific mixture of alkaloids to the ants is not clear, at least they are useful for the chemotaxonomist because they provide a distinctive label to a species. The venom of the old world species *M. pharaonis* is particularly distinctive in containing, in addition to four dialkylpyrrolidines, two indolizidines [13,39][112,156].

A minor constituent, methyl 4-methylpyrrole-2-carboxylate [11], identified in the venom of *Atta texana*[106], *A. cephalotes*[107] and *Acromyrmex octospinosus*[86] is a component of their trail pheromone. Similarly 3-ethyl-2,5-dimethylpyrazine [12] from the venom of *Atta sexdens* [108] and many species of *Myrmica*[109,110], releases trail following behaviour on the respective worker ants. Although many trail pheromones that originate from the poison glands (Table 2) are not yet chemically identified, they can well be expected to be nitrogenous compounds of a similar nature. Skatole [36] and anabaseine [37] are two further alkaloids isolated from two myrmicine ants, *Pheidole fallax*[164] and *Aphaenogaster fulva*[165] respectively. The venom of the myrmicine ant *Myrmicaria natalensis* is distinctively aberrant and unusual. The reported[170] presence of monoterpene hydrocarbons, such as α-pinene, camphene, β-pinene, sabinene, β-myrcene, α-phellandrene, α-terpinene, limonene and terpinolene, in the poison gland of *M. natalensis* may puzzle anyone interested in the phylogeny of the venom gland.

The very highly volatile constituents present in nanogram quantities in the poison glands of *Myrmica rubra* and *M. scabrinodis* were reported to be simple alkanes, alcohols and carbonyl compounds containing one to five carbon atoms[171].

All reported evidence given so far clearly show the diversity of ant venoms and it would not be far from the truth to conclude that the ant venom gland is the most versatile biosynthetic tissue that has been evolved by social hymenopterans.

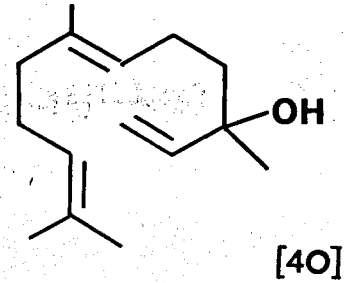
1.6.3 Dufour gland substances

In 1841, Dufour first described this gland in ants and bees[172]. In most of the social Hymenoptera the gland is superficially stereotyped and appears like an elongated sac, lined with simple cuboidal or columnar cells. The Dufour gland of ants is a remarkable hydrocarbon biosynthetic unit. Over 50 alkanes and alkenes have been identified in the Dufour gland and the

reports up to 1974 have been reviewed comprehensively by Blum and Hermann 70,147.

Aliphatic hydrocarbons within the range C_9 to C_{27} are present in the Dufour glands of myrmeciine[173], ponerine[173], pseudomyrmecine[70], myrmicine[70], formicine[70] and dolichoderine[70] ants. The Dufour glands of ants are typically filled with linear hydrocarbons but not exclusively. Branched chain hydrocarbons and many oxygenated compounds can also be encountered as Dufour gland constituents of many ants. The formicine ants are specially noted for their ability to produce a variety of oxygenated compounds in their Dufour gland. These compounds produced by a number of species of *Formica*, *Lasius* and *Camponotus*[174-178] include, a variety of primary aliphatic alcohols (C_{10}-C_{16}), simple ketones (C_{13}-C_{19}), alkylacetates (C_9OAc-C_{18}OAc) and a few terpenoid derivatives like farnesyl acetate[175] and geranylgeranyl acetate[179]. The presence of highly volatile oxygenated compounds such as simple alcohols, aldehydes and ketones in the C_1-C_4 range, have also been reported in myrmicine ants of the genus *Myrmica*[171].

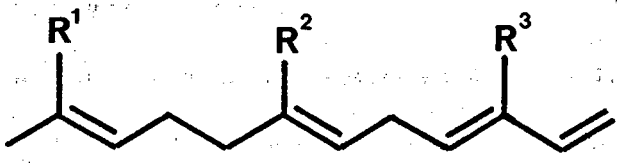
The Dufour gland contents of a number of species of the genus *Myrmica* have been thorougly investigated[71,180,181]. Beside the general linear hydrocarbon theme, a few species of *Myrmica* also have terpenoid hydrocarbons, sometimes even as the major components in the Dufour gland[180]. These terpenoid hydrocarbons were identified by Morgan and Wadhams, on mass spectral evidence as farnesene, homofarnesene and bishomofarnesene[181]. Subsequently, a trishomofarnesene had also been described from *M. scabrinodis*[180]. Parry[182] identified the farnesene isomer from the *Myrmica* ants as (*Z*,*E*)-α-farnesene [17] by the comparison of its mass spectrum and GC retention times, with those of a mixture of six farnesene isomers prepared from the dehydration of nerolidol [40].



(E)-nerolidol

[40]

The structure was recently confirmed by total synthesis[183]. On the basis of their mass spectra, structures [**41**] and [**42**] have been proposed by Morgan and Wadhams[181] for the homofarnesene and bishomofarnesene isolated from the Dufour gland of *Myrmica* ants.



[**41**] $R^1=R^3=Me$, $R^2=Et$

[**42**] $R^1=R^2=Et$, $R^3=Me$

The primary functions of the Dufour gland appear to be defence and communication. Blum[184] has suggested the diverse chemical compounds found in the Dufour gland could function to overstimulate the olfactory receptors of predators, hence act as a deterrent. According to Bergstrom and Lofqvist[176] these compounds are often used as alarm pheromones. Bradshaw *et al.*[185] have demonstrated in the African weaver ant *Oecophylla longinoda*, how undecane from the Dufour gland and formic acid from the Poison gland act synergistically to release a "mass attack" reaction. The Dufour glands of slave keeping formicine ants such as *Formica subintegra*, *F. pergandei* and *F. sanguinea* produce large quantities of C_{10}-C_{14} acetates, which are sprayed during slave raids to excite and attract the slave maker-ants but panic and disperse the slave-defender species[174,186]. Cammaerts *et al.*[71] have demonstrated that the ants of the genus *Myrmica* use their Dufour gland contents as a territorial marking pheromone.

In addition to the roles discussed above, some ant species utilize trace components of the Dufour gland as trail pheromones (Table 2 and 3). The few compounds identified from the Dufour glands as trail releasers include one sesquiterpenoid aldehyde, faranal [**15**][90] and a few sesquiterpenoid alkenes [**20-16**][118,120].

It is possible to conclude that the Dufour gland has evolved a

long way from the suggested original functions, such as providing a lubricant for the sting or eggs during oviposition[187]. And it has assumed the novel duties to act as an important social organ to carry out a number of functions in defence and communication.

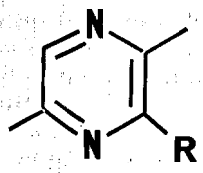
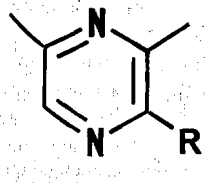
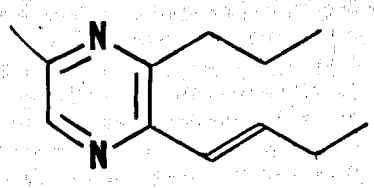
1.6.4 Mandibular gland substances

Mandibular glands are found in most insects and probably in all ants. Apart from the variation in size (in *Camponotus* it is remarkably large and extends up to the abdomen[188]), the mandibular glands appear to be similar in anatomy in all ant species. The glands are associated with the mesal side of the mandibular base and consists of a reservoir and a true glandular region.

The mandibular gland secretions are also utilized for both defensive and pheromonal functions. A variety of natural products have been identified in the mandibular glands of the ants. Blum and Hermann[70] have reviewed the reported compounds up to 1974. The different subfamilies of ants appear to biosynthesize different classes of compounds in their mandibular glands.

The dolichoderine ant, *Iridomyrmex humilis* produces three trisubstituted pyrazines in its mandibular glands[189]. No other information is available about the chemistry of dolichoderine mandibular glands. The ponerine ants are also well known for their ability to produce pyrazines in their mandibular glands. Recently a novel alkylpyrazine [43] was reported from the mandibular glands of a myrmicine ant, *Aphaenogaster rudis*[190]. The alkylpyrazines reported from the mandibular glands of ants are listed in Table 9. The ability to biosynthesize alkylpyrazines does not appear to be unique to ants because many other insects, like wasps[190] and flies[195] also produce a variety of alkyl pyrazines. Some ponerine ants have the ability to produce sulfides[196], ketones[197], alcohols[197] and a salicylate ester[70] in their mandibular glands. The general theme of mandibular gland contents of

Table 9. Alkylpyrazines identified from mandibular glands of ants

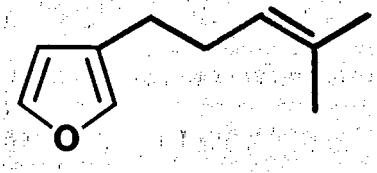
Structure	Source-species	Subfamily	References
			
R=Pr	*Iridomyrmex humilis*	Dolichoderinae	189
R=isobutyl	*Anochetus sedilloti*	Ponerinae	192
R=sec-butyl	*A. sedilloti*	Ponerinae	192
R=pentyl	*A. sedilloti*	Ponerinae	192
R=isopentyl	*Odontomachus hastatus*	Ponerinae	191
	O. clarus	Ponerinae	191
	O. troglodytes	Ponerinae	192
	Ponera pennsylvanica	Ponerinae	193
	Hypoponera opacior	Ponerinae	193
	Iridomyrmex humilis	Dolichoderinae	189
	Rhytidoponera metallica	Ponerinae	194
R=(E)-styryl	*Iridomyrmex humilis*	Dolichoderinae	189
R=citronellyl	*Rhytidoponera metallica*	Ponerinae	194
			
R=Et	*Odontomachus brunneus*	Ponerinae	191
	O. troglodytes	Ponerinae	192
R=Pr	*O. brunneus*	Ponerinae	191
R=butyl	*O. brunneus*	Ponerinae	191
	O. troglodytes	Ponerinae	192
	Anochetus sedilloti	Ponerinae	192
	Brachyponera sennaavensis	Ponerinae	192
R=isobutyl	*Anochetus sedilloti*	Ponerinae	192
R=sec-butyl	*A. sedilloti*	Ponerinae	192
R=pentyl	*Odontomachus brunneus*	Ponerinae	191
	O. troglodytes	Ponerinae	192
	B. sennaavensis	Ponerinae	192
R=hexyl	*O. troglodytes*	Ponerinae	192
	*Aphaenogaster rudis*	Myrmicinae	190

[43]

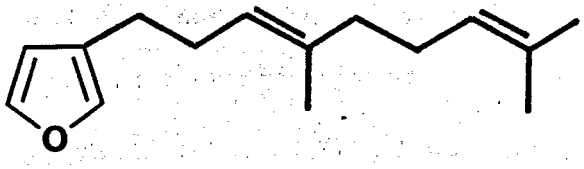
doryline[70] and pseudomyrmecine[70] ants is aliphatic ketones.

The mandibular gland secretions of myrmicine and formicine ants have been more extensively studied than those of other ant subfamilies. The myrmicine ants biosynthesize an abundance of homologous 3-alkanones and the corresponding 3-alkanols of the C_6-C_{11} range. The 4-methyl-3-heptanone in the mandibular glands of *Atta texana* is biosynthesized with an stereospecific exactitude, to yield the (S)-(+)-isomer only[29]. But the 4-methyl-3-heptanol found in the same gland was a racemate[29]. However the 4-methyl-3-hexanol found in *Tetramorium impurum* is only the (3R,4S)-optical isomer[198]. Many 3-alkanols were reported from the myrmicine ants but no information was available about their chiral composition at the beginning of the present study. Although not particularly characteristic, the presence of compounds such as citral[199], citonellol and geraniol[200] in a few attine ants and some aromatic carbonyl compounds in *Veromessor pergandei*[201], demonstrates the biosynthetic versatility of the mandibular glands.

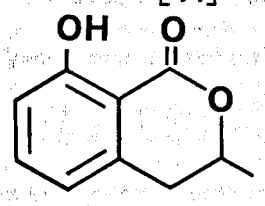
The formicine mandibular gland is also a rich source of natural products. Besides the alkanones and alkanols, the presence of distinctive terpenes[175], lactones and a few aromatic compounds are also reported. Particularly distinctive compounds are perillene [**44**][202], dendrolasin [**45**][203], geranylcitronellal[175] (a diterpene), mellein [**46**][204] and massoilactone [**47**][205].



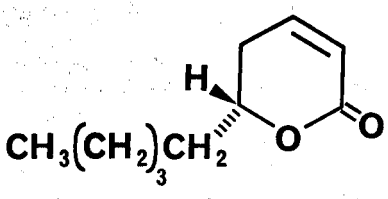
[**44**]



[**45**]



[**46**]



[**47**]

In the present study the chemical composition of the mandibular gland contents of *Myrmica albuferensis* and the chirality of 3-octanol from

the mandibular glands of few species of *Myrmica* were investigated.

1.6.5 Postpharyngeal gland substances

The postpharyngeal glands of the ants are a pair of glove-shaped structures overlying the brain. These glands can occupy a large portion of the head and open separately into the posterior portion of the pharynx[206] (Figure 1). The function of the postpharyngeal gland has been the subject of numerous reports but the information available is insufficient to make any definite conclusions. Bugnion[207] had suggested that the postpharyngeal glands play a role in larval feeding. Forbes and Mcfarlane[207] have speculated that the glands serve in the digestive activities of the individual ant. The lipase activity has been found to be very low therefore the glands are not involved in lipid digestion[209]. Phillips and Vinson[210] had claimed the glands function as a cephalic caecum and the major lipid components come from the food but Thompson *et al.*[211] recently discovered that the major class of compounds in the postpharyngeal glands of *Solenopsis invicta* queens were hydrocarbons. The suggestion of Thompson *et al.*[211] that the postpharyngeal gland may be important in overall colony organization, caste determination, food exchange and queen and brood tending, awaits proof by appropriate bioassays. The 10-fold increase of the gland weight per unit body length in queens compared with that of minor worker of *S. invicta* suggests a special function for the gland in queens[206]. Also Glancey[211] had observed the glands become disproportionately large in virgin queens and are filled with fluid prior to their nupital flight.

Very few chemical analyses have been reported on the composition of the postpharyngeal gland. Usually the contents are simply described as a yellow oil. Vinson *et al.*[212] found the composition of the hexane soluble material of postpharyngeal glands of newly mated *S. invicta* queens to be, 63% hydrocarbons, 19% free fatty acids, 13% glycerol esters, 6% steroids and a trace of wax esters. The hydrocarbon fraction was analysed by Thompson *et*

al.[211], who found four major methyl-branched hydrocarbons of the C_{28}-C_{29} range. Vander Meer *et al.*[213] found that the total hydrocarbon content of the gland showed a marked increase at 15 days after mating, which suggested that the queen has the biosynthetic capacity to produce these materials herself. The increase of hydrocarbon levels in the postpharyngeal glands coincides with wing muscle histolysis. The hydrocarbon level decreased to the original level after 15 days and during this period the free fatty acid and triacylglycerol concentrations remained the same.

In the present study the postpharyngeal gland contents of *S. geminata* workers were investigated.

1.7 Methodology

1.7.1 General

Butenandt *et al.*[3], needed more than 500,000 female silkworm moths to isolate 6 mg of bombykol [1]. Even in 1971, Tumlinson *et al.*[134] required 3.7 kg of leaf cutting ants, *Atta texana* to identify its trail pheromone. This amount of material and work involved are no longer representative because of the availability of modern and more sophisticated techniques. For example, the identification of the sex pheromone of the artichoke plume moth, *Platyptilia carduidactyla* required only 20-30 virgin female moths[214]. Similarly the trail pheromone of the ant, *Myrmica rubra* has been identified using only 50 worker ants[109].

The main difficulty encountered by the pheromone chemist is the elucidation of structures of compounds, present in ng to μg quantities among a large excess of extraneous material. Although the strategy that needs to be adopted to identify a pheromone depends heavily on the particular example, two distinctive approaches can generally be recognized. These two analytical schemes are shown in Figure 2. The scheme I is the more conventional approach, although it requires bulk quantities of insect material, it may yield sufficient pure pheromone components for complete spectral identification. However, sometimes it may be very difficult to accumulate the required quantity of insect material. In such circumstances Scheme II becomes more useful because its requirements are extremely economical in both biological and chemical material.

1.7.2 Bioassay

The chemical investigation of insect pheromones is dependent on appropriate bioassays that can be carried out conveniently in the laboratory. The bioassay provides the analyzing chemist with a method to monitor the biological activity of a substance qualitatively and quantitatively, using

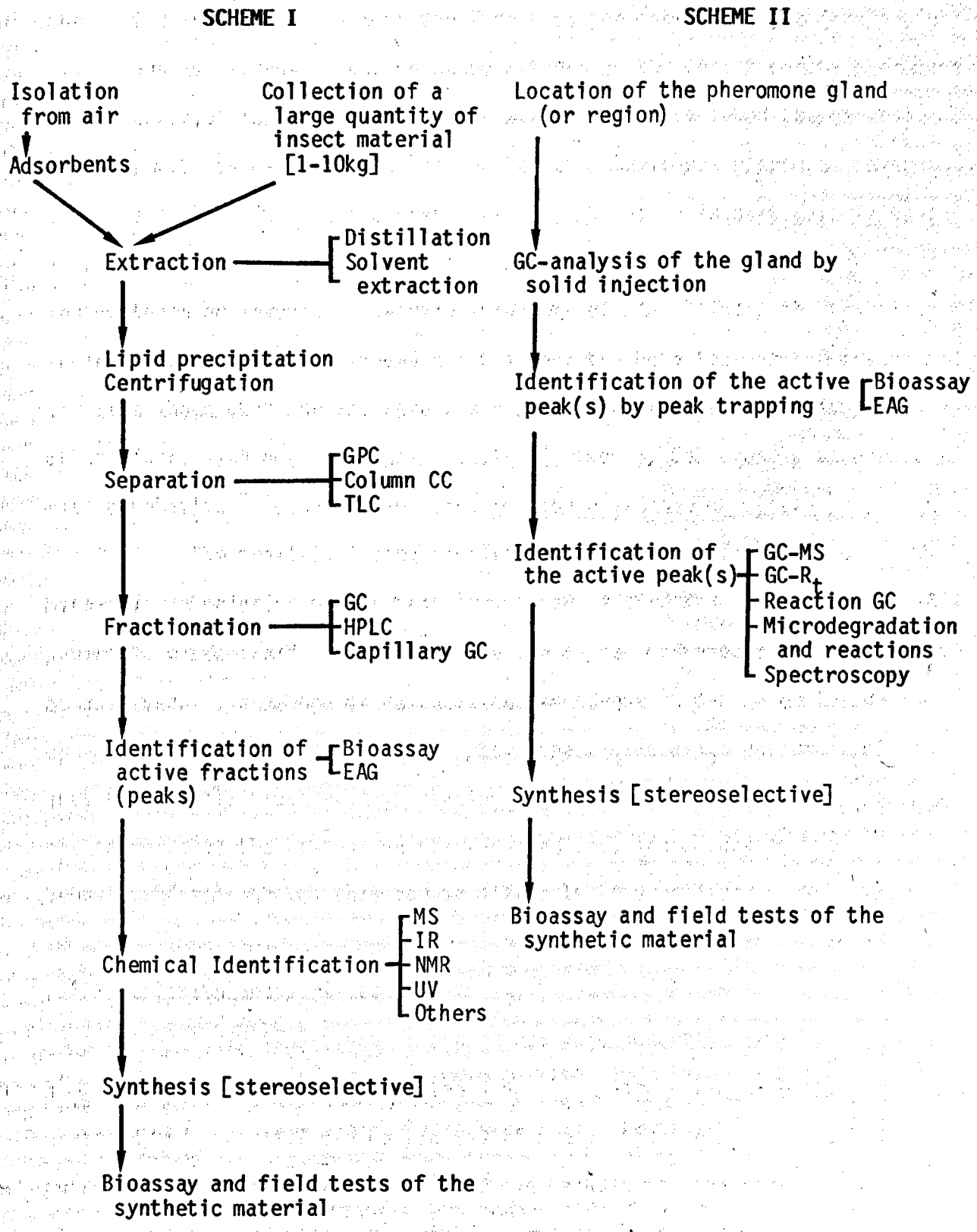


FIGURE 2. General schemes adapted for the isolation and identification of pheromones.

the living insect or a part of the insect as the detector. The develpoment of a bioassay depends on the response pattern of the insect to the pheromone under investigation. Young and Silverstein[215] have reviewed the general bioassay methods available to monitor insect behavioural responses toward semiochemicals. The electroantennography (EAG) has been particularly useful as a convenient bioassay, especially in the characterization of many lepidopteran pheromones. The main drawbacks of EAG include, its inability to indicate how an active compound might affect the behaviour of the insect and the likelihood that the antennae may respond to irritants, inhibitors and other odours with no pheromonal activity. However, EAG closely integrated with a behavioural assay is an asset for the pheromone chemist.

The trail-following activity is relatively simple to monitor by bioassay. A solution of the test sample can be streaked over a surface of paper[216], cardboard[106] or glass[217] and the degree of response of insects can be observed. The method of Pasteels and Verhaeghe[218] can be employed conveniently to bioassay ant trail pheromones. The alarm pheromones of the ants are usually bioassayed by placing solutions of test samples on paper at the entrance of the nest or on the foraging area and observing the behaviour[29,219], though this can be difficult to quantify.

1.7.3 Isolation and purification

Early isolations of pheromones were generally made by homogenizing a large number of whole insects[220] [Scheme I]. A large excess of unwanted body material can be avoided if the particular part where the pheromone is localized in the insect, can be used for the isolation [Scheme II]. The amputated parts or excised glands may be rinsed or extracted with solvent. The volatile pheromones can also be collected by steam distillation or short-path vacuum distillation[181]. When the pheromones are stored in distinctive glands the contents can sometimes be discharged by force ("*milking*")[85] or the glandular liquid can be withdrawn into glass

micro-capillaries[221]. The other methods of collection of pheromones include, isolation from faeces and frass, passing air over live insects (aeration) into a cold trap, solvent or absorbent (Porapak, Tenax), and extraction of the absorbent surfaces used to line the cages of insects[215].

The pheromonesmust be purified before they can be identified. Numerous chromatographic methods have been used for the purification and fractionation of pheromone extracts. The chromatographic methods, besides the separation also provide some information about the size and polarity of the substances under investigation.

The relatively new method of gel permeation chromatography (GPC) offers a way to prepurify the extracts. GPC is generally used to get rid of higher molecular weight impurities (1000 and above) but with suitable columns it can even be used to separate substances of molecular weight between 100 and 300[222]. Klun *et al.*[222] used Styragel 100 μA° to isolate the sex attractant of the European corn borer *Ostrina nubilalis*, achieving separations of compounds of molecular weight between 235 to 275. Similarly Sephadex LH20 and Poragel 60A (37-75 μm) have been used to isolate the sex pheromones of the cockroach, *Periplanata americana*[223] and the tobacco budworm, *Heliothis Virescens*[224] respectively. In the latter example, they were able to obtain a 20 ml fraction that contained all the active pheromone from a crude extract of 2500 *Heliothis Virescens* females and the fraction was pure enough for direct gas chromatographic separations[224].

Column chromatography is often used in the first steps of purification. Since many pheromones are olefinic, silver nitrate impregnated columns (Argentation chromatography) have proved useful in the separations of alkenes according to the number of double bonds. The (E)-isomer always elutes before the (Z)-isomer.

High pressure liquid chromatography (HPLC) has become one of the most effective method of pheromone purification in recent years[224]. Two different kinds of HPLC methods are recognized. In the normal phase

technique a polar bound phase and an non-polar mobile phase are used and as a result the less polar compounds are eluted first. In the reverse phase technique a non-polar bonded phase and a polar mobile phase (e.g. methanol, acetonitrile, water) are used, hence the more polar substances will first leave the separation system. One limitation of the HPLC technique is the unavailability of a sensitive universal detector. The detection limit of a refractometer detector is about 1 μg. Therefore sometimes it is necessary to derivatize the compounds before separation.

Thin layer chromatography (TLC) is a simple and fast procedure that can often be used for isolation and purification of insect pheromones. This method has been improved recently to yield high precision separations (HPTLC)[225].

Gas chromatography (GC) is almost universally used in pheromone isolation and purification. GC can be used on a micro-preparative scale by splitting the effluent after separation and collecting one part. The various collection methods available have been reviewed[39,215,226]. The collection devices described by Brownlee and Silverstein[227] and Baker *et al.*[228] permit efficient trapping of substances from the GC effluent. The pheromone glands or distinct parts can be directly introduced in to the gas chromatograph by solid injection techniques[229-232]. The solid injection technique has some definite advantages over the usual method which uses solutions. The advantages include no dilution, no impurities can come from the solvent, no peak overlapping due to solvent tailing and the gas chromatograph can be operated at its maximum sensitivity if necessary.

1.7.4 Structure elucidation and identification

Rapid progress in the identification of pheromones has been made in the past few years due to the improvement in analytical instrumentation and special micromethods.

a. *Chromatographic methods*

The Kovats[233] system of GC retention indices can provide considerable information about the polarity, molecular size and presence of certain functional groups in an unknown compound. The comparison of known and unknown compounds by GC, TLC and HPLC can be helpful to establish the identity of the unknown compound. The capillary gas chromatography is especially useful for this purpose because of its high resolution capabilities.

The direct separation of (Z)- and (E)- isomers of alkenes by gas chromatography offers considerable difficulties. Polar stationary phases incorporated with silver nitrate gave good results but allow temperatures only up to 65 °C and is therefore applicable only for small molecules[234,235]. The use of highly polar phases like diethyleneglycol succinate (DEGS) as the liquid phase on a capillary column allows base line separations of (Z)- and (E)- isomers of alkenes[236]. Argentation HPLC has been employed to separate (Z)- and (E)- isomers[224] even on preparative scales[237]. An interesting modification of this technique uses chemically bonded reversed stationary phase and isopropanol /water/ $AgNO_3$ as the eluent system[231].

Direct resolution of optical isomers by gas chromatography has been achieved using optically active stationary phases[239], but it has been widely applied only to polar nitrogen-containing compounds especially amino acids[240]. Oi *et al.*[241] have prepared a variety of low-molecular weight chiral phases which show stereo-selectivity for alcohols, but the retention times of the compounds studied so far are very long. Recently, Konig *et al.*[242] have resolved isopropyl urethane derivatives of chiral alcohols using a glass capillary column coated with a chiral stationary phase.

The direct separation of enantiomers has also been achieved by HPLC methods. These methods either use a chiral stationary phase[243-247] or an optically active reagent in the mobile phase[248,249]. Audebert[250] has reviewed the literature concerning the direct resolution of enantiomers by

liquid chromatography. Recently, Wainer *et al.*[251] have separated enantiomers directly by TLC. The TLC plate has been continuously developed with a chiral absorbent and subsequently used to separate (±)-2,2,2-trifluoro-1-(9-anthryl)-ethanol.

Reaction gas chromatography can yield useful information about the chemical structure of an unknown compound. In this technique the injected chemicals are transformed or retained during the gas chromatographic process.

A commonly employed reaction gas chromatographic technique is termed *carbon-skeleton chromatography*[252,253]. In this method an appropriate catalyst is used at an elevated temperature (200-300 °C) in a pre- or post-column reactor with hydrogen as the carrier gas [227,254,255]. The compounds essentially undergo hydrogenation and hydrogenolysis to strip off all functional groups to yield the parent hydrocarbon or its next lower homologue. Carbon skeleton chromatography has been extended to study even large molecules such as steroids[256]. The GC-hydrogenation of olefinic bonds can be performed under milder conditions (usually 1% Pd on support, 150-250 °C)[257]. By combining with mass spectrometry, hydrogenation can be used to determine the degree of unsaturation and the number of rings present in an unknown compound. Many other reaction gas chromatographic methods are available[258,259], although not widely applied in the study of pheromones.

The use of "*subtraction loops*" placed in the GC pathway can indicate the presence or absence of certain functional groups in the unknown compound[260]. If a peak disappears after passing a ZnO-loop, the presence of an acid is indicated, while with boric acid the signals of primary and secondary alcohols are eliminated[261,262]. Benzidine removes carbonyl compounds and O-dianisidine aldehydes[260]. Benzidine is a potent carcinogen and its use is now banned. But non-mutagenic 3,3',5,5'-tetramethylbenzidene may be a reliable substitute. The structure of disparlure [2] was partly manifested when it was subtracted by phosphoric acid which had been known to

remove epoxides[5]. The substances with oxygen containing functional groups such as alcohols, aldehydes, ketones, esters and epoxides can be subtracted with $LiAlH_4$[261]. The ketones can be distinguished from esters by means of sodium trimethoxy borohydride[262]. The subtraction methods, initially used for packed columns have been extended for the use with capillary columns as well[263].

Reaction thin layer chromatography combined with bioassay can also be useful. Bierl *et al.*[264] used reaction TLC for the determination of epoxide position and configuration in various epoxides including disparlure [2]. Argentation-TLC is useful for the comparison of R_f values of unknown compounds with those of (Z)- and (E)- isomers of structurally related substances.

b. *Microchemical methods*

The information that can be obtained from microchemical reactions is often useful for the determination of the total structure of an unknown compound. Simple microchemical tests performed on an active fraction or even crude extracts coupled with a sensitive bioassay can indicate the functional groups present in the pheromone[265,266]. For example, if a pheromone loses activity after saponification or $LiAlH_4$ treatment and regains it upon acetylation the presence of an acetate group is indicated[267]. Similarly loss of activity after hydrogenation or bromination indicates unsaturation. Inscoe *et al.*[268] have reviewed the chemical reactions that can be carried out on a micro-scale.

The determination of double bond position and geometry is of great importance in pheromone chemistry. Considerable progress has been made recently to develop microtechniques to determine the location of unsaturation. Although a mass spectrum of such a compound could be obtained with a few nanograms of material the double bond position is difficult to determine even with the chemical ionization technique. Suitable derivatives

can often be prepared and the location of the double bond can be subsequently determined by mass spectrometry. These methods include methoxymercuration-demercuration[269-273], deuteration[274,275] and formation of derivatives such as acetonides[276], hexafluoroacetonides[277], silyl ethers[278], methyl ethers[279], N,N-dimethylhydroxyamines[280], ketones[281], dimethyl disulphide adducts[282] and epoxides[283,284]. The alkene can also be oxidised with osmium tetroxide to yield a 1,2-glycol which can be reacted with phenylboronic acid to yield a cyclic boronate. Cyclic boronates are also useful to locate the double bond position by MS[285].

Micro-ozonolysis has been particularly useful to determine the double bond position by examination of the carbonyl fragments produced[286,287]. The main drawback of ozonolysis in a solvent is that it is difficult to identify the small molecules formed as products. A variety of solvent systems has been studied to find the suitable solvents for ozonolysis and subsequent analysis by GC[286,288].

The formation of diastereomeric derivatives with optically pure derivatizing agents are frequently useful in the study of optical isomeric composition of chiral pheromones. The diastereomeric derivatives prepared can be studied by GC, HPLC or NMR analysis. Halpern[289] has reviewed the derivatives available for chromatographic resolution of optically active compounds. A number of chiral pheromones are alcohols. Among the most effective diastereomeric derivatives for alcohols are the N-trifluoro-acetyl-(S)-(+)-alanyl esters [290], N-trifluoroacetyl-(S)-(-)-prolyl esters[291], (R)-(-)-menthyl carbonates[292], (+)-*trans*-chrysanthemoyl esters[293], (S)-acetoxypropionyl esters[198] and (R)-(+)-1-phenyl urethanes[294]. The enantiomeric composition of sulcatol [4] was determined by NMR analysis of the corresponding (R)-1-methoxy-1-trifluoromethylphenylacetate[295].

c. *Spectrometric methods*

The pheromone chemist can frequently elucidate structures using microgram quantities or less of material because of recent advances in spectroscopic techniques.

Mass spectrometry (MS), usually combined with gas chromatography is almost universally employed in pheromone studies. Modern mass spectrometers are capable of giving spectra of one ng or less of sample. The electron impact ionization mass spectrum (EI-MS) can sometimes give the molecular weight of the compound as the most important single piece of information available from MS. The presence of certain functional groups and branching in molecules can often be deduced from the fragmentation pattern of the MS. If the compound is already known a comparison of its MS with the compiled MS literature[296] or computer MS data banks may lead to its identification. High resolution MS can determine the mass of the molecular ion (if visible) and other fragment ions up to three or four decimal places. An accurate molecular formula can be derived from this information. When the molecular ion is unstable or short lived, no direct information can be obtained about the molecular weight of the compound by EI-MS. In these circumstances chemical ionization mass spectrometry (CI-MS) becomes useful. CI-MS may use a reagent gas as the carrier gas in the GC-MS system, which may abstract or add a proton from or to the investigated substance. The "quasi molecular ion" thus formed can be observed in the MS. CI-MS technique has been extended to locate the double bond positions in compounds[297]. Vinyl methyl ether can be used as a reagent gas and the fragmentation pattern of the 4-centered addition complex can locate the double bond position[298-300]. Mass fragmentography, or single or multiple ion monitoring method can improve the sensitivity of the instrument to subnanogram levels. However, no complete MS is obtained but the intensity of a particular ion(s) is monitored at the expected retention times. Apart from studying the actual mass spectrum of a pheromone, sometimes more information can be derived by

examining the MS of a particular derivative prepared from it. The general derivatives available were mentioned under microchemical methods[301].

Ultraviolet spectroscopy (UV) can show absence or presence of conjugation in a compound. A number of pheromones have conjugated systems therefore purification and fractionation of such compounds by HPLC can be monitored by highly sensitive UV-detectors.

Infrared spectroscopy (IR) is valuable in functional group analysis and by mean of micro IR-cells and Fourier transform IR-spectrophotometers it is now possible to obtain spectra of submicrogram samples[107,223,302]. Beside the general IR-absorptions diagnostic of functional groups like carboxyl, hydroxyl etc., the band at 970 cm^{-1} is especially useful for confirming the E-configuration of olefinic bonds.

Nuclear Magnetic Resonance spectroscopy (NMR) is probably the most useful method for structural determinations but until recently its application in pheromone identification has been restricted because of the requirement for a relatively large sample (100 µg). The use of micro-NMR-sample tubes (75 µl sample cavity),computer accumulation of large number of spectra (CAT) and pulse-Fourier transform technique have considerably improved the NMR technique to reduce the required sample size. In the recent literature, there are examples of usable NMR spectra obtained from 1-2 µg of sample[223,224]. The interpretation of NMR spectra with overlapping peaks is often difficult but the use of lanthanide shift reagents can sometimes lead to simplified spectra[303]. NMR has been used to determine optical isomeric composition of chiral pheromones either directly by the use of a chiral shift reagent[303-305], or indirectly by the preparation of a diastereomeric derivative and subsequent examination with an achiral shift reagent[303,305]. ^{13}C-NMR has become a powerful tool in structure elucidations but at present the large sample sizes required (1 mg or more) limits its use in pheromone studies. However, it has proved useful in the investigation of structure and purity of synthetic pheromones[306]. Once the lack of

sensitivity will be overcome, ^{13}C-NMR will undoubtedly be a primary method of microstructure elucidation in future.

d. *Other physical techniques*

Although a few other techniques are available for structural studies, these methods are not frequently used in pheromone work because relatively large samples are required. If the required amount of sample can be supplied the following methods are useful in structural studies. Melting point and optical rotation are characteristic of a particular compound. Optical Rotatory Dispersion (ORD) and Circular Dichroism (CD) can predict the absolute stereochemistry of a chiral centre or a molecule. X-ray or electron diffraction methods are valuable to study extremely complex crystalline molecules.

1.7.5 Synthesis

To confirm a proposed structure for a pheromone and test its activity it is necessary to synthesize it. Synthetic samples are also required for field tests in order to check its viability as an insect control agent. Insect pheromones generally have simple structures. However, many of the pheromones contain double bonds and/or chiral centres. Because of this fact, synthetic methods with high degree of stereochemical purity and selectivity are required for pheromone synthesis. Many recent and comprehensive reviews are available on insect pheromone synthesis[39-41,307-310].

DISCUSSION

The main objective of the present study was to investigate the volatile compounds produced by some myrmicine ants. The details of that study are discussed in the later sections. The interest in the volatile chemicals arose from the fact that ants utilize chemicals as their major channel of communication. These substances serve to regulate the colony organization and social order. They may also act as defensive secretions, to repel as well as to cripple or kill their predators. The identification, synthesis and test of the ethological activity of the substances produced in the exocrine glands of the ants can be useful in the integrated control of pest species. Furthermore, the information on the composition of the exocrine glands of the ants is useful to differentiate between morphologically similar species. The result can also be used as an indicator of the phylogeny of a particular species.

The handling and identification of minute amounts of substances (μg to ng) from the exocrine glands can be facilitated by micro-methods and it has been the policy in this laboratory to work with single insect glands as far as possible.

A part of the present study was devoted to develop novel micromethods and improve already known techniques. Subsequently, these techniques were applied to investigate the volatile chemicals found in some myrmicine ants.

2.1 Micro-analytical Techniques used with GC

The direct injection of a piece of biological tissue, such as an insect gland, into the gas chromatograph has a number of advantages over using a solution. The solventless technique avoids dilution and introduction of impurities via the solvent. Furthermore, because there is no solvent tail, the GC can be operated at its maximum sensitivity. A number of solid injection techniques are available[191,229,231,232]. The method of Morgan and Wadhams[229] has been particularly useful and was used throughout the present study.

2.1.1 Trapping and rechromatography

A solvent is usually required to trap a single substance from the GC effluent for bioassay or for rechromatography in a different system[228]. The substance eluting from the column is either passed directly into the cooled solvent or trapped in metal or glass tubes and washed subsequently with solvent[311-313]. The solvent can introduce impurities that may completely obscure the desired compound present in nanogram quantities. Furthermore, the solvent prevents the rechromatography of the total sample efficiently.

In order to overcome these difficulties a method has been developed for trapping minute samples and reinjecting them efficiently and totally onto the column. A splitter[228] was fitted to the end the column and the substances from the GC effluent were trapped in glass capillaries. After sealing both ends of the capillary tube it was reintroduced into the gas chromatograph by the solid injection technique[229]. A mixture of tetradecane and pentadecane in hexane was used to determine the efficiency of trapping. Figure 3 shows typical chromatograms obtained from trapping and rechromatography of the pentadecane peak. The split ratio was 95:5 (outlet:FID) as determined by the peak areas with and without the use of the splitter. It was found that the trapped material can be rechromatographed

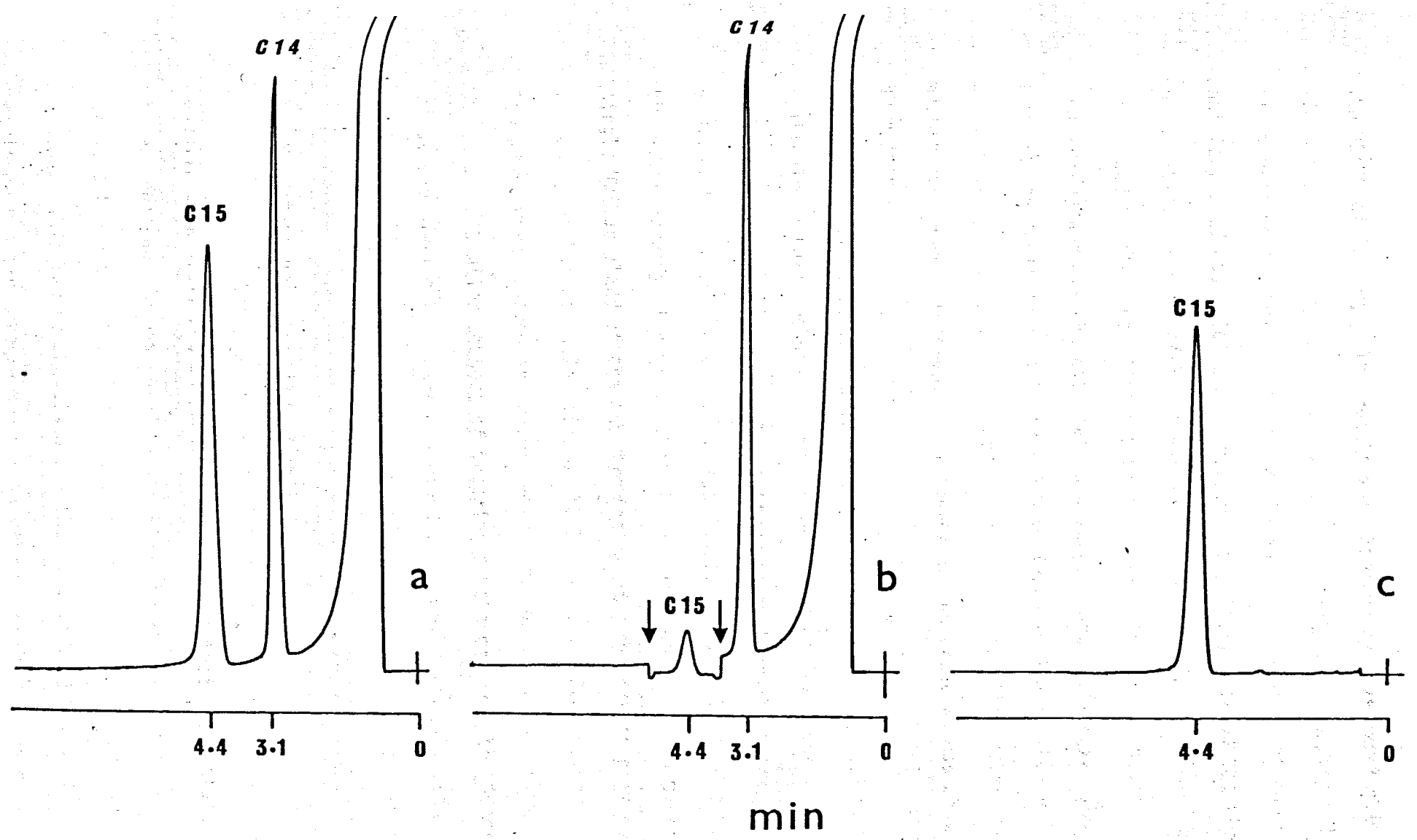


FIGURE 3. Efficiency of trapping and rechromatography of a GC peak. . Chromatograms on SE-30 column at 145 °C: (a) 250 ng each of tetradecane and pentadecane in 0.5 µl of hexane; (b) same amount injected and effluent between the arrows was trapped; (c) total trapped material was reinjected.

with 85±5% efficiency. The result was obtained by five replicate determinations, using pentadecane. But obviously the trapping efficiency will depend on the boiling point of the sample. The size of the glass capillary used was critical. A narrower capillary altered the flow rate and split ratio, and more effluent was vented to the FID. A larger capillary introduced problems of efficient trapping, and it was not possible to rechromatograph all the material in a large, long capillary. The optimum size of the capillary was 45 mm x 0.5 mm (0.45 mm i.d.). Lower flow rates of the carrier gas gave better trapping efficiencies. Flow rates above 50 ml/min decreased the trapping efficiency and no trapping was observed above 80 ml/min.

Nanogram to microgram quantities of pure material were made available by this technique for microreactions or for rechromatography on a different stationary phase. The latter was employed to check the homogeneity of GC peaks. Figure 4 shows the application of this technique to demonstrate the heterogeneity of a particular GC peak in a chromatogram obtained from a single Dufour gland of the ant *Myrmica rubra*. The single peak corresponding to 200 ng at the retention time of 14.0 min on the PEGA column, split into two peaks when trapped and rechromatographed on the SE-30 column.

Similar methods of trapping substances from the GC effluent into glass capillaries have been described by Brownlee and Silverstein[227], Stanley and Kennet[314], and Cronin and Gilbert[315].

2.1.2 Melting points of trapped solids

The trapped substances from the GC effluent were restricted usually to a small region (usually about 1 cm) inside the glass capillary tube. This was demonstrated by the trapping of solid substances. Figure 5 shows a few micrograms of myristic acid trapped from the effluent of a FFAP column. This led to the discovery that the melting points of the solids that could be gas chromatographed can be determined with great accuracy using only

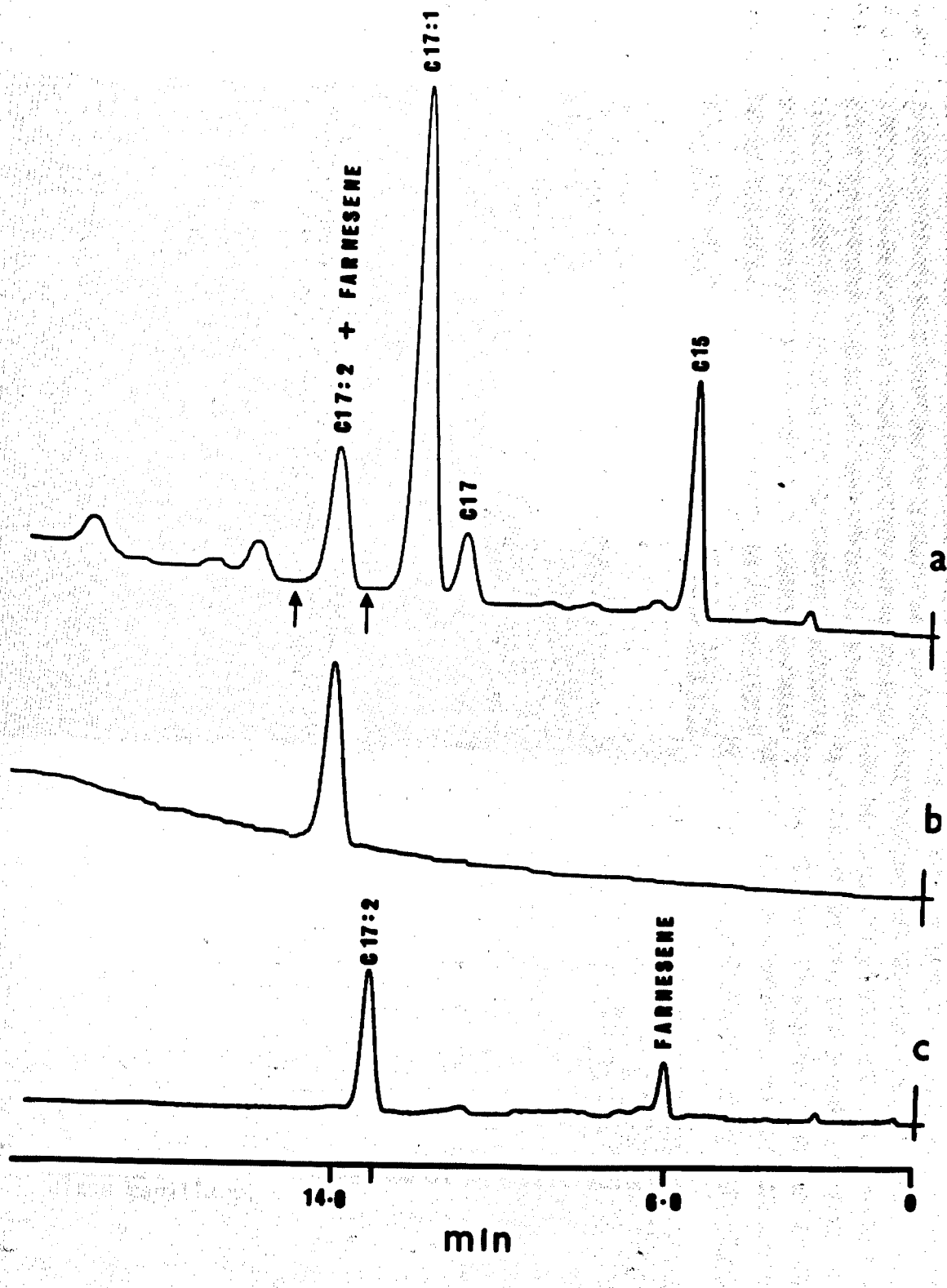


FIGURE 4. **Determination of homogeneity/heterogeneity of a GC peak.** (a) Chromatogram of one Dufour gland of Myrmica rubra with the oven temperature programmed from 125 to 162 °C at 2 °C/min, on 10% PEGA column. (b) Effluent between the arrows of (a) was trapped and rechromatographed on 10% PEGA column. (c) Same as (b) but rechromatographed on 5% SE-30 column with temperature programmed from 140 to 192 °C/min.

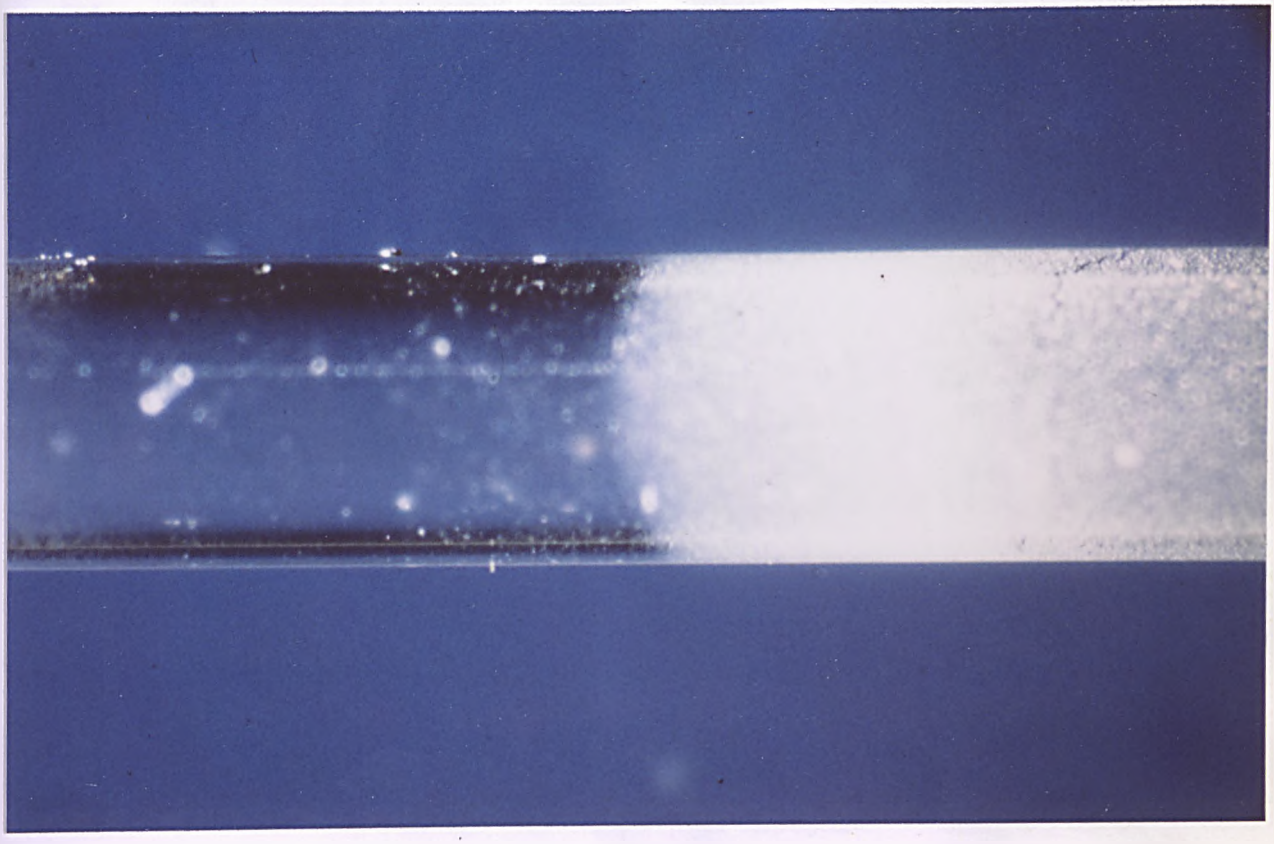


FIGURE 5. A few micrograms of myristic acid trapped from GC effluent, in a glass capillary.

a few micrograms or less of material. The glass capillary with the trapped substance was placed directly on the hot stage of the melting point apparatus. The melting pointsobserved were very sharp because of the high purity of the samples collected from the GC effluent.

A few of the reported insect pheromones are solids[39,316]. Therefore this method can be applied to determine the melting points of micro samples of solid pheromones and similar natural products. Furthermore, it gives a novel, non-destructive method to compare micro amounts of natural products with authentic material.

2.1.3 Epoxidation

One of the methods available to determine the position and geometry of olefinic bonds is epoxidation. The epoxides can be readily prepared by the reaction of the alkene with m-chloroperbenzoic acid[317]. EI-MS of simple epoxides can locate the position of the oxirane ring[283]. For more complicated epoxides, CI-MS has been successfully employed[284]. Furthermore, epoxidation has been used to determine unsaturation in polymers[318].

Epoxidation of alkenes by peracids is known to take place via a stereospecific cis addition mechanism. The direct separation of (E)- and (Z)- isomers of alkenes by GC has been limited to special conditions and compounds[234,236]. The conversion of the (E)- and (Z)- alkenes into the corresponding epoxides allows base line separations even on packed columns[3119]. The trans epoxide has been found to elute faster than the cis epoxide when chromatographed on polar or nonpolar stationary phases. The MS can not be used to distinguish *cis* and *trans* epoxide-isomers because only slight intensity differences exist between them. However, the geometric isomers of epoxides can be distinguished readily by their NMR spectra[283].

The epoxidation reaction has been used generally to examine mg quantities of alkenes. In the present study µg quantities of alkenes were

readily epoxidized by passing a particular region of the GC effluent directly into a solution of m-chloroperbenzoic acid. The reaction was quantitative and completed within ten min at room temperature. The aromatic alkenes were more difficult to epoxidize and required a longer time. The reaction took place most readily in dichloromethane but carbon tetrachloride and hexane were also successful.

The technique was refined to epoxidize nanogram quantities of alkenes, keeping the total reaction volume at one µl. The alkenes were trapped in glass capillary tubes (0.45 mm i.d.) and 1 µl of m-chloroperbenzoic acid in hexane was injected onto the trapped material using a syringe fitted with a fine stainless steel needle (0.23 mm o.d.). The solution was withdrawn back into the syringe and introduced into the GC using an on-column injection system. Hexane (B.Pt. 69 °C) was found to be the more suitable solvent for on-column cold injection (40 °C), dichloromethane was too volatile for this purpose. This method was used to determine the (E)- and (Z)- isomer ratio of a number of synthetic alkenes. The results obtained with a mixture of heptadecane and 8-heptadecene are shown in Figure 6. When the mixture was trapped and epoxidized the 8-heptadecene peak completely disappeared to yield the two corresponding epoxides, while the heptadecane peak remained unreacted. Similarly, when 8-heptadecene from the Dufour gland of the ant *Myrmica rubra* was epoxidized, only a single peak was obtained (Figure 6c). This confirmed that 8-heptadecene from *M. rubra* was 100% (Z)- isomer only.

2.1.4 Cleavage of epoxides

Epoxide groups are present in many biologically important natural products. The juvenile hormones [59-62] are one group of important epoxides encountered in insects. Disparlure [2] and (Z,Z)-3,6-cis-9,10-epoxyheneicosadiene [48] found in the gypsy moth[5] and the saltmarsh caterpillar moth[320] respectively, are two examples of epoxide sex pheromones.

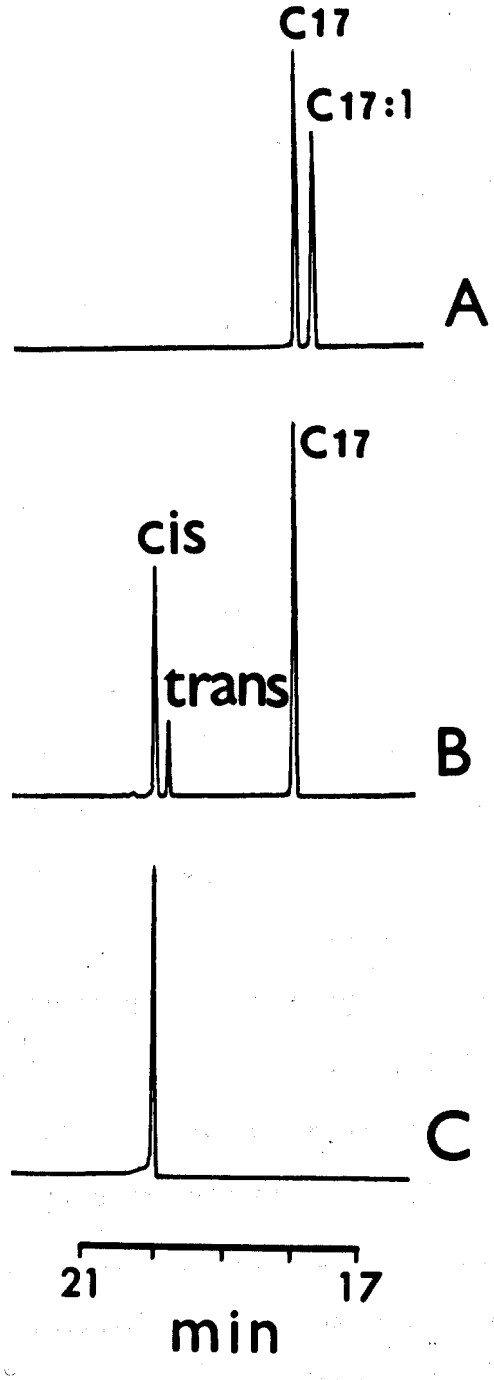
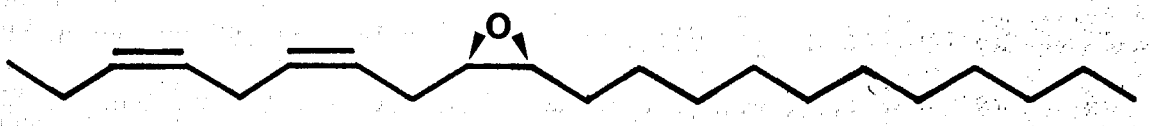


FIGURE 6. Separation of (E)- and (Z)-8-heptadecenes by epoxidation. Chromatograms on OV-1 capillary column with the oven temperature programmed from 40 to 300 °C at 10 °C/min; (A) approximately 100 ng each of heptadecane and (Z)- and (E)-8-heptadecenes in 0.5 µl of hexane; (B) both peak of (A) were trapped, epoxidized and reinjected; (C) (Z)-8-heptadecene from Myrmica rubra Dufour gland was trapped, epoxidized and injected.



[48]

The techniques to locate the epoxide position are of great importance to the natural products chemist. When MS facilities are available, EI-MS[283] and particularly CI-MS[284] are useful to locate the position of the oxirane ring. As an alternative to MS, simple microchemical methods are often employed to determine the epoxide positions. Bierl *et al.*[264] performed this by the cleavage of 1-100 μg samples with periodic acid in a chlorinated solvent, and subsequent examination of the carbonyl products by GC. A column of periodic acid on calcium sulfate has been used by Schwartz *et al.*[321], to cleave micromole amounts of epoxides to aldehydes. Similarly, Mizuno *et al.*[322] used HIO_4 in anhydrous ether and subsequently analyzed the carbonyl products formed by GC.

In the present study, a simple reaction gas chromatographic technique was developed to locate the oxirane position in nanogram quantities of unknown epoxides. Periodic acid was used to cleave the epoxides into the corresponding carbonyl compounds by reaction gas chromatography. Initially, the epoxides trapped in glass capillaries were sealed in glass tubes containing anhydrous periodic acid. The tubes were placed in the solid injector, inside the injection port of the GC for a few minutes before crushing. Later, it was discovered that a precolumn packing of 10% periodic acid on Chromosorb W was more efficient to cleave the epoxides.

The commercially available periodic acid (H_5IO_6) contained water and was dried in a drying pistol. A number of preliminary trials were made to determine the best formulation. A 10% w/w loading of HIO_4 upon 5% OV-101 on Chromosorb W, 100-120 mesh gave the best results. A higher loading of

HIO_4 increased by-product formation. After conditioning the pre-column, pre-injection of a solvent such as hexane helped to reduce adsorption of the products. The pre-column could cleave epoxides quantitatively at any oven temperature between 150 to 220 °C but preferably, it was operated at the highest temperature suitable to observe the expected product peaks. The pre-column material did not show any loss of activity on storage for months but always over-night conditioning was essential before the use of the pre-column. The pre-column material needed to be changed when the activity decreased but usually more than 100 injections of one microgram samples could be made before any decrease of activity was seen.

The HIO_4 pre-column on top of either a Porapak Q or a PEG 20M column was used to determine the epoxide position of a number of known compounds. The compounds were either trapped in glass capillaries and solid injected or injected as solutions. Figure 7 shows the results obtained from the injection of three low mass epoxides on a Porapak Q column. 1,2-epoxypropane (propylene oxide) and 2,3-epoxy-2,3-dimethylbutane yielded ethanal and propanone respectively, whereas 2,3-epoxy-2-methylbutane gave both ethanal and propanone. The reactions were quantitative. Many solvents were tried and carbon tetrachloride was found to be useful to examine low mass products up to C_6 on the Porapak Q column. Table 10 summarises the results of many other compounds which produced smaller fragments on the Porapak Q column. When ethanal was a product, trace amounts (<5%) of ethanoic (acetic) acid were always observed due to oxidation. A peak corresponding to methanal was not observed perhaps due to the poor stability or poor response factor of methanal towards the FID. The technique clearly demonstrated in samples of 200 ng, the presence of an ethyl and a methyl group attached to the epoxide ring of juvenile hormones I and II by the production of butanone. In the case of juvenile hormone III propanone was produced to indicate the presence of two methyl groups attached to the epoxide ring.

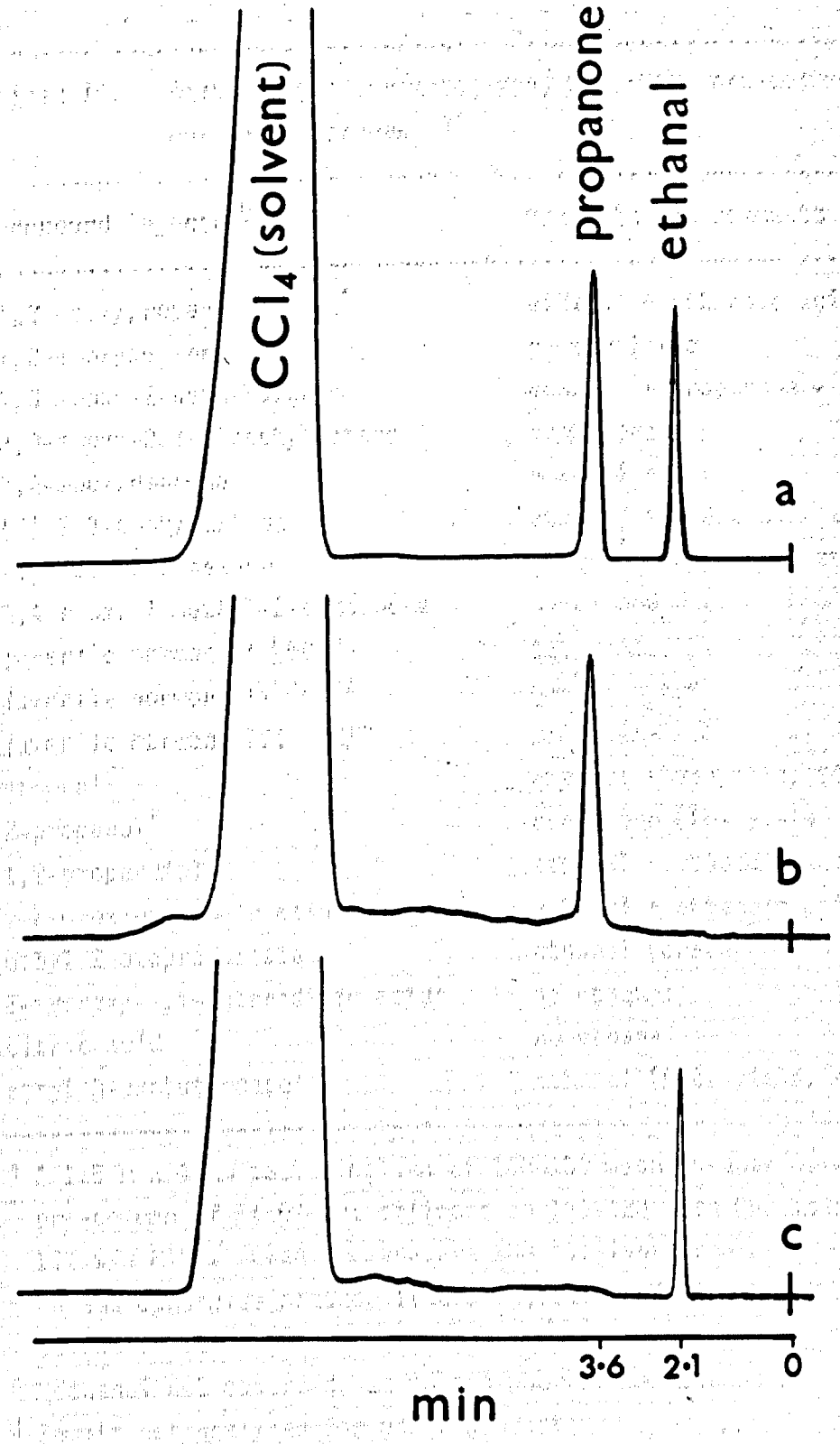


FIGURE 7. **Cleavage of epoxides by a periodic acid pre-column.**
Chromatograms on Porapack Q column with a periodic acid pre-column, at 205 °C: Approximately 200 ng each of (a) 2,3-epoxy-2-methylbutane (b) 2,3-epoxy-2,3-dimethylbutane and (c) 1,2-epoxypropane were injected as solutions in carbon tetrachloride.

Table 10. Reaction gas chromatography by a HIO_4 pre-column on a packed column of Porapak Q[a].

Compound injected[b]	Products and comments
1,2-epoxypropane	ethanal + ethanoic acid (trace, <5%) + c
1,2-epoxybutane	propanal + c
2,3-epoxy-2-methylbutane	ethanal + propanone + ethanoic acid (trace)
2,3-epoxy-2,3-dimethylbutane	propanone
1,2-epoxypentane	butanal + c
(Z)-2,3-epoxypentane	ethanal + propanal + ethanoic acid (trace)
(Z)-2,3-epoxyhexane	ethanal + butanal + ethanoic acid (trace)
3,4-epoxy-4-methyl-2-pentanone	propanone + d
juvenile hormone I [60][e]	butanone + d
juvenile hormone II [61][e]	butanone + d
juvenile hormone III [62][e]	propanone + d
ethanol[f]	ethanal (low yield, 20%)
2-propanol[f]	propanone (low yield, 30%)
1,2-propandiol	ethanal + ethanoic acid (trace)
2-hydroxypropanoic acid	ethanal + ethanoic acid (trace)
ethyl 2-oxopropanoate	ethanal (trace)
2-hydroxy-1,4-butanedioic acid[f]	no ethanal
citric acid	no ethanal
ethyl 3-oxobutanoate	ethanal (poor yield, <5%)

[a] A 1.5 m x 4 mm packed column of 100-150 mesh Porapak Q, with a 70 mm x 4mm pre-column of 5% OV-101 silicone on 100-120 mesh Chromosorb W loaded with 10% w/w HIO_4. Oven temperature 200 °C, isothermal.

[b] Unless otherwise stated, liquid injection of 200-300 ng samples in carbon tetrachloride.

[c] Methanal not observed due to its poor flame response.

[d] Sample not analysed for other products.

[e] Solid injection of 200 ng samples trapped in glass capillaries.

[f] Liquid injection of 300 ng samples as aqueous solutions.

The method was not specific to epoxides. Therefore it can be extended to study other substances capable of being oxidized by a HIO_4 pre-column. For example, 1,2-propandiol (propylene glycol) and 2-hydroxypropanoic acid (lactic acid) were both cleaved to yield ethanal (Table 10). One of the methods currently employed to determine lactic acid in blood and other biological tissues is to convert lactic acid to ethanal by HIO_4 oxidation and determine the ethanal by GC[323]. The HIO_4 pre-column has the potential to determine lactic acid in a more efficient and convenient manner. Similarly, the method can be extended for the estimation of propylene glycol and other vicinal diols.

A HIO_4 pre-column placed before a Carbowax 20M column was used to study compounds that produce large fragments on cleavage. Table 11 summarises the compounds studied. The HIO_4 pre-column cleaved cis-7,8-epoxy-2-methyloctadecane (disparlure [2]), quantitatively into 6-methylheptanal and undecanal (Figure 8). Epoxyethylbenzene produced phenylethanal as a minor product, along with the expected major product benzaldehyde. The pre-column was able to oxidize 3-octanol to 3-octanone to demonstrate its nonspecificity but the yield was poor (<30%, for 200 ng samples). The yield improved to 60% when sample size was made smaller, to 50 ng. Surprisingly, the pre-column was capable of cleaving alkenes directly but the yields were poor. 8-Heptadecene and 9-nonadecene were split to yield the corresponding aldehydes but the yield was below 40%.

Alkenes can be better examined by this method by converting them first to epoxides using m-chloroperbenzoic acid[317]. The crude reaction mixtures in carbon tetrachloride, dichloromethane or hexane can be directly injected onto the HIO_4 pre-column. However, the decomposition of the reagent mixture gives some impurity peaks, therefore a blank GC run of reagent mixture without the alkene was found to be useful to identify the product peaks. This method was employed to examine the alkylidene terminals (the part of a molecule between its end and the first double bond) of some

Table 11. Reaction gas chromatography by 10% HIO_4 precolumn on a packed column of Carbowax 20M[a].

Compound injected[b]	Products and comments
(Z)-8,9-epoxyheptadecane	octanal + nonanal
(E)-8,9-epoxyheptadecane	octanal + nonanal
(Z)-9,10-epoxynonadecane	nonanal + decanal
(E)-9,10-epoxynonadecane	nonanal + decanal
(Z)-9,10-epoxytricosane	nonanal + decanal
methyl (Z)-9,10-epoxyoctadecanoate	nonanal + aldehydeester[c]
1,2-epoxyethylbenzene	benzaldehyde + phenylethanal(minor product)
(E)-2,3-epoxy-3-phenylpropanal	benzaldehyde + few other products
(E)-2,3-epoxy-3-phenylpropanoic acid	benzaldehyde
(Z)-1,2-epoxy-1,2-diphenylethane	benzaldehyde
1,2-epoxy-2-phenylpropane	acetophenone + 5% starting material
1,2-epoxy-1,1,2-triphenylethane	benzophenone + benzaldehyde
2,3-epoxy-3-phenylpropanoic acid	benzaldehyde
(Z)-7,8-epoxy-2-methyl octadecane (disparlure)	6-methylheptanal + undecanal
8-heptadecene	octanal + nonanal (low yield, <40%)
9-nonadecene	nonanal + decanal (low yield, <40%)
3-octanol	3-octanone (low yield, <30%)

[a] A 2.75 m x 4 mm packed column of 10% Carbowax 20M with a 70 mm x 4 mm pre-column of 5% OV-101 silicone on 100-120 mesh Chromosorb W loaded with 10% w/w HIO_4. An appropriate isothermal, oven temperature between 150-220 °C was used according to the sample.

[b] 200-300 ng samples injected as solutions in hexane or dichloromethane

[c] No synthetic sample was available for comparison, but R_t of the peak corresponds to the expected products.

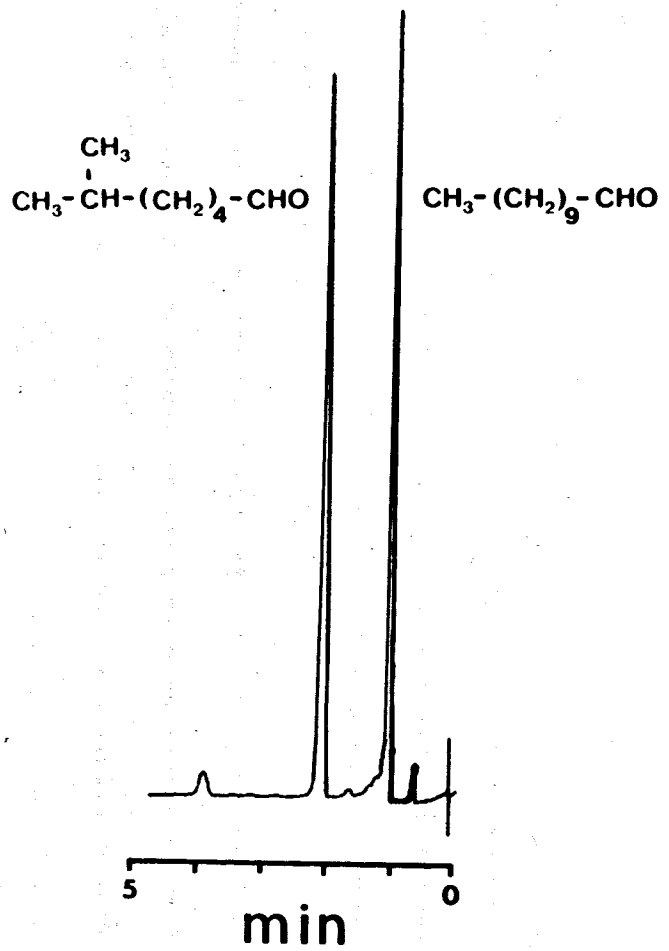


FIGURE 8. Cleavage of disparlure by a periodic acid pre-column.
A chromatogram on PEG 20M column with a periodic acid pre-column, at 180 °C. Disparlure (200 ng) collected in a glass capillary was introduced by the solid injection technique.

terpenes. The results are presented in Table 12. The production of propanone by citronellal, farnesol, citral and pulegone readily demonstrated the presence of an isopropylidene group in their structures. Limonene did not produce propanone hence demonstrated the absence of an isopropylidene group in its structure.

The periodic acid pre-column appears to posses a great potential in reaction gas chromatography. Perhaps the method can be extended for the analysis of sugars as well[324].

2.1.5 Ozonolysis

Ozonolysis of alkenes has been a particularly useful technique for the determination of the location of unsaturation. In the frequently adapted method for micro-ozonolysis, the alkenes are disolved in a solvent and ozone is directly bubbled through the solution. The ozonides formed are reduced to yield the corresponding carbonyl compounds and the resulting solution is examined by GC. Therefore, ozonolysis in solution is not a reaction gas chromatographic technique. The main drawback of ozonolysis in a solvent, as pointed out by others who have sought alternative methods[284,315], are the difficulty in identifying the small molecules formed, introduction of impurities, and the impossibility to use the total reaction product for GC. A variety of solvent systems have been investigated to find the best solvent for ozonolysis and subsequent analysis of the products by GC[286,288]. Ma and Ladas[325] have reviewed ozonolysis methods in general.

The ozonolysis products of 1 µg of methyl oleate and other compounds in solution were identified by Beroza *et al.*[286,288] by injecting volumes as large as 20 µl in to the GC. The structure of vulpinic acid was determined by White *et al.*[326] by ozonolysis of 25 µg in 100µl of ethyl acetate and subsequent injection of 10 µl of the reaction mixture into the GC. The huge solvent peak thus obtained extended over 12 min. Such large solvent peaks, even if the solvent was scrupulously pure, would render the

Table 12. Analysis of terpenes by reaction gas chromatography by a 10% HIO_4 pre-column on a packed column of Porapak Q[a]

No	Terpene component of the mixture injected[b]	Products and comments
1	citronellal	propanone + c
2	farnesol	propanone + c
3	limonene	no propanone + c
4	citral	propanone + c
5	pullegone	propanone + c

[a] A 1.5 m x 4 mm packed column of 100-150 mesh Porapak Q with a 70 mm x 4 mm pre-column of 5% OV-101 silicone on 100-120 mesh Chromosorb W loaded with 10% w/w HIO_4. Oven temperature 200°c.

[b] 1µl of the crude reaction mixture of terpene (5µg) and m-chloroperbenzoic acid (100µg) in carbon tetrachloride (10µl)

[c] Sample not analyzed for other products

method not particularly useful to identify the small molecules produced.

The terminal methylene groups produce methanal on ozonolysis. However, no peak corresponding to methanal was observed on analysis of ozonolysis products of alkenes with terminal double bonds (Table 13). This problem has been encountered by many other workers[286,327]. The difficulty arises due to near-zero response factor shown by methanal, in the flame ionization detector (FID). Moore and Brown[327] have developed a successful method to derivatize methanal with cyclohexane-1,3-dione to give a pyran derivative amenable to GC. Dialdehydes, commonly encountered as ozonolysis products of polyenes are also not amenable to GC. The dialdehydes have been derivatized with o-phenylenediamine to form quinoxalines, suitable for GC analysis[315,327].

In the present investigation a solventless ozonolysis technique was developed. The alkene substances were collected in a glass capillary after chromatographing a mixture, such as a pheromone mixture. The collected material was treated with ozone and the ozonides immediately rechromatographed. The decomposition of the ozonides in the injection port of the GC gave quantitative yields of the corresponding carbonyl compounds. Figure 9 shows the ozonolysis of trapped heptadecane and (Z)-8-heptadecene. The (Z)-8-heptadecene peak completely disappeared upon ozonolysis to yield octanal and nonanal, while the heptadecene peak remained unreacted. Though 350 ng samples were conveniently used, analysis of samples as small as 50 ng were readily achieved. This technique was used to determine the double bond positions in a number of known and natural alkenes isolated from ants. The results are summarized in Table 13. The method was particularly useful to examine the pentadecenes from the Dufour gland of *Tetramorium caespitum*. Solventless ozonolysis of the pentadecene peak yielded hexanal, heptanal, octanal and nonanal to show the peak was a mixture of 6- and 7- pentadecenes.

The method was found to overcome most of the difficulties encountered in ozonolysis in a solvent. The alkylidine group analyses in

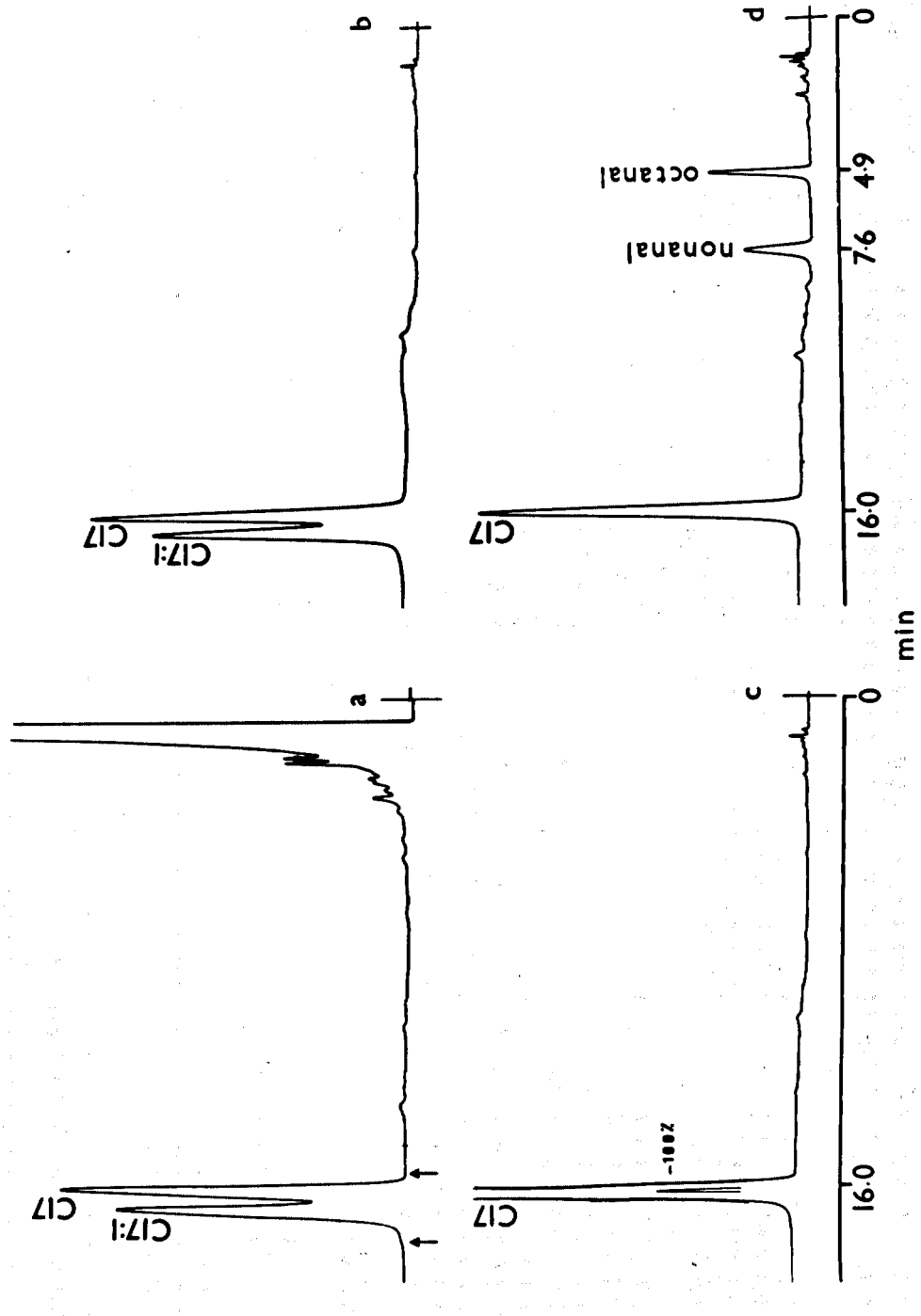


FIGURE 9. Solventless ozonolysis and hydrogenation of (Z)-9-heptadecene. Chromatograms on 10% PEG 20M column, temperature held at 130 °C for 8 min and programmed 40 °C/min to 170 °C; (a) 350 ng each of heptadecane and 8-heptadene in 0.5 µl hexane. The effluent between the arrows was trapped and (b) reinjected (c) reinjected and hydrogenated. (d) ozonized and reinjected.

Table 13. Results of Ozonolysis of Alkenes

no.	compound[a]	source[b]	products and comments: higher mol wt products[c]	products and comments: lower mol wt products[d]
1	2-methyl-2-butene	C		ethanal + acetone (minor products, acetic acid)
2	2,3-dimethyl-2-butene	C		acetone
3	(*Z*)-2-pentene	C		ethanal + acetone (minor product, acetic acid)
4	(*Z*)-2-heptene	C		ethanal + pentanal (minor product, acetic acid)
5	(*Z*)-8-heptadecene	N (*Myrmica rubra*)[e]	octanal + nonanal	
6	(*Z*)-9-nonadecene	N (*Myrmica rubra*)[e]	nonanal + decanal	
7	(*Z*)-9-tricosene	N (*Solenopsis geminata*)[e]	nonanal + tetradecanal	
8	methyl (*Z*)-9-octadecenoate	C	nonanal + aldehyde ester	
9	phenylethene	C	benzaldehyde + *f*	
10	1-methyl-1-phenylethene	C	acetophenone + *f*	
11	(*E*)-3-phenyl-2-propenal	C	benzaldehyde + *g*	
12	(*Z*)-1,2-diphenylethene	C	benzaldehyde	
13	1,3-diphenyl-1-butene	C	benzaldehyde + 2-phenylpropanal	
14	(*Z,E*)-α-farnesene (I)	N (*Myrmica scabrinodis*)[e]	4-oxopentanal + *f, g*	acetone
15	homofarnesene (II)	N (*Myrmica scabrinodis*)[e]	4-oxohexanal + *f, g*	acetone
16	bishomofarnesene (III)	N (*Myrmica scabrinodis*)[e]	4-oxohexanal + *f, g*	butanone
17	(*Z*)- or (*E*)-nerolidol (IV)	C	4-oxopentanal + *f, g*	propanone

[a] Sample size 200–350 ng. [b] C = commercial, N = natural (source). [c] Products larger than hexanal were identified on 10% PEG 20M column. [d] Products smaller than pentanal were identified on Porapak Q column. [e] Samples collected from the Dufour glands of the ants by preparative GLC. [f] Methanal not observed due to its poor flame response. [g] Sample not analyzed for other products.

large terpenoid molecules were carried out using samples as small as 50 ng. Even the products of low mass were detected conveniently. The loss of material was minimal as no evaporation was involved. The products were introduced to the chromatograph as ozonides rather than the more volatile carbonyls. It can be concluded that the solventless ozonolysis has many advantageous over the method that uses solvent.

2.1.6 Hydrogenation

Hydrogenation of olefinic bonds can be performed on analytical or preparative scales by reaction gas chromatography. The degree of unsaturation and the number of rings present in an unknown compound can be determined by hydrogenation. The saturated analogues thus obtained provide simpler mass spectra that may yield information about the branching of the molecules. Furthermore, only a few nanograms of material are required to derive this information.

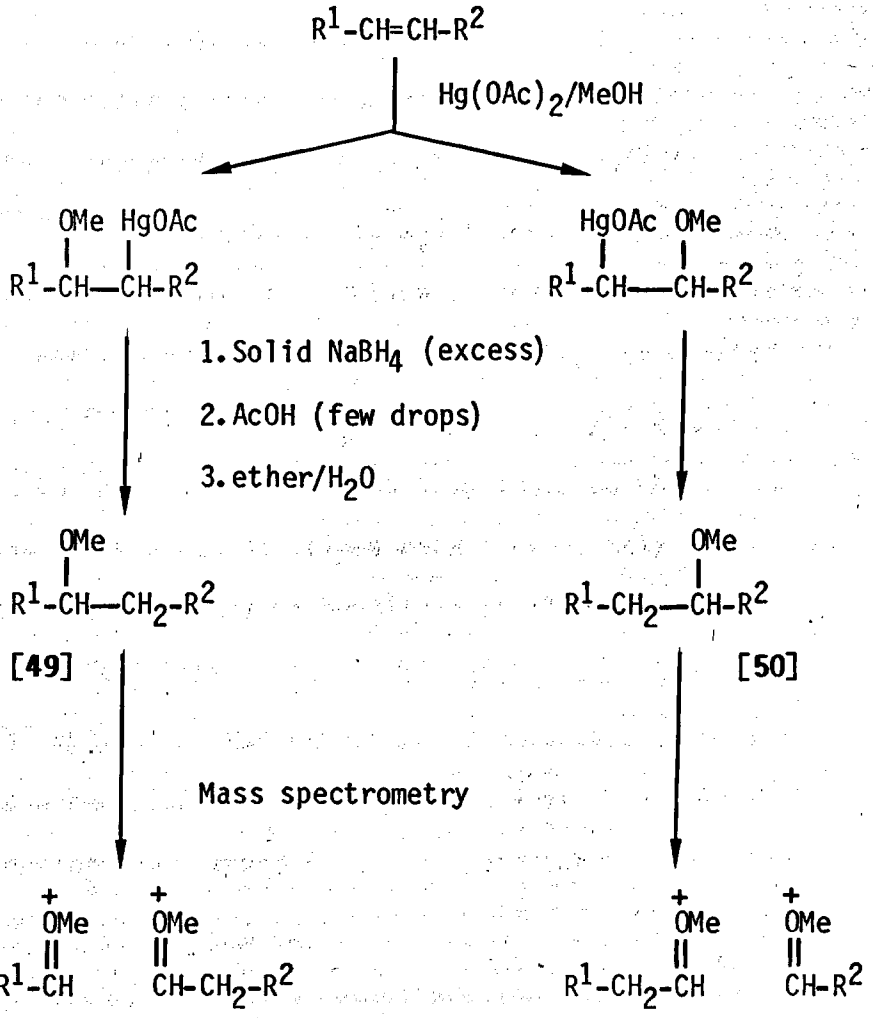
The procedure adapted for the GC-hydrogenation in the present study was based on the methods described by Mounts and Dutton[328] and Beroza and Sarmiento[257]. Stanley and Kennet[314], and Cronin and Gilbert[315] have also described similar hydrogenation procedures. A pre-column of 1% palladium catalyst on Chromosorb W was made to replace the first 60 mm of packing in the GC column and hydrogen was used as the carrier gas.

This procedure was able to hydrogenate alkenes readily. The alkenes were either solid injected in glass capillaries or injected as solutions. Hexane was found to be a convenient solvent for the injections. Chlorinated solvents, such as carbon tetrachloride were avoided because they gave a number of unexpected products. The pre-column was able to hydrogenate microgram quantities of alkenes, even with three double bonds instantaneously and quantitatively. Hydrogenation of (Z)-8-heptadecene is shown in Figure 9. No significant loss of activity of the catalyst was observed over several weeks. The oven temperature did not appear to be crucial for the activity of

the catalyst, no difference of efficiency was observed at oven temperatures between 140 and 220 °C.

2.1.7 Methoxymercuration-demercuration

A further method available to locate the position of the double bonds is methoxymercuration-demercuration. Abley *et al.*[269] first described the application of GC-MS to the methoxy derivatives obtained from the sodium borohydride reduction of methoxymercuration products of alkenes.



The two methoxy derivatives [**49,50**] are not usually separated by GC, as a result, a mixed mass spectrum is obtained by GC-MS. The fragmentation of the methoxy derivatives [**49,50**] in the mass spectrometer results in cleavage on either side of the methoxy group to yield four characteristic fragment ions. These ions give intense peaks that can be used to locate the original double

position.

Many application of this technique to locate the alkene position of insect pheromones have been reported[270-273]. The main drawback of the methoxymercuration-demercuration procedure is the final solvent extraction step. The methoxy derivatives produced are partitioned between water and ether[273] or hexane[272] before the non-aqueous layer is examined by GC. For the solvent extraction step, usually 50 µl or more of the non-aqueous solvent is required to allow convenient withdrawal of the upper layer. Use of such a large volume of the non-aqueous solvent can be considered wasteful as only a small part of it can be injected into the GC.

In the present study, a special glass vial (Figure 10) was developed for the convenient extraction of substances into solvent-volumes as small as 5 µl. The reaction was performed in the bottom chamber (75 µl). The reactants were placed in the reaction chamber using a syringe fitted with a fine needle. For the extraction of the products, the reaction mixture was shaken with hexane (5 µl) or ether and the upper layer was pushed into the narrow neck region by adding water with a syringe, to the bottom layer. The air bubbles that got trapped sometimes were conveniently removed with the syringe. Fine glass beads may be equally suitable to push the upper layer into the neck. The solution in the neck region was withdrawn with a syringe for GC and GC-MS analysis. Furthermore, crude mixtures were filtered directly into the reaction chamber by placing a block of glass or cotton wool in the neck, pouring the mixture into the upper chamber and withdrawing air from the bottom chamber with a syringe.

The methoxymercuration-demercuration technique with the modified extraction step was successfully used to determine the double bond position of the tricosene isomer isolated from the postpharyngeal gland of the ant, *Solenopsis geminata*. The vial has a great potential as a useful piece of equipment for the micro-chemist.

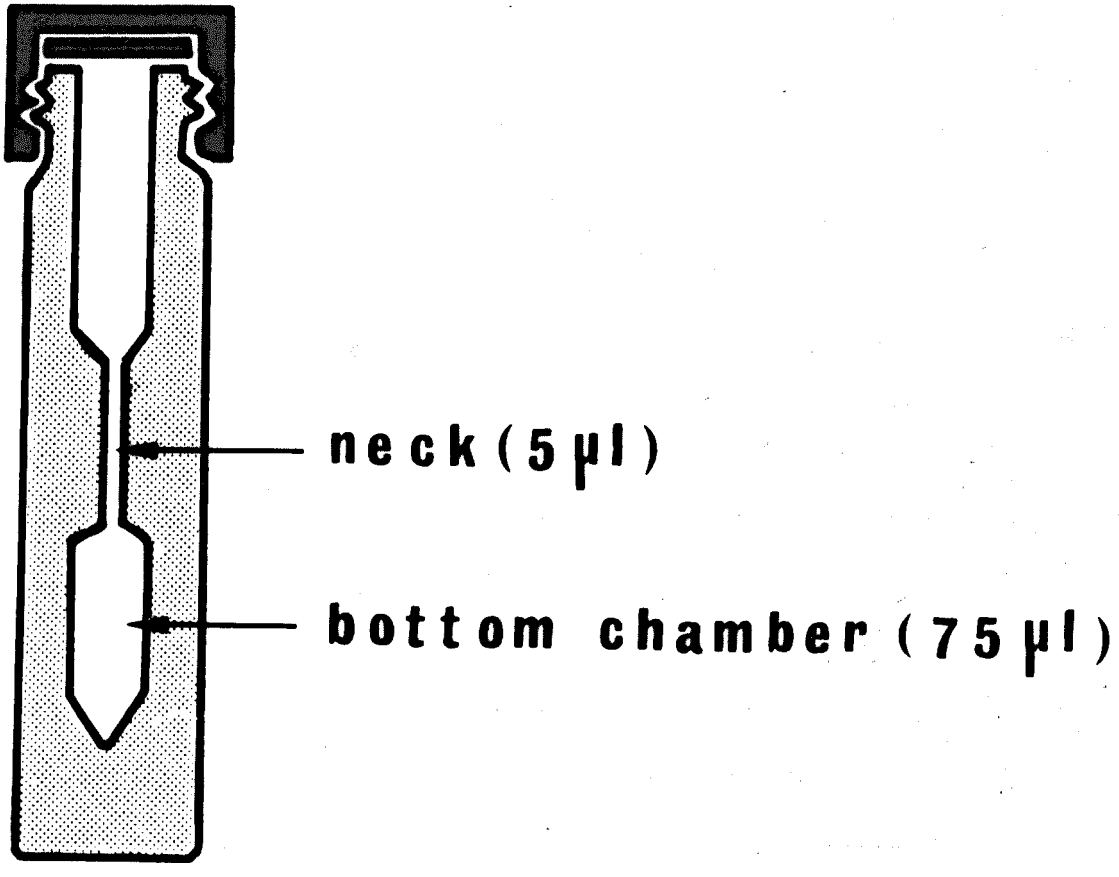


FIGURE 10. Micro-reactor vial

2.1.8 Sodium borohydride reduction

The identification of aldehydes and ketones has been aided by the reduction to alcohols with sodium borohydride and subsequent GC analysis of the products[329]. A saturated solution of $NaBH_4$ in ethanol or water has been used to reduce 1 μg samples in the syringe barrel and products larger than hexanol have been identified[330]. The method cannot be applied to monitor small molecules because the solvent may mask them. Solid $NaBH_4$ has been used to reduce the total mixture of volatile compounds from three heads or six poison reservoirs of the ant *Myrmica rubra*[221,331]. Similarly, micro-synthesis of alcohols, from ketones trapped from GC effluent has been done on a preparative scale using solid $NaBH_4$[332].

In the present study, carbonyl compounds trapped separately in glass capillaries were reduced to corresponding alcohols using solid $NaBH_4$. Surprisingly, no secondary hydrolysis step by the addition of water to liberate the alcohols was found to be necessary. The 3-octanone peak obtained from a single head of *M. rubra*, as represented in Figure 11 represented approximately 400 ng. The 3-octanone peak was trapped in a glass capillary and sealed in a glass tube containing solid $NaBH_4$. Although the tube was allowed to stand at room temperature for 15 min, the reduction probably occurred between the alcohol vapour and the solid reagent when tube was at the injection port of the GC, before crushing. The 3-octanone peak was quantitatively reduced to 3-octanol. 3-Heptanol, a minor component of the mandibular gland of *M. rubra*, which appeared as a shoulder on the 3-octanone peak was trapped together with the 3-octanone peak. However, this minor peak became clearly visible once the major 3-octanone peak was reduced to 3-octanol (Figure 11).

As an alternative, the reduction can be performed using a solution of $NaBH_4$ by taking the advantage of the on-column cold injection technique on capillary columns, which prevents the tailing of the solvent peak. Water and ethanol were found not suitable for on-column injection.

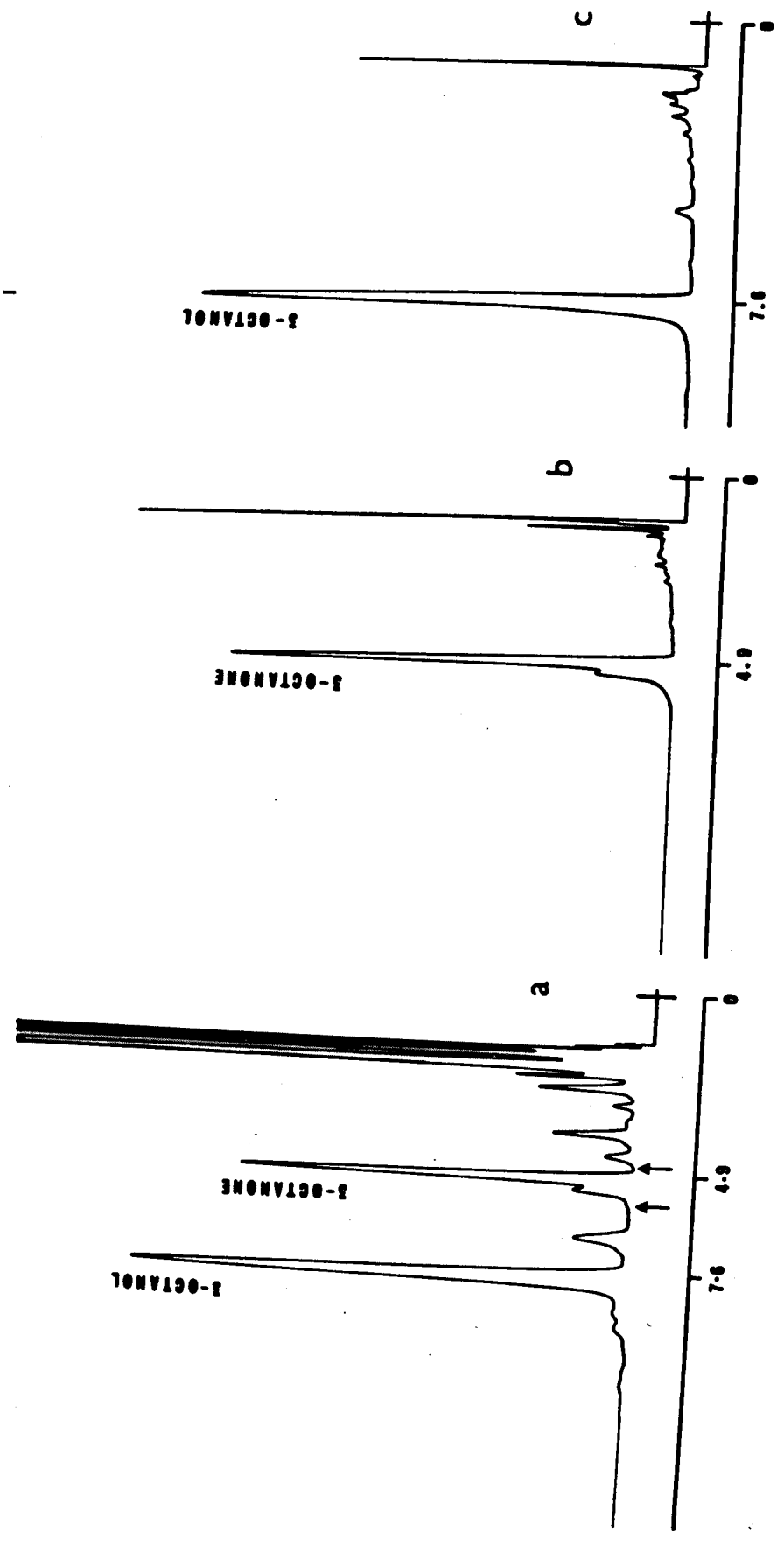


FIGURE 11. **Reduction of 3-octanol by sodium borohydride.** Chromatograms on 10% PEG 20M column at 125 °C: (a) the total volatiles of the mandibular gland of Myrmica ruginodis from one individual head; (b) the effluent between the two arrows was trapped and reinjected; (c) the effluent was trapped and injected with sodium borohydride.

Tetrahydrofuran (THF) was better suited although the solubility of $NaBH_4$ in THF is poor (100 mg/100g). Perhaps dimethoxyethane may be still better (solubility 5.5 g/100g). A saturated $NaBH_4$ solution (1 µl) in THF was injected, using a syringe with a fine needle onto the trapped 3-octanone in a glass capillary. The capillary was kept for 10 min at room temperature and the solution was withdrawn back into the wet syringe and injected immediately on a cold (40 °C) OV-101 capillary column. The reduction of the 3-octanone peak to yield 3-octanol was over 90% efficient.

2.1.9 Bromination

A method to identify the unsaturated peaks in a GC profile is valuable to the pheromone chemist. The bromination results in the complete elimination of all unsaturated peaks from the GC trace, leaving the saturated peaks intact. Morgan and Wadhams[229] have used Br_2 in CS_2 to brominate the components of the Dufour gland of *Myrmica rubra*. The bromination can be achieved readily by adding Br_2 in CS_2 on to the gland placed inside a glass tube with one end sealed. After the reaction was over and excess bromine evaporated the tube can be sealed and injected onto the GC. Many workers have found the method to be useful[333,334]. O'keefe *et al.*[335] have utilized bromination in solution to subtract unsaturated fatty acid methyl esters.

The bromination was extended in the present study to material trapped in glass capillaries. The products were examined by capillary GC using on-column cold injection method. Hexane was suitable for on-column injection, therefore bromine in hexane was used as the reagent. The reagent was injected onto nanogram to microgram quantities of material trapped from the GC effluent into glass capillaries. The disappearance of the yellow colour can be observed directly under the microscope if the trapped material was unsaturated. Bromine in hexane was added until slight yellowish colour persisted, the solution was withdrawn back into the syringe and injected onto the cold capillary column. The unsaturated peaks were readily recognized by

the comparison of the chromatograms with those obtained without bromination.

2.1.10 Esterfication with diazomethane

Lower members of aliphatic monocarboxylic acids are sufficiently volatile to be separated directly by GC on a stationary phase like FFAP. However, adsoption onto the chromatographic support due to the highly polar character of carboxyl function, often leads to tailing of peaks. A number of procedures are available in the literature to chromatograph non-esterified fatty acids[336-338]. Nevertheless, in general practice esterification is frequently used to convert relatively non-volatile and polar carboxylic acids into less polar derivatives having better GC characteristics. The esters can be chromatographed at reasonable temperatures and they give better separations, peak shapes and mass spectra than their parent acids.

Darbre[339] and, Ma and Ladas[325] have reviewed the wide variety of technique available for the esterification of fatty acids. Diazomethane is commonly employed to prepare methyl esters of fatty acids. A number of methods are available for the small scale preparation of diazomethane[339-341].

The method of Fales *et al.*[339] was modified in the present study, to prepare microgram quantities of diazomethane.
N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used as the precursor for diazomethane. MNNG was convenient to use because of its stability and crystalline nature. However, due care was taken to handle it because of its potent mutagenic nature. A solution of diazomethane in tetrahydrofuran (THF) was conveniently made in a Reacti-vial. MNNG was covered with a water and a THF layer, and upon the addition of aqueous KOH the THF layer immediately became yellow due to the liberation of diazomethane. The CH_2N_2 in THF layer was withdrawn with a syringe to esterify the carboxylic acids.

The fatty acids were trapped separately in glass capillaries from the GC effluent off a FFAP column. The CH_2N_2 in THF solution (1 µl) was injected onto the trapped material using a syringe fitted with a fine needle.

The solution was drawn back immediately and injected on-column on OV-101 capillary column. The method was tested with myristic, palmitic and stearic acids. The esterification reaction was instantaneous and quantitative.

Analysis of fatty acids is important in natural product chemistry. The present method refines the existing technique to be applied to nanogram quantities of material. Huwyler *et al.* used a similar method to identify the fatty acids found in the trail pheromone of *Lasius fuliginosus*[121].

2.2 Trail Pheromone of *Tetramorium caespitum*

A large number of ant species are known to employ trail pheromones as a means of communication but only in a very few cases has the pheromone been chemically identified. The first trail substance to be identified was methyl 4-methylpyrrole-2-carboxylate [**11**] from *Atta texana*[106]. The same compound was subsequently demonstrated to be active to evoke trail following in *A. cephalotes*[107] and *Acromyrmex octospinosus*[86]. 3-Ethyl-2,5-dimethylpyrazine [**12**] has been shown to be the major component of the trail pheromone of *Atta sexdens rubropilosa*[108] and the same compound has since been identified as the single component of the trail pheromone of eight species of *Myrmica*[109]. In the beginning of the present study only the aforementioned two compounds were known as trail substances that originate from the poison glands. Faranal [**15**], a terpenoid that originates from the Dufour gland has been identified as the major trail pheromone of *Monomorium pharaonis*[90]. There is a controversy about the composition of the trail pheromone of *Solenopsis invicta*. Williams *et al.*[118] have reported it to be Z,Z,Z-allofarnesene [**16**], while Vander Meer *et al.*[120] describe it as a mixture of Z,E and E,E-α-farnesenes [**17,18**] and Z,Z and Z,E-homofarnesenes [**19,20**]. A mixture of C_6 to C_{12} and C_{14} to C_{20} fatty acids are reported to be the active trail following mixtures for *Lasius fuliginosus*[121] and *Pristomyrmex pungens*[97] respectively. In *Iridomyrmex humilis* Z-9-hexadecenal has been identified as one of the components of its trail pheromone[125].

The above summary illustrates that the information available about the chemistry of trail pheromones is very limited. Some of the artificial trails laid with the above mentioned single substances were not species-specific although the natural trails showed a much higher degree of species-specificity. Although many trail pheromones had been recognized as multicomponent, the true quantitative and qualitative compositions of none of them were known at the beginning of this study. In the present project the

trail pheromone of *Tetramorium caespitum* was investigated and its composition was determined.

Blum and Ross[99] first reported that the trail pheromone of *T. caespitum* originates from the poison gland. As a preliminary survey, the work of Blum and Ross was repeated in the present study. Artificial trails were laid on a circular track with extracts obtained from the poison gland and the Dufour gland. The extract from the poison gland evoked high trail following activity on the worker ants whereas the Dufour gland extract was completely inactive. This result confirmed the observations of Blum and Ross.

The poison gland and the associated Dufour gland are attached to the sting. The poison gland contents are dispensed through the sting to lay trails as a means of communication during food gathering and change of nest sites. The two filaments of the poison-producing glands open into a spherical venom reservoir (Figure 12). The average diameter of the spherical reservoir was measured. The average volume was calculated (assuming it to be a sphere) to be about 14 nl.

Little or no work had been done on the chemistry of the *T. caespitum* poison gland, apart from the report by Blum and Ross[99]. They detected trace quantities of some free amino acids (aspartic acid being the major component) by paper chromatography.

The preliminary characterization of the trail pheromone was achieved by TLC. The contents of two poison glands were chromatographed on a silica gel plate. The silica gel was cut into ten bands, each representing a R_f difference of 0.1. When the bands were eluted with hexane and tested by bioassay, it was found three bands of R_f values between 0.3-0.4, 0.4-0.5 and 0.5-0.6 elicited high activity (Table 14). Initially, it was difficult to conceive the reason why the activity was spread over a range of R_f values. The reason became clear when the silica gel was cut into narrower bands and the activity of each band was tested. Two regions of high activity were

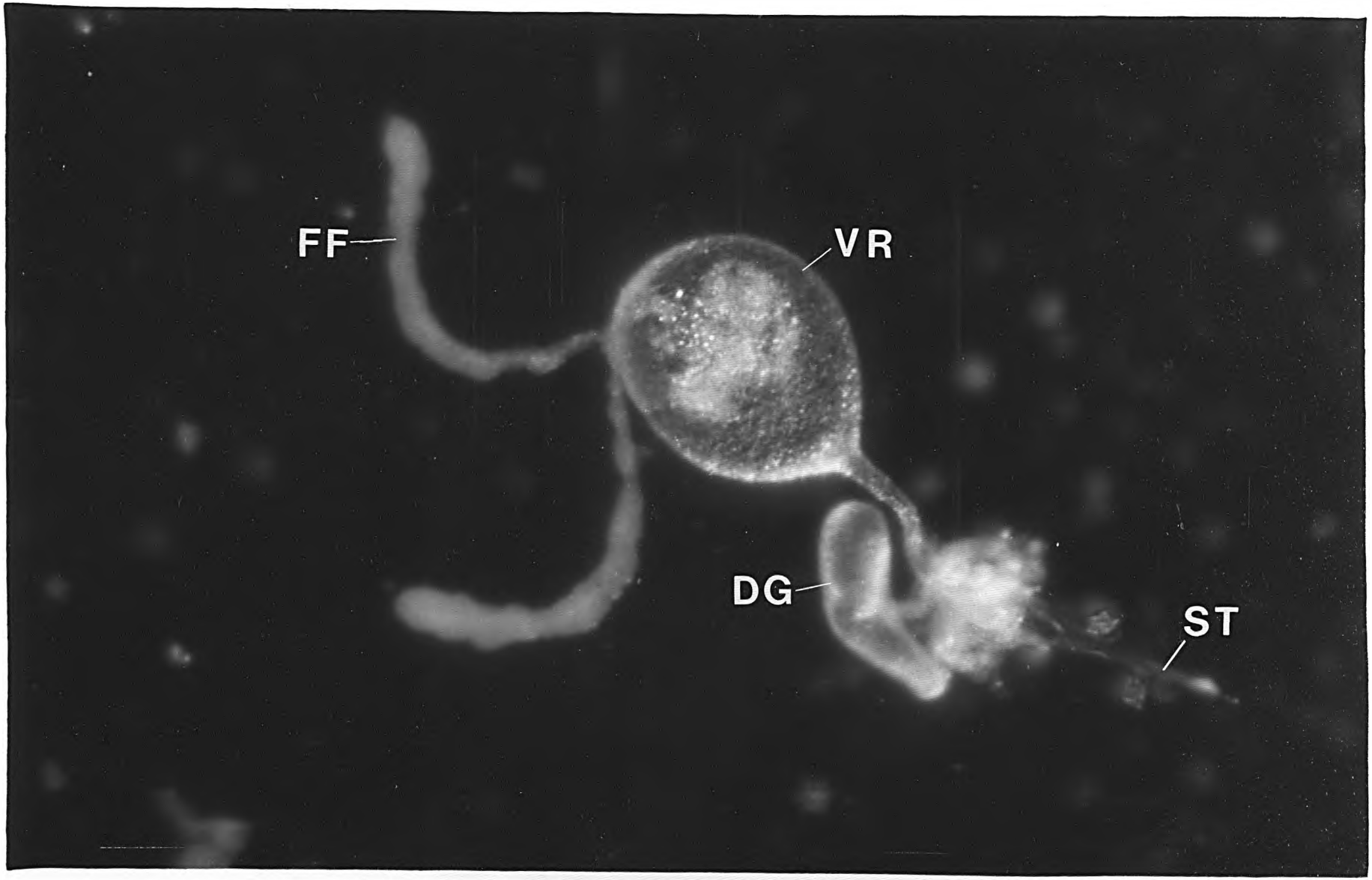


FIGURE 12. Venom apparatus of Tetramorium caespitum. FF: free filaments of the poison gland, VR: venom reservoir, DG: Dufour gland, ST: sting.

Table 14. Trail following activity evoked by extracts of various bands of silica gel, after TLC of two poison glands of *T. caespitum*

R_f range	Activity[a]
0.0 - 0.1	0
0.1 - 0.2	0
0.2 - 0.3	0
0.3 - 0.4	17
0.4 - 0.5	22
0.5 - 0.6	14
0.6 - 0.7	0
0.7 - 0.8	0
0.8 - 0.9	0
0.9 - 1.0	0

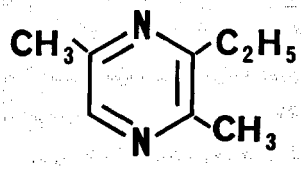
[a] Two poison glands were chromatographed (60:40, hexane:acetone). The bands were cut, eluted with hexane and the activities were tested as usual.

evident with a valley in between (Figure 13). Therefore it was possible to infer that the trail pheromone of *T. caespitum* was composed of as least two components of moderate polarity.

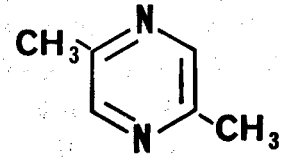
The chemical treatment of the glandular extracts and subsequent TLC separation and bioassay showed the trail pheromone components are basic because the activity was destroyed by acid treatment. Furthermore, the activity was unaffected by Br_2 in hexane, showing the absence of unsaturation.

GC examination of the contents of three poison glands on the PEG 20M column showed the presence of two major components (Figure 14). Only a narrow fraction of the GC effluent containing these two components was able to evoke trail following activity when the GC effluent was split, trapped and bioassayed. The retention indices of the two peaks on the PEG 20M column were 1370 and 1450 respectively. Similarly those on the PEGA column were 1437 and 1545 respectively. This indicated the compounds were moderately polar and their approximate molecular weight range to be between 100 and 150.

The identification of the two major components present in the poison gland was achieved by GC-MS. The mass spectra were obtained using 50 cleanly dissected poison vesicles sealed in a glass vial. The sample was injected by the solid injection technique on a 5% SE-30 column and the mass spectra were recorded by GC-MS. The mass spectrometer was operated at a very high sensitivity, therefore it was necessary to record the back ground spectra in between the peaks of interest. The mass spectra of the two major components obtained after the manual subtraction of the background are given in Figure 15.



[12]



[51]

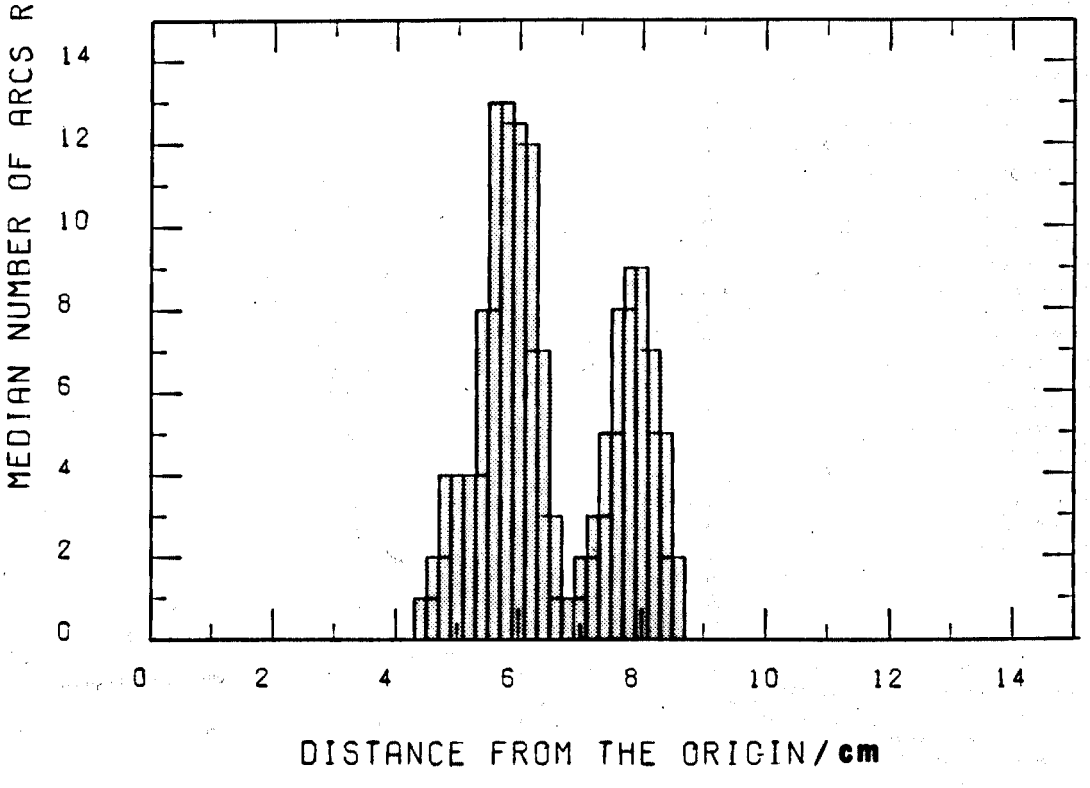


FIGURE 13. TLC-bioassay of poison gland contents of Tetramorium caespitum. An extract of two poison glands was chromatographed using hexane-acetone (60:40) as the eluent. The solvent front was allowed to run 15 cm and the silica gel was cut into narrow bands (2 mm). The trail releasing activity (stippled bars) of each band was measured by bioassay.

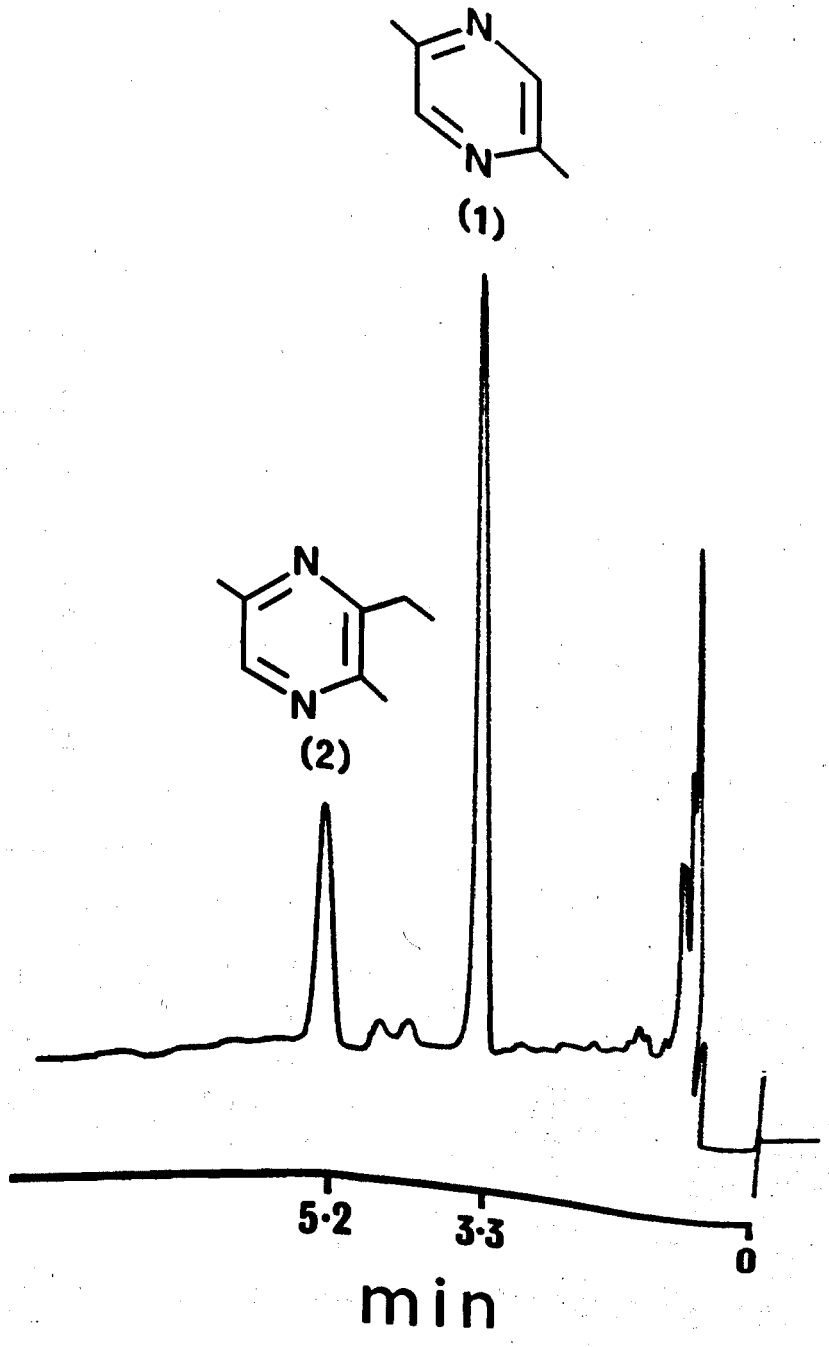
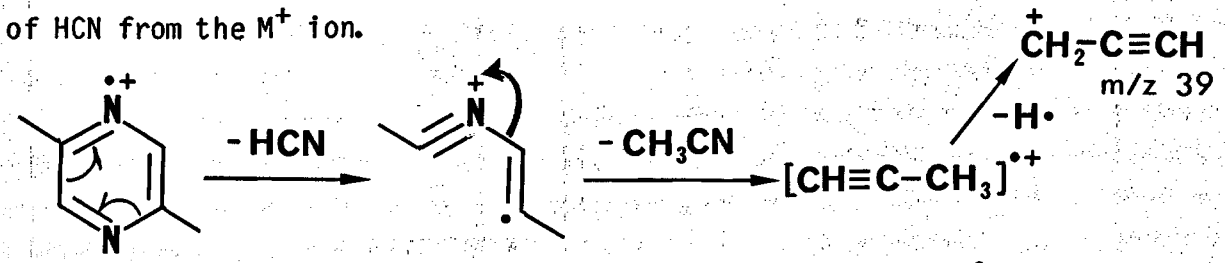
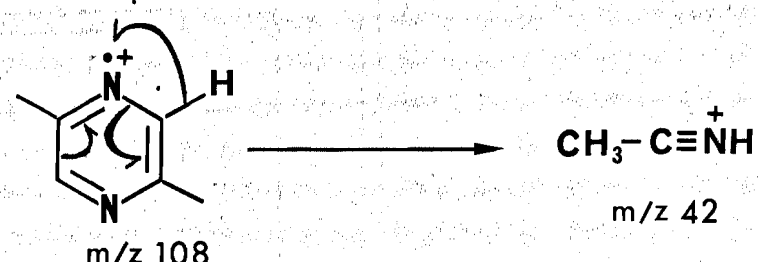


FIGURE 14. A gas chromatogram of three poison vesicles of Tetramorium caespitum. The glands were solid injected on a 2.75 x 4 mm packed column of 10% PEG 20M on Chromosorb W, at 130 °C (attenuation x 50).

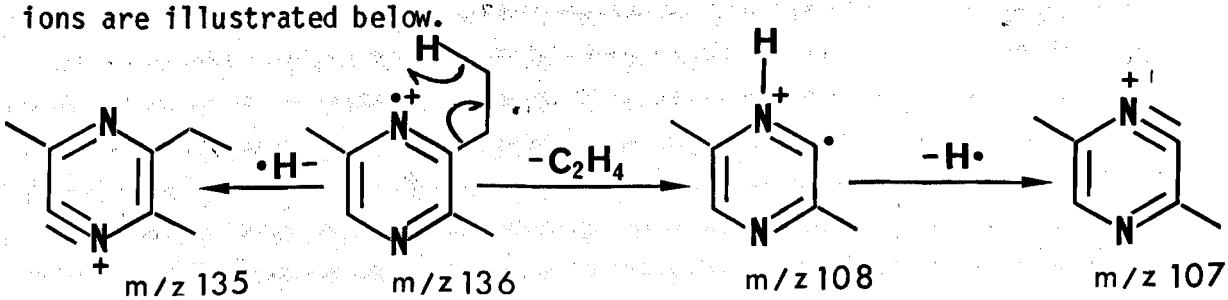
The mass spectrum of the peak with lower retention time was identified as 2,5-dimethylpyrazine [51] by comparison with published data[296,342]. Three positional isomers are possible for dimethylpyrazine, however the mass spectrum of each isomer is characteristic although those of 2,5- and 2,6-isomers are very similar. Therefore it was possible to deduce the structure to be 2,5-dimethylpyrazine and not the 2,3-or 2,6-isomers. The mass spectra of synthetic samples of the three isomers were recorded under identical conditions and only the spectrum of the 2,5-isomer corresponded to that of the natural material. The molecular ion was the base peak of the spectrum of 2,5-dimethylpyrazine. The peak at m/z 81 resulted from the loss of HCN from the M^+ ion.



The abundant fragment of m/z 42 was probably the protonated acetonitrile ion. The existence of such a species can be supported by the presence of M+1 peaks in the spectra of aliphatic nitriles[343].



The mass spectrum of the peak with higher retention time (Figure 15) was identified to be 3-ethyl-2,5-dimethylpyrazine [12]. It was identical with that obtained under the same conditions from the synthetic material. Furthermore the spectrum agreed well with that published in the literature[109,296]. The key fragmentation patterns that lead to the abundant ions are illustrated below.



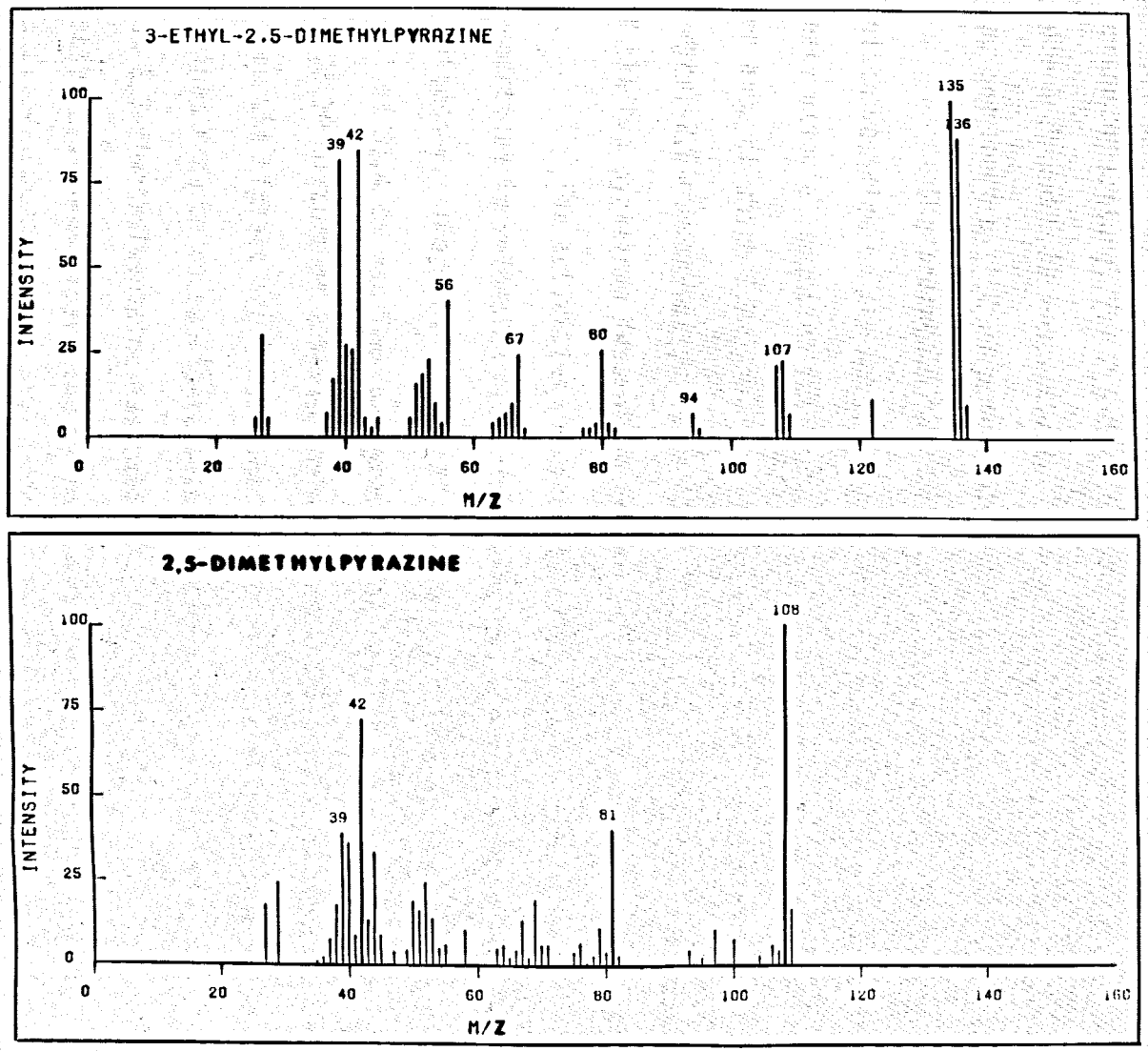


FIGURE 15. Mass spectra of the pyrazines from the poison gland of Tetramorium caespitum.

The confirmation of the two major components as 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine was achieved by the comparison of their chromatographic properties with authentic samples. The corresponding gas chromatographic retention times on three different GC phases were identical. Furthermore, the R_f values obtained on TLC (60:40, hexane in acetone) for 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were 0.38 and 0.52 respectively. These R_f values corresponded to the active regions shown by TLC of poison gland extracts (Figure 13).

The average total amount of the two pyrazines present in the poison vesicles of the worker ants was quantified by the comparison of the GC peaks using a computing integrator. The total amount was found to be 3.9 ng per ant, of which 2,5-dimethylpyrazine was 2.7±0.4 ng (70±4%) and 3-ethyl-2,5-dimethylpyrazine was 1.15±0.25 ng (30±4%). The absolute amounts of the two pyrazines determined in ten worker ants are listed in Table 15.

The activities of mixtures of the two pyrazines in different proportions in releasing trail following behaviour in worker ants were tested by bioassay. The total amount of the two pyrazines applied to the circular trail of 31.4 cm was four nanograms. The results are shown in Figure 16. The 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine showed the highest activity. The results given are the median values obtained from three replicate determinations (Table 16). Figure 16 shows that 2,5-dimethylpyrazine has twice the activity of 3-ethyl-2,5-dimethylpyrazine to evoke trail following behaviour when used in identical concentrations and conditions.

The most significant fact that can be seen from Figure 16 is that 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine makes a synergistic mixture with highest activity. When 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were tested separately using concentrations of 2.8 and 1.2 ng/31.4 cm trail the activities observed were 13.5 and 5.1 respectively. If one presumes the activities were additive, the activity of

Table 15. Absolute amounts of 2,5-dimethylpyrazine (DMP) and 3-ethyl-2,5-dimethylpyrazine (EDMP) present in the poison glands of ten *T. caespitum* workers.

Replicate number	Absolute amount (ng/ant)		Percentage by weight	
	DMP	EDMP	DMP	EDMP
1	2.4	0.75	76	24
2	3.2	0.8	80	20
3	2.3	1.2	66	34
4	3.1	1.5	67	33
5	2.6	1.2	68	32
6	3.1	1.3	70	30
7	2.4	1.4	63	37
8	3.3	1.2	73	27
9	2.4	0.95	72	28
10	2.4	1.1	68	32
Mean	2.7	1.15	70	30
SD	0.4	0.25	4	4

Table 16. Trail following activity evoked by mixtures of the two pyrazines in different proportions compared with that of one poison vesicle[a].

Test solution	DMP : EDMP ratio[b]	Trail following activity[c]			
		I	II	III	mean
Pyrazine mixture	10 : 0	14	16	15	15
	9 : 1	18	16	19	17.7
	8 : 2	26	24	25	25
	7 : 3	31	34	29	31.3
	6 : 4	28	29	26	27.7
	5 : 5	18.5	20	17	18.5
	4 : 6	15	17	14	15.3
	3 : 7	14	17	13	14.7
	2 : 8	10	9	9	9.3
	1 : 9	7	9	9	8.3
	0 : 10	7	9	5	7
Single poison gland		31	36	32	33
Blank (hexane)		0	0	0	0

a The trails were applied as hexane solutions (100 µl) on a circle of 5 cm radius marked with 1 cm arcs. The number of arcs run along the trail by each worker ant was recorded for 20 min and the median value was determined.

b The total concentration of 2,5-dimethylpyrazine (DMP) + 3-ethyl-2,5-dimethylpyrazine (EDMP) was 4 ng/trail.

c Mean values obtained from three replicate determinations.

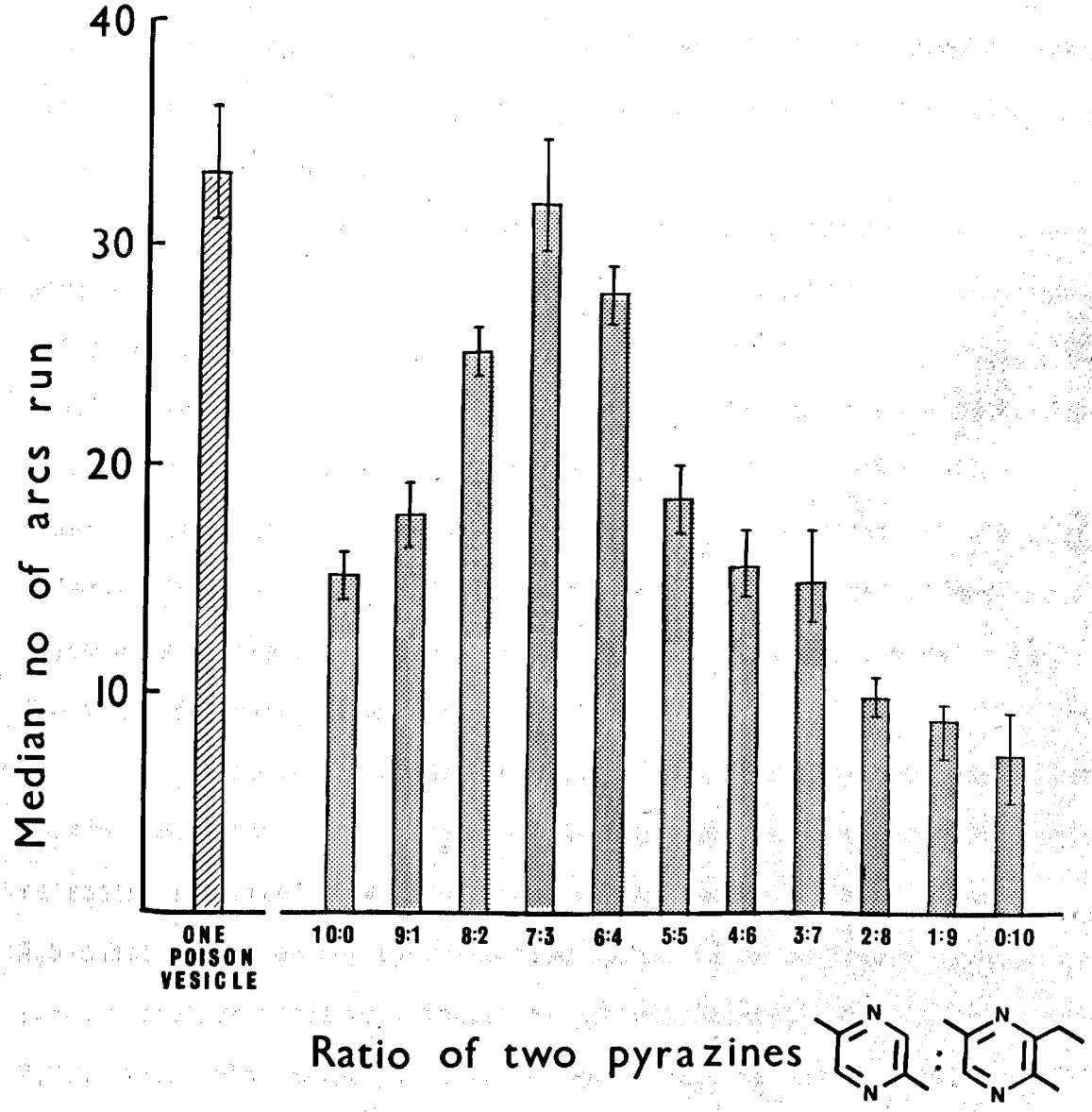


FIGURE 16. Trail following activity evoked by mixtures of the two pyrazines (4 ng/trail) in different proportions (stippled bars) compared with that of one poison vesicle (hatched bar). The trails were applied as hexane solutions (100 µl) with a 0.8 mm "Standardgraph" pen, on a circle of 5 cm radius, marked with 1 cm arcs, drawn on graph paper. The number of arcs run along the trail by each individual worker were recorded for 20 min. Each bar represents mean from three determinations and the vertical line indicates the range of values.

the mixture can be expected to be 18.6. But the 70:30 mixture demonstrated an activity of 31.3, nearly twice as great, demonstrating clearly that synergism operates. Furthermore the 70:30 mixture showed no significant difference in activity when compared with a trail made of a single poison vesicle. Although at least one multi-component trail pheromone of ants has been reported[120], this is the first complete identification of the composition of such a pheromone mixture.

A range of concentrations of the two pyrazines were presented separately to the ants to determine the amounts of material that evoke most efficient trail following behaviour. Concentrations between 1-10 ng/31.4 cm trail released highest activity (Figure 17). The ants were able to detect concentrations as low as 0.3 pg/cm but showed difficulty in following any lower concentrations. Moreover relatively high concentrations also failed to release efficient trail following behaviour. When concentrations above 3 ng/cm were applied, the ants were alarmed and confused, and exhibited a very low trail following behaviour.

A number of related compounds were tested by bioassay in order to obtain some information concerning the stereochemical requirements for trail releasing activity. The results are summarized in Table 17. The 2,5-substitution on the pyrazine ring appear to be important because 2,3- and 2,6-dimethylpyrazines were inactive. It was interesting to find that 2,3,5-trimethylpyrazine was able to evoke weak trail following in *T. caespitum* and the three species of *Myrmica* tested. *T. impurum* did not respond to any of the chemicals tested indicating its trail pheromone to be different from any of the tested compounds.

It needs to be mentioned that the bioassays were performed always under identical condition as far as possible. Therefore the results obtained in any given experiment, conducted within a short period of time were comparable. Nevertheless significant differences in absolute values can occur from time to time because the activity of ants is dependent on many

FIGURE 17. BIOASSAY OF THE TWO PYRAZINES

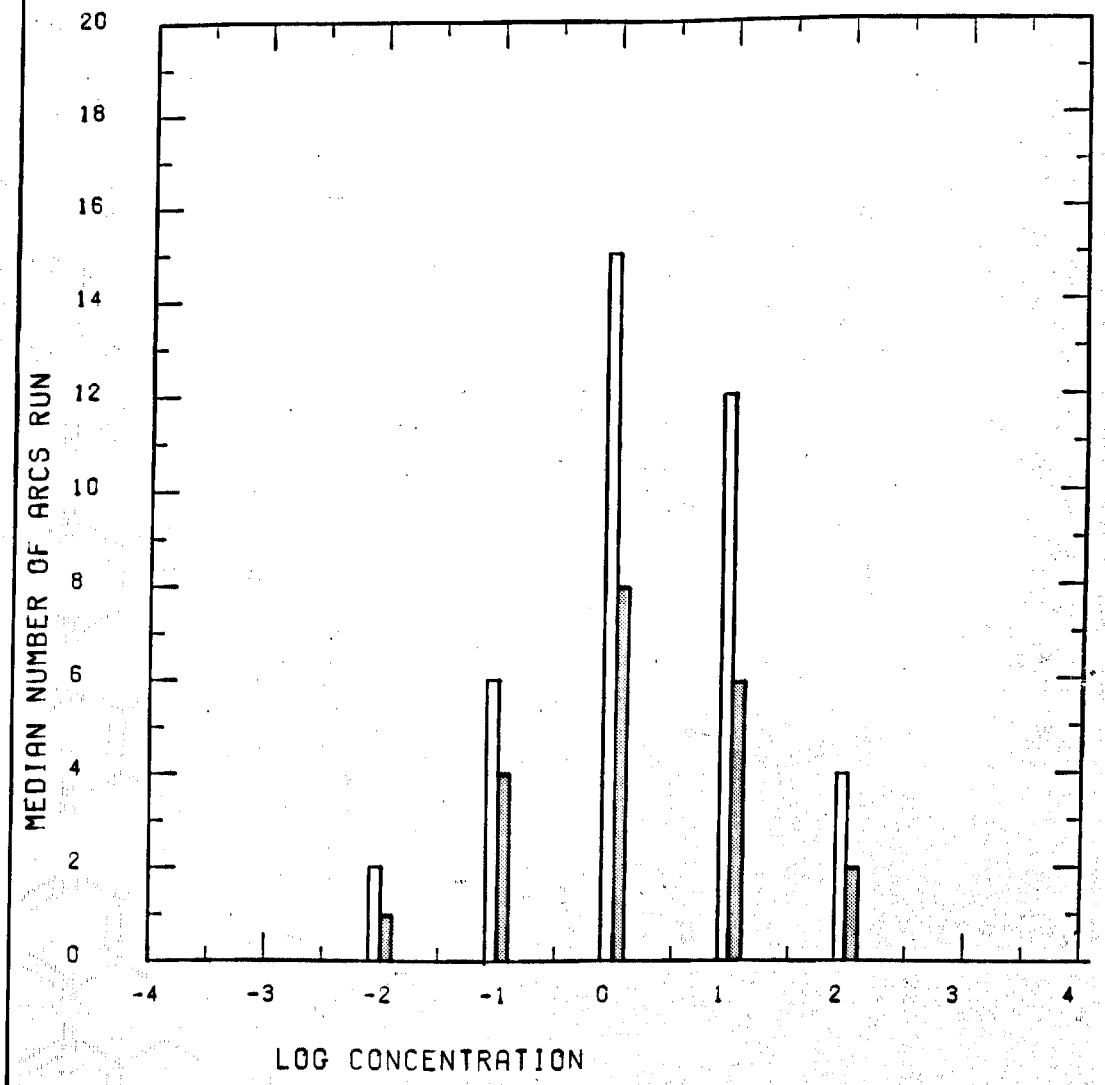
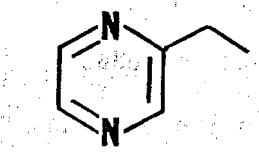
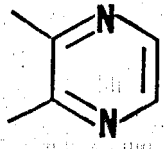
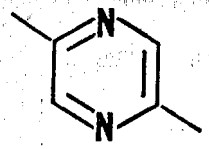
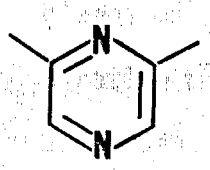
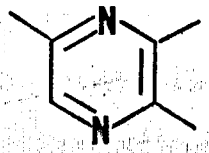
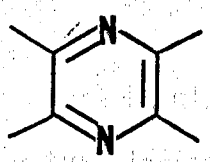
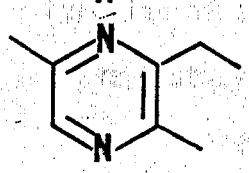
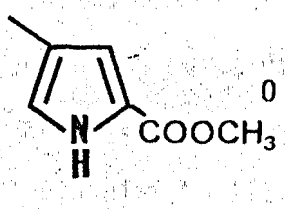


FIGURE 17. Bioassay of the two pyrazines over a range of concentrations. 2,5-dimethylpyrazine (empty bars) and 3-ethyl-2,5-dimethylpyrazine (stippled bars) were applied as hexane solutions of different concentrations on a circular trail (5 cm radius). The number of arcs run along the trail by each individual worker were recorded for 20 min and median value was obtained.

Table 17. Trail following activity evoked by various substituted pyrazines and a pyrrole on a few species of *Tetramorium* and *Myrmica*[a].

Compound	Test species				
	T. caespitum	*T. impurum*	*M. rubra*	*M. ruginodis*	*M. scabrinodis*
	1	0	0	0	0
	0	0	0	0	0
	14	0	0	0	0
	0	0	0	0	0
	6	0	3	4	3
	0	0	0	0	0
	8	0	10	10	12
	0	0	0	0	0

[a] Trails were applied as hexane solutions (4 ng/31.4 cm) on a circle of 5 cm radius marked with arcs (1 cm). The number of arcs run along the trail by each worker ant was recorded for 20 min and the median value was calculated.

factors such as sunlight, temperature, humidity, time of the day, etc.

Pyrazines have been identified from a variety of sources in nature[344]. 2,5-dimethylpyrazine has been identified as a flavour component of potato chips[342]. It is also found in fusel oil, black tobacco and in the smoke of nonfilter cigarettes made from these tobaccos[344]. 3-ethyl-2,5-dimethylpyrazine is a component important to the aroma of baked potato[345] and coffee[346]. Maga and Sizer[347] have published a review listing the extensive occurrence of alkylpyrazines in foods. The review published by Brophy and Cavill[348] provides the mass spectra of the pyrazines. A number of trisubstituted alkyl pyrazines have been reported from the mandibular glands of some subfamilies of ants (Table 9). Many other insects like wasps[190], flies[195] and some beetles also have the ability to biosynthesize pyrazines.

A wide variety of alkaloids have been identified from the poison glands of ants (Table 8) but 3-ethyl-2,5-dimethylpyrazine was the only pyrazine that had been identified prior to this study. It has been shown to be present in the trail pheromone of *Atta sexdens rubropilosa*[108] and eight species of *Myrmica* ants[109]. The 2,5-dimethylpyrazine identified in *Tetramorium caespitum* is novel because it is the simplest and the only disubstituted pyrazine yet reported from ants.

Blum[69] has postulated that the trail pheromones of myrmicine ants were originally trace constituents of the venom that were exploited as the function of the gland changed into its present form. The results of the present study clearly showed that the trail pheromone components of *T. caespitum* are indeed trace constituents. The pyrazines occupy only 0.03% of the volume of the poison gland. The poison reservoir contents have been reported to be rich in free amino acids[99], which probably can react enzymatically to form pyrazines[349]. Morgan (unpublished) has postulated a mechanism to illustrate the biosynthesis of pyrazines from amino acid precursors.

As the same 3-ethyl-2,5-dimethylpyrazine had been identified as

the trail pheromone of eight species of *Myrmica*, it was anticipated that *T. caespitum* and species of *Myrmica* may follow each others trail. Such a transposition study has not been carried out previously. Therefore a cross-activity study was carried out with poison gland extracts of *T. caespitum* and *M. rubra*. It was found that they indeed followed each others trails. The results are given in Table 18. The chemical composition of the poison glands can explain their behaviour. *T. caespitum* will follow the trails of *Myrmica*, moderately because of the presence of the 3-ethyl-2,5-dimethylpyrazine. On the other hand the trail pheromone of *Myrmica* ants has only one component. Poison glandsof *M. ruginodis* were examined carefully and a peak corresponding to 2,5-dimethylpyrazine was not found in the GC traces (Figure 18). *Myrmica* ants did follow poison gland extracts of *T. caespitum* because of the presence of 3-ethyl-2,5-dimethylpyrazine. Similar observations in inter-specific trail following behaviour have been reported with the leaf-cutting ants *Acromyrmex octospinosus* and *Atta sexdens*. The major components of their trail pheromones are methyl 4-methylpyrrole-2-carboxylate and 3-ethyl-2,5-dimethylpyrazine respectively. However *Acromyrmex octospinosus* will follow the trails of *Atta sexdens* because the pyrrole is also present as a trace component in the venom of the latter. But *A. sexdens* will not follow a trail of pure synthetic pyrrole.

Bolton[350] has suggested on the ground of habitats and morphology that *Tetramorium* and *Myrmica* have most probably decended from the same ancestral stock . The similarities of the chemical composition of the poison glands and the trail following behaviour, as found in the present study appear to support the postulation of Bolton, which considers the tribes *Myrmicini* and *Tetramoriini* to be closely related.

Table 18. Trail following activity evoked by individual poison glands of two species of Myrmicinae subfamily[a].

Source species	Test species	
	T. caespitum	*M. ruginodis*
Tetramorium caespitum	31	14
Myrmica ruginodis	12	19

[a] The trails were applied as hexane solutions (100 µl) containing one poison gland equivalent of material, on a circle of 5 cm radius marked with 1 cm arcs. The number of arcs run along the trail by each worker ant was recorded for 20 min and the median value was determined.

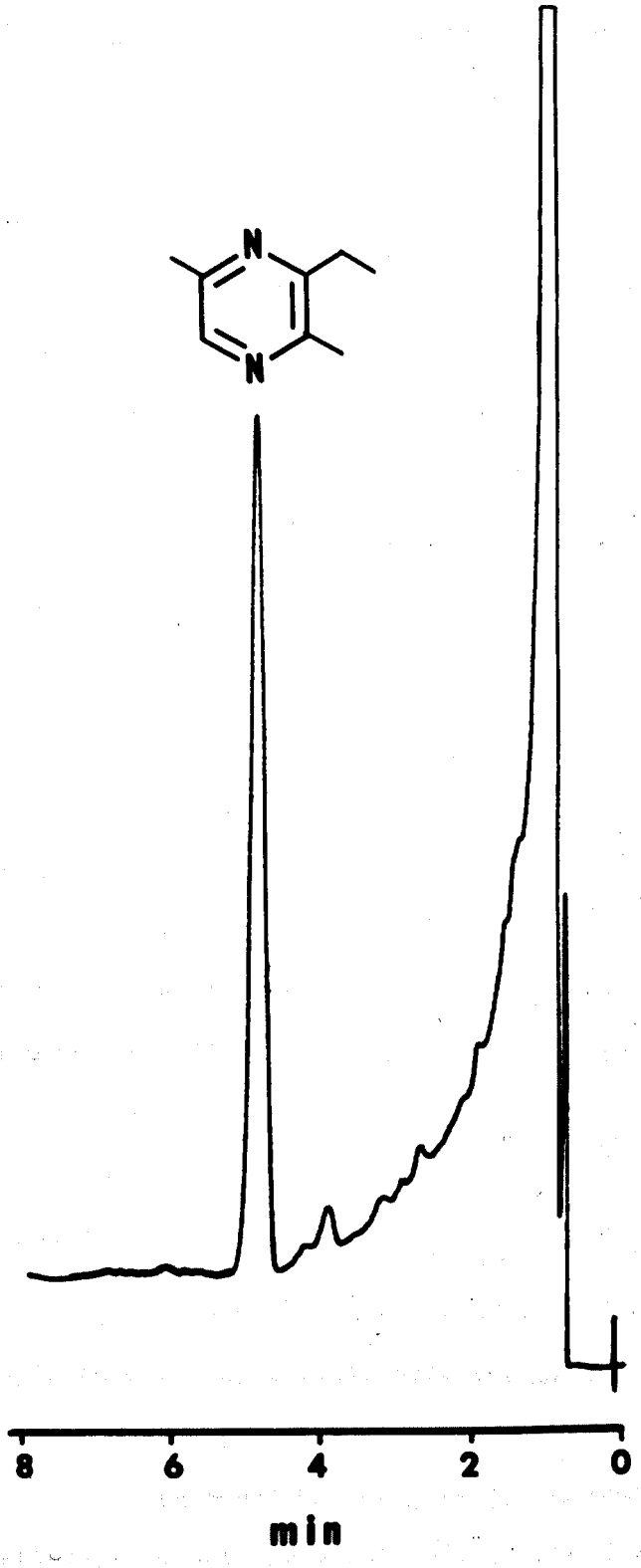


FIGURE 18. A gas chromatogram of three poison vesicles of Myrmica ruginodis. The glands were solid injected on a 2.75 x 4 mm packed column of 10% PEG 20M on Chromosorb W, at 130 °C (attenuation x 20).

2.3 Chemical Investigation of *Solenopsis geminata*

Solenopsis geminata is a circum-tropical ant species generally known as a fire ant because of the potency of its venom. In Sri Lanka it is sometimes known as the cobra ant (because of its violent sting) and also as the oil ant (because it infests oil cake used as cattle feed). Species of fire ants are considered as important economic pests, particularly in the southern United States, where every conceivable method of control has been utilized by some property owners to get rid of them. Besides being a domestic nuisance, recent medical studies indicate that the fire ants are assuming a greater importance as a public health hazard. Their venom exhibits pronounced necrotic[167] and hemolytic[168] activities. In 1970, 12,438 patients were treated for fire ant stings in the Mississippi, Alabama and Georgia areas[351].

Toxic baits have been used since 1957 for the control of fire ants[351]. The first highly effective toxicant discovered was mirex[352]. However, the ill-effects due to indiscrimate use and aerial spraying of these toxicants soon became evident. In 1962, Rachel Carson[353] in her controversial book "Silent Spring" severely criticized the use of toxicants to eradicate fire ants. Although the use of mirex has been banned, since 1978 in the United States, because of the environmental pollution and suspected carcinogenicity, many states are still seeking to reintroduce it as the fire ants are continuing to spread[354].

In order to avoid environmental damage caused by persistent pesticides, novel and more effective insect control methods have been sought recently. The use of semiochemicals to manipulate and control the behaviour of insects has gained popularity as a more acceptable means of insect control.

Although the research on the behaviour of fire ants has increased dramatically in the past few years, the information available on their

chemical communication system is still very elementary. Here, the chemical compositions of postpharyngeal, poison and Dufour glands of *S. geminata* were investigated in order to gain a better understanding of its chemical communication system.

2.3.1 Postpharyngeal gland

The functions of the postpharyngeal gland remain unknown although numerous suggestions have been made (1.6.5). Most of the reported work has been carried out only on the postpharyngeal glands of the queens although the glands are present in males and workers as well[206]. Vinson *et al.*[212] have found that the postpharyngeal glandectomized queens of *S. invicta* were treated normally by the workers but the queens continuously lost weight and died within two months. The chemical analysis of the glands has been carried out only for the queens of *S. invicta*[211]. Hydrocarbons have been reported as the major component (63%) and significant amounts of free fatty acids and glycerol esters (32%) were also present. No chemical information was available about the postpharyngeal glands of worker ants, therefore the glands of *S. geminata* workers were investigated in the present study.

The examination of the excised postpharyngeal glands by GC revealed that the major components were hydrocarbons. Figure 19 shows a typical chromatogram obtained from a single postpharyngeal gland. The comparison of the gas chromatograms obtained from excised glands with those of the individual heads revealed that the volatile chemicals of the heads do originate essentially from the postpharyngeal glands. Three components represent 85% of the volatile material present in the gland (Table 19). These three components were identified as heneicosane ($M^+296, C_{21}H_{44}$), tricosene ($M^+322, C_{23}H_{46}$) and tricosane ($M^+324, C_{23}H_{48}$) by their mass spectra (Figure 20) obtained by GC-MS. Peak 2, 5 and 7 lie on one straight line when the logarithms of retention times from an isothermal run were plotted against their carbon numbers.

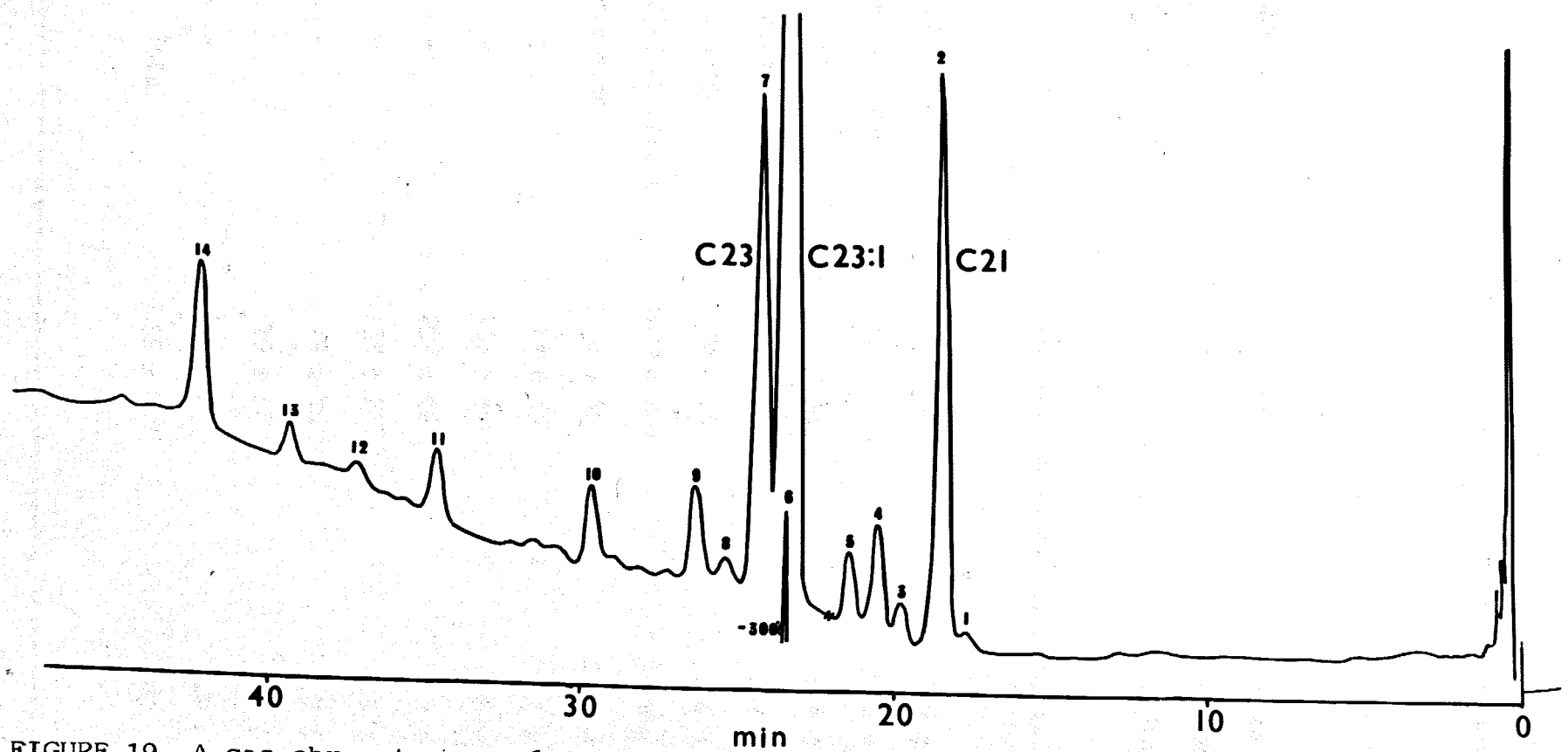


FIGURE 19. A gas chromatogram of postpharyngeal gland contents of Solenopsis geminata. A gland was solid injected on a 1.5 mm x 4 mm packed column of 5% SE-30 on Chromosorb W. The oven temperature was programmed from 147 to 260 °C at 3 °C/min. The attenuation was x500 upto 22 min and x1000 for the rest. The peak numbers refer to Table 19.

Table 19. Composition of the volatile chemicals on the postpharyngeal gland of *Solenopsis geminata*

No[a]	Compound	Mean composition[b] by weight (ng/ant ± S.D.)	Mean % by weight ± S.D.
1	(Heneicosene ?)	62 ± 29	0.7 ± 0.3
2	Heneicosane	2215 ± 356	23.2 ± 2.2
3	-	81 ± 56	0.8 ± 0.4
4	(Docosene ?)	162 ± 111	1.7 ± 0.9
5	Docosane	94 ± 24	1.1 ± 0.5
6	(Z)-9-tricosene	4672 ± 604	49.9 ± 4.1
7	Tricosane	1057 ± 89	11.4 ± 0.6
8	-	64 ± 24	0.7 ± 0.2
9	-	155 ± 45	1.6 ± 0.4
10	-	202 ± 48	2.2 ± 0.5
11	-	132 ± 22	1.5 ± 0.3
12	-	79 ± 35	1.0 ± 0.3
13	-	70 ± 17	0.8 ± 0.2
14	-	315 ± 57	3.4 ± 0.8
	Total	9356 ± 980	100

[a] numbers refer to the peaks of Figure 19.

[b] numbers of replicates =10

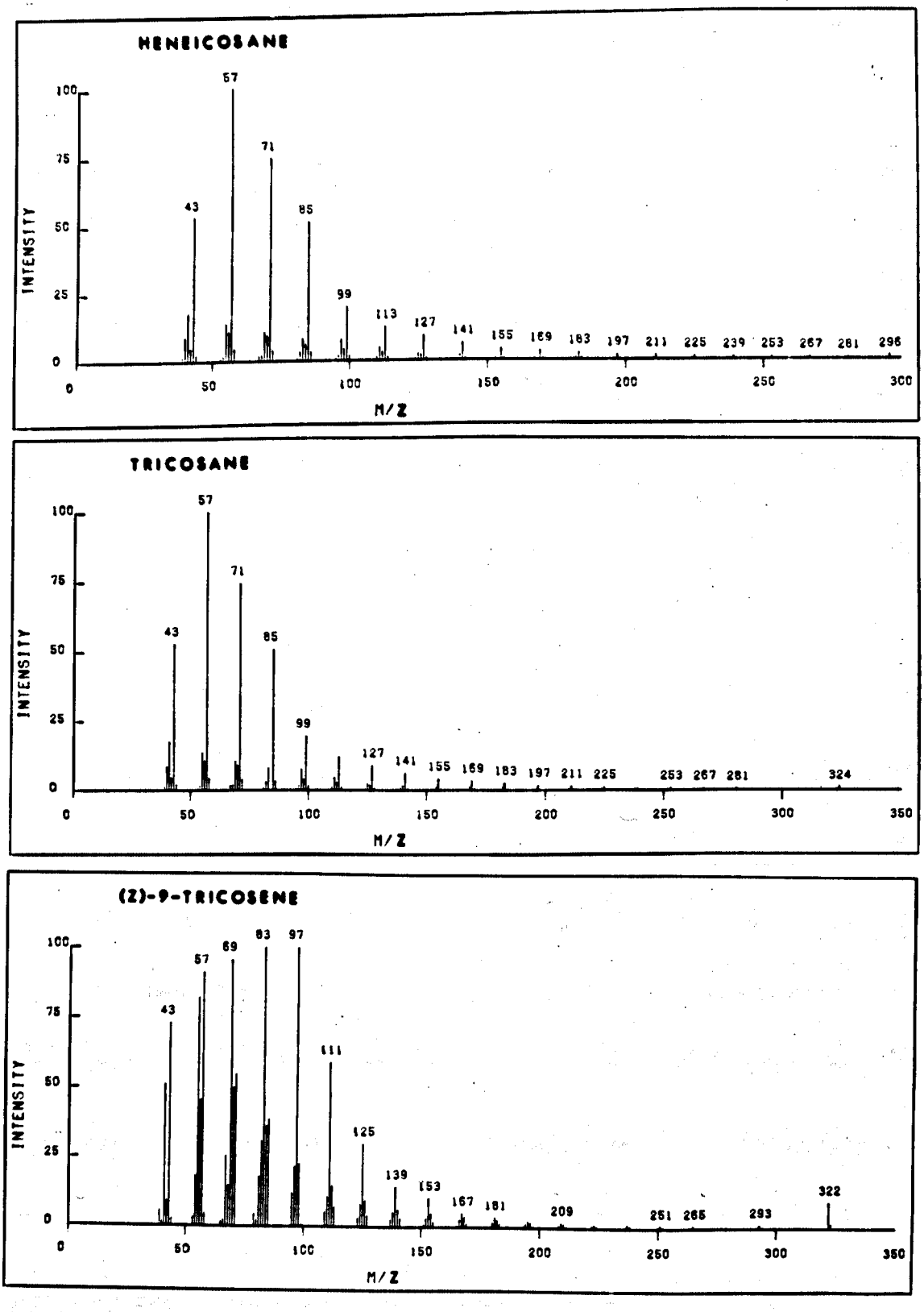
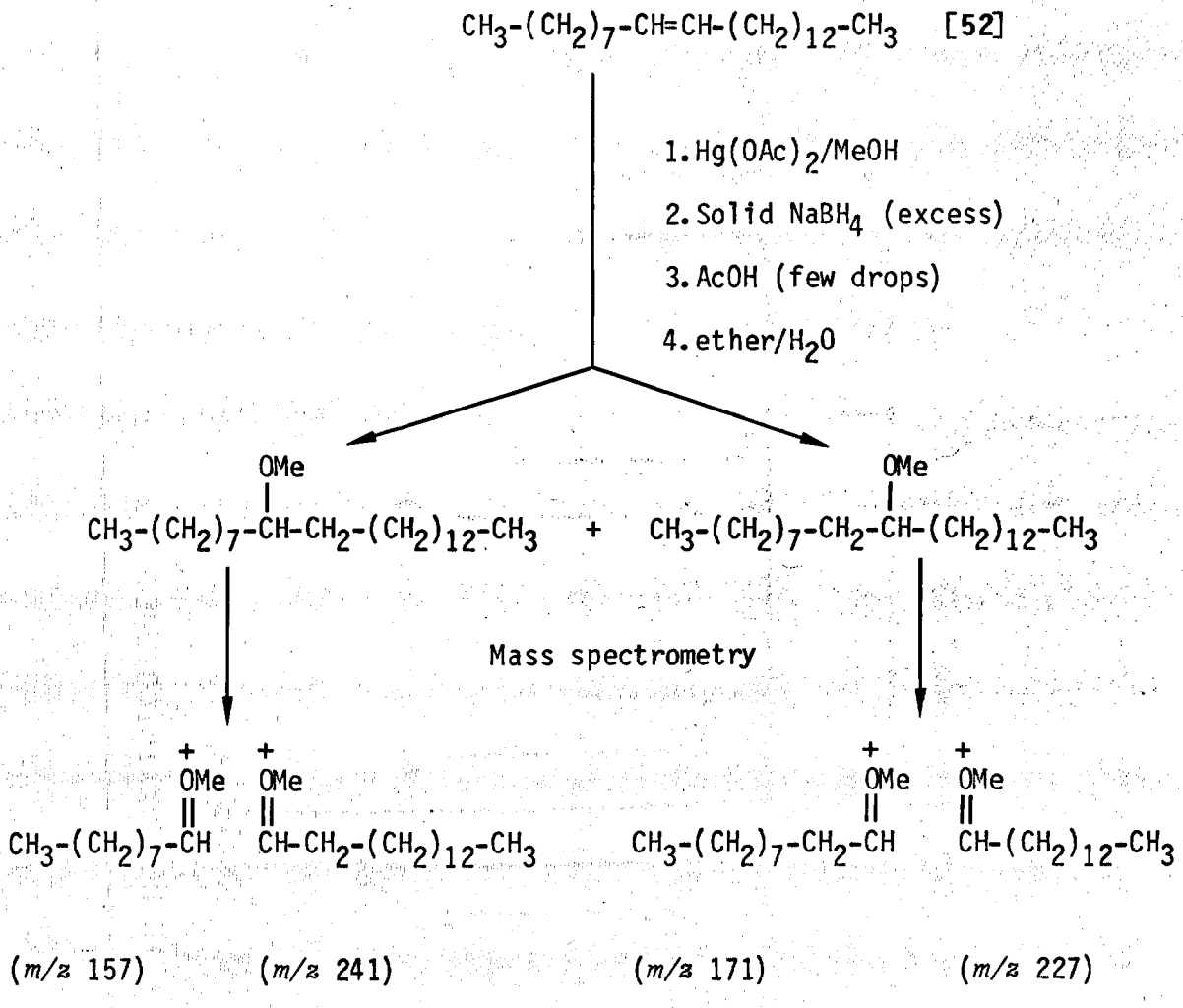


FIGURE 20. Mass spectra of heneicosane, tricosane and (Z)-9-tricosene from the postpharyngeal gland of Solenopsis geminata.

The location of the double bond position of the tricosene isomer was achieved by methoxymercuration-demercuration. The two methoxy derivatives thus obtained from tricosene was subjected to GC-MS. The mixed mass spectrum of the two derivatives gave intense peaks at *m/z* 157, 171, 227 and 241 values (Figure 21). The fragmentation of the two methoxy derivatives takes place preferentially on the either side of the methoxy groups as illustrated below. The formation of these ions clearly showed that the double bond was located at the C9 position.



The configuration of the double bond of the 9-tricosene from the postpharyngeal gland was established as the (Z)-isomer by argentation-TLC. The substance from the ants gave a single spot corresponding to a R_f value of 0.55 when chromatographed using a 1% diethylether in light petroleum solvent system. The spot from authentic (Z)-9-tricosene gave the identical R_f value

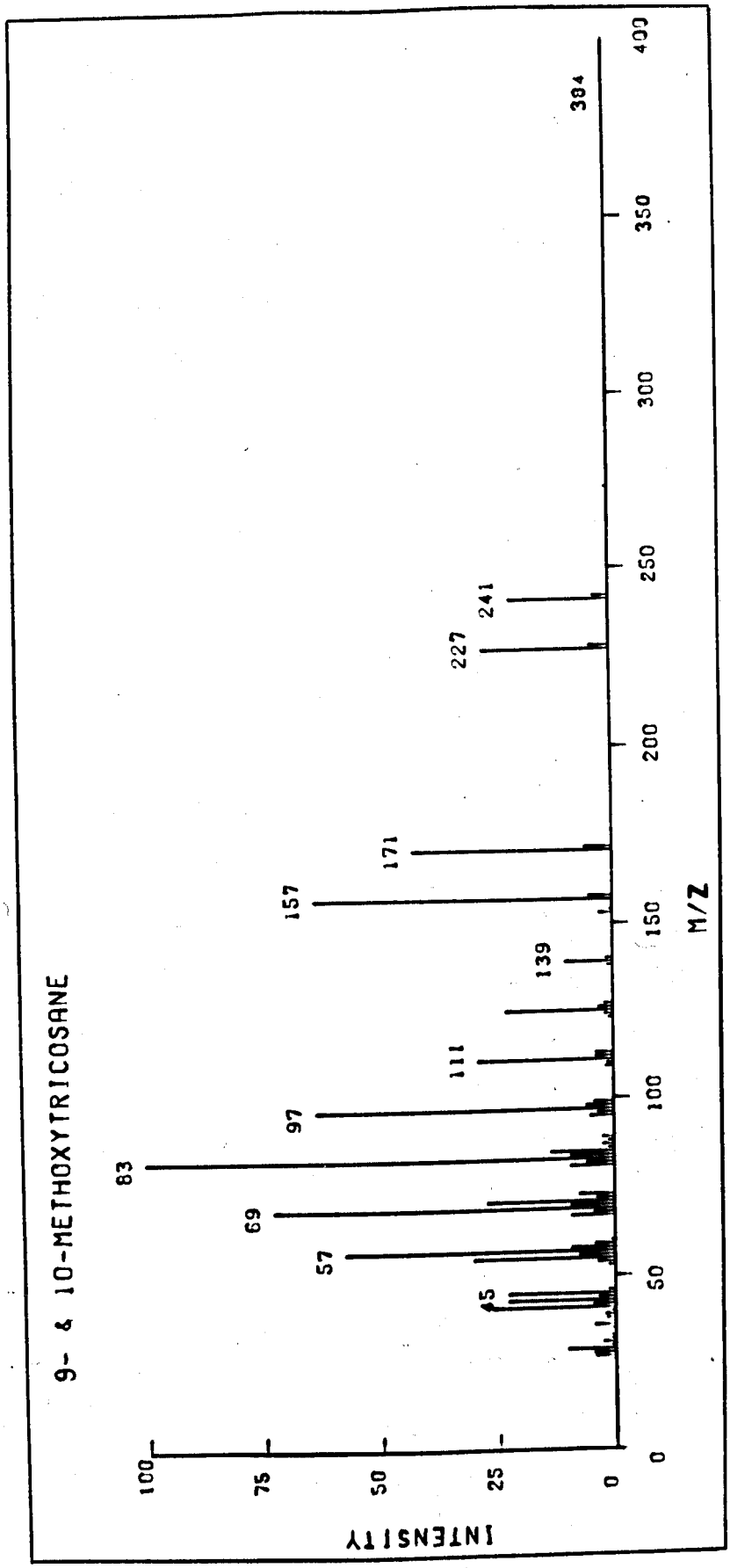


FIGURE 21. A mixed mass spectrum of 9-methoxytricosane and 10-methoxytricosane.

under the same conditions. The mixture of synthetic (Z)-and (E)-9-nonadecene gave two spots at R_f values 0.55 and 0.75 corresponding to the (Z)- and (E)-isomers respectively. The natural compound clearly has the (Z)-configuration.

The three major components, which comprised 85% of the volatile material of the gland, identified as eicosane, tricosane and (Z)-9-tricosene had the same chromatographic and mass spectrometric properties as their corresponding authentic materials. No significant behavioural response was observed when (Z)-9-tricosene was presented to the worker ants. However, Thompson *et al.*[211] have reported in a similar study that worker ants of *S. invicta* clustered around a sample of hydrocarbons from the postpharyngeal gland of the queens.

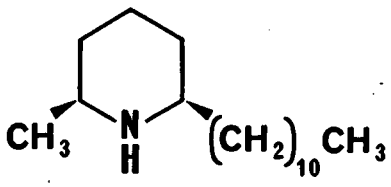
Thompson *et al.*[211] discovered that the major class of compounds in the postpharyngeal gland of *S. invicta* queen was hydrocarbons. The hydrocarbon fraction composed of four uncommon methyl-branched compounds, namely 13-methylheptacosane, 3-methylheptacosane, 13,15-dimethylheptacosane and 3,9-dimethylheptacosane. The published GC-traces show no major peaks in the C_{22} to C_{23} region[206]. It is evident that the composition of the postpharyngeal gland of *S. invicta* queen and *S. geminata* workers are significantly different from each other. However, the information available is too incomplete to make any conclusions whether the difference is species or caste specific.

The general theme of myrmicine mandibular gland secretions is 3-alkanones and 3-alkanols of the C_6 to C_{11} range (1.6.4). Surprisingly, the mandibular glands of *S. geminata* workers showed no evidence of any similar compounds at the sensitivities examined (*ca.* 10 ng of any compounds would have been detected). A slow temperature programme was used to scan the region between 600 to 2000 of the retention indices scale but no significant peaks were apparent.

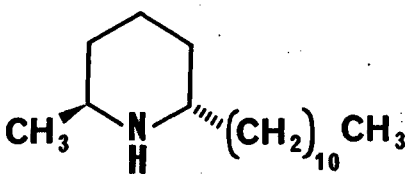
2.3.2 Poison gland

The poison gland, venom reservoir and Dufour gland are the major glandular components of the venom apparatus of *S. geminata* (Figure 22). The two filamentous glands are attached to the convoluted gland found within the venom reservoir. The Dufour gland and the venom reservoir open into the poison bulb at the base of the sting where the contents of either can be emitted from the tip of the sting.

The members of the genus *Solenopsis* are known to possess alkaloid-rich venoms (Table 8). The characteristic compounds of the venom of the sub-genus Solenopsis are the *cis*- and *trans*- 2-alkyl-6-methylpiperidines. Brand *et al.*[163] have examined the venom of *S. geminata* collected from Texas, U.S.A.. They found *cis*- and *trans*-2-methyl-6-undecylpiperidine [53,54] were the major components of the venom with trace amounts of *cis*-2-methyl-6-tridecylpiperidine and *cis*-2-methyl-6-(Z-4-tridecenyl)-piperidine. In the present study the venom of *S. geminata* from Sri Lanka was examined and compared with the published data for *S. geminata* from Texas in order to determine how such a vast geographical separation may affect the composition of the venom.



[53]



[54]

The result of the study clearly showed that there is no significant difference in the composition of the position gland of *S. geminata* from Sri Lanka and Texas. The gas chromatography of poison glands of *S. geminata* workers from Sri Lanka showed the presence of two major components (Figure 23). The mass spectra of both peaks obtained by GC-MS were identical (Figure 24). The two peaks were identified as *cis*-

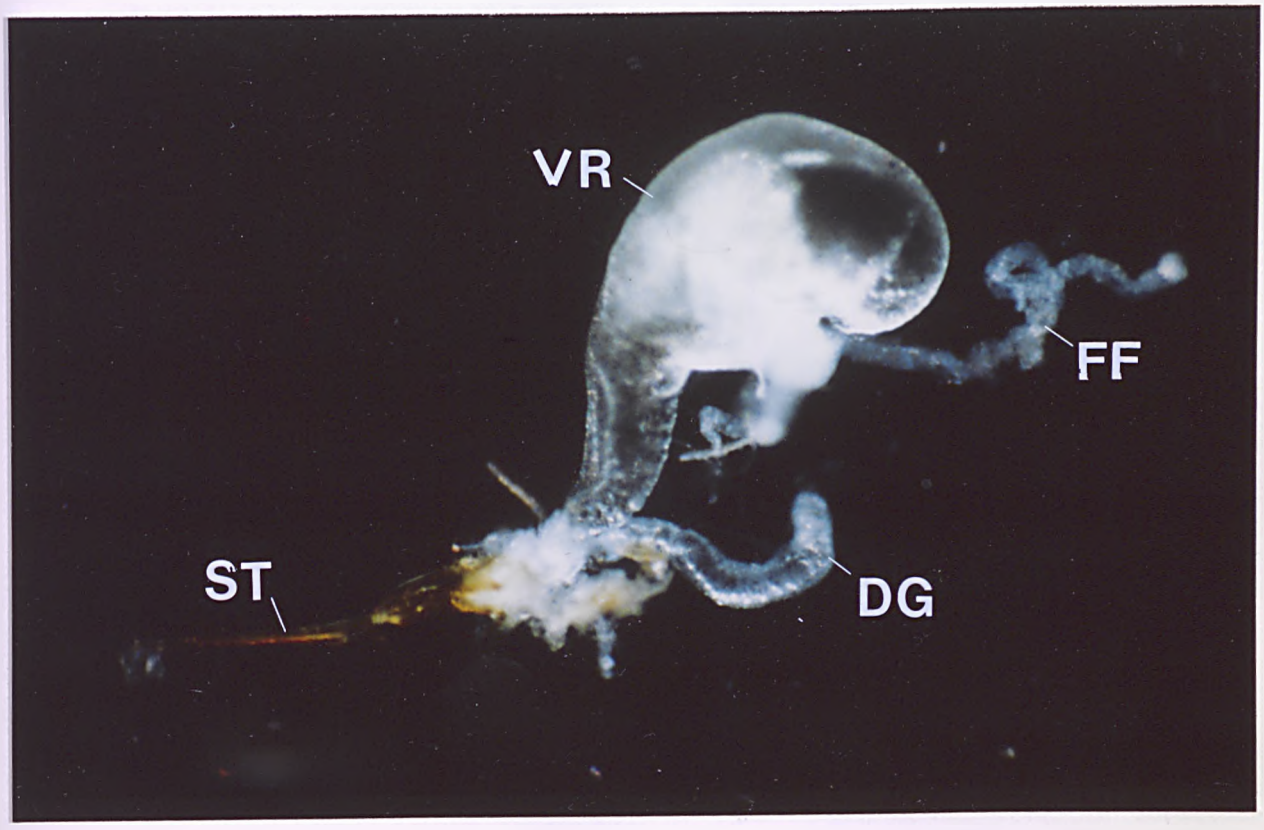


FIGURE 22. **Venom apparatus of Solenopsis geminata.** FF: free filaments of the poison gland, VR: venom reservoir, DG: Dufour gland, ST: sting.

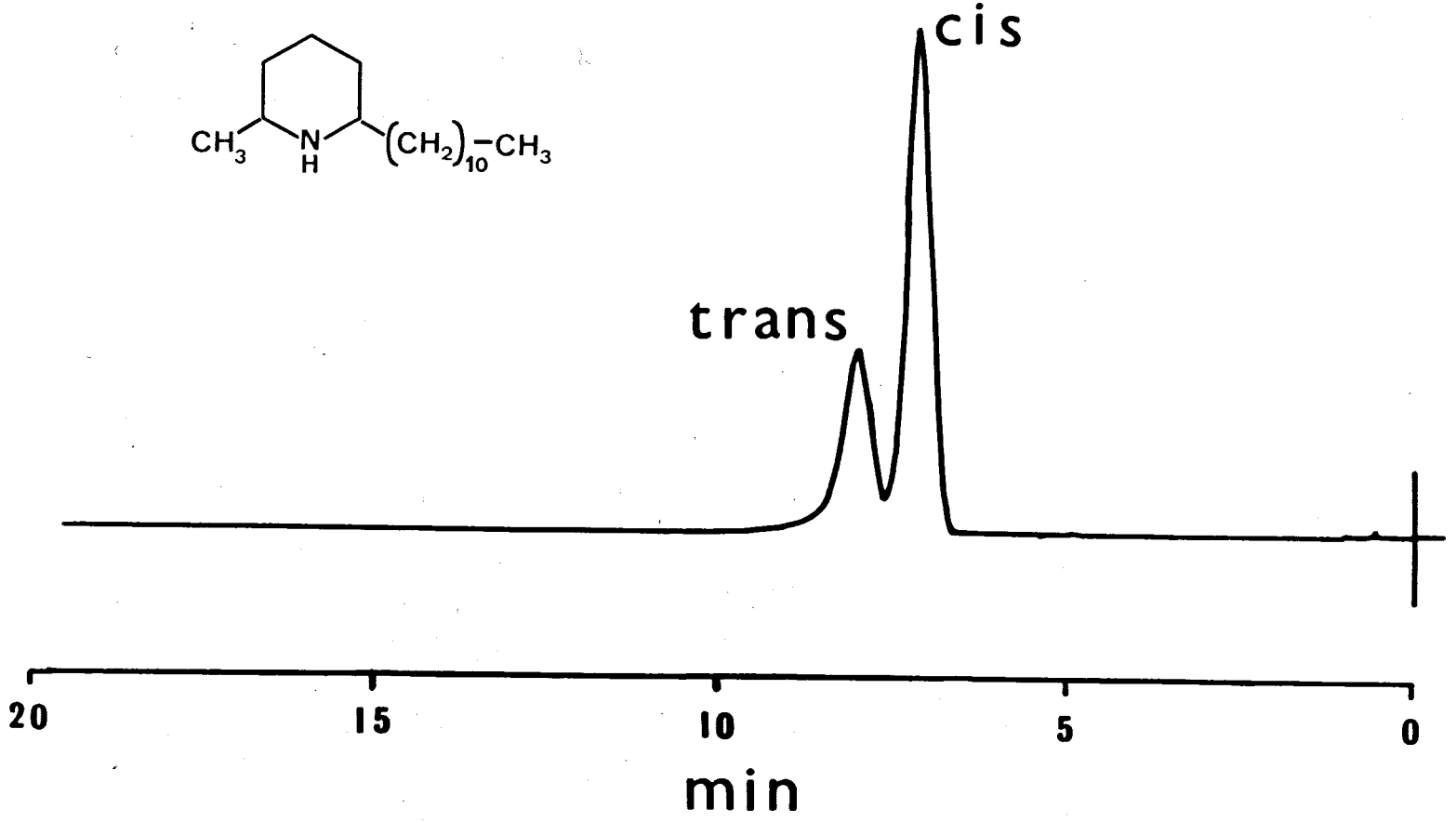


FIGURE 23. A gas chromatogram of poison gland contents of Solenopsis geminata. One gland was solid injected on a 2.75 x 4 mm packed column of 10% PEG 20M on Chromosorb W, at 185 °C (Attenuation x 10,000).

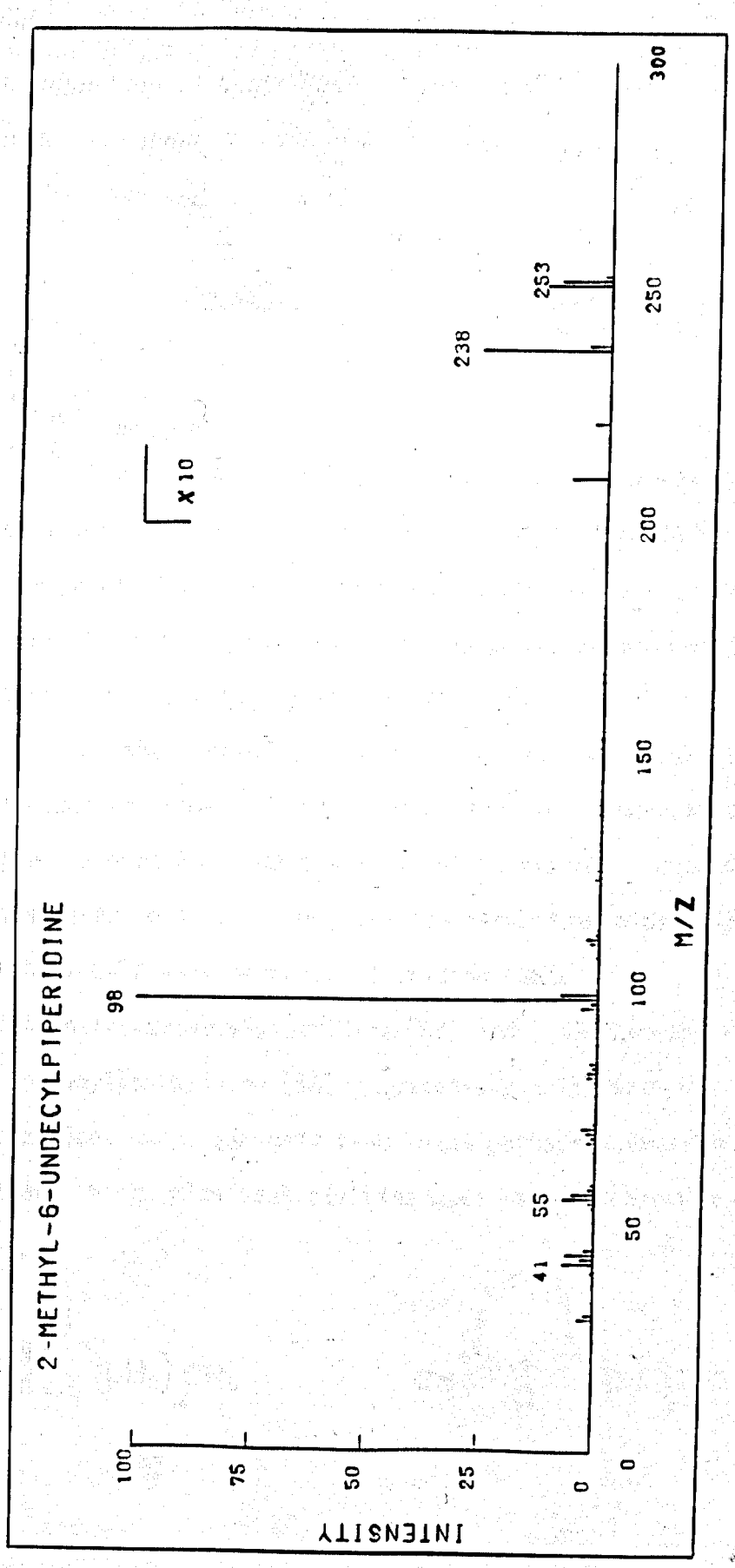
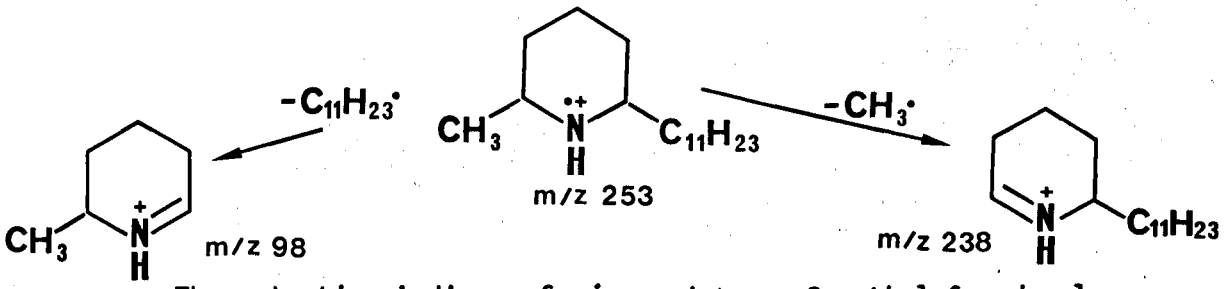


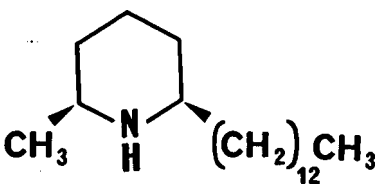
FIGURE 24. Mass spectrum of 2-methyl-6-undecylpiperidine

2-methyl-6-undecylpiperidine [53] and *trans*-2-methyl-6-undecylpiperidine [54] by the comparison of their mass spectra with the published data[162]. The main fragmentation pathways responsible for the abundant ions are illustrated below. The base peak at *m/z* 98 is characteristic of methyl piperidines.

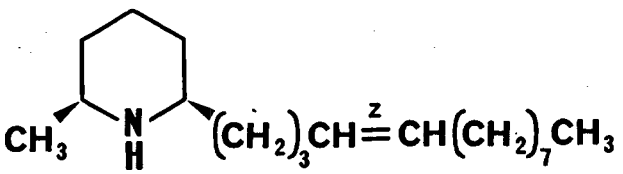


The retention indices of *cis*- and *trans*-2-methyl-6-undecyl piperidine were 1994 and 2055 on PEG 20M and 1840 and 1870 on OV-1 stationary phases respectively. The chromatographic and mass spectroscopic data of the two natural products agreed well with those obtained under the same conditions from authentic synthetic materials.

Further analysis of the venom for trace constituents on the OV-1 capillary column showed the presence of four more components in minute quantities (Figure 25). On the basis of GC retention data the four peaks were considered to be *cis*-2-methyl-6-tridecylpiperidine [55], *cis*-2-methyl-6-(Z-4-tridecenyl) piperidine [56], *trans*-2-methyl-6-tridecylpiperidine [57] and *trans*-2-methyl-6-(Z-4-tridecenyl)piperidine [58] respectively. The last two compounds had not been recognized in *S. geminata* from Texas perhaps because Brand *et al.*[163] did not operate at the high sensitivities used in the present work.



[55]



[56]

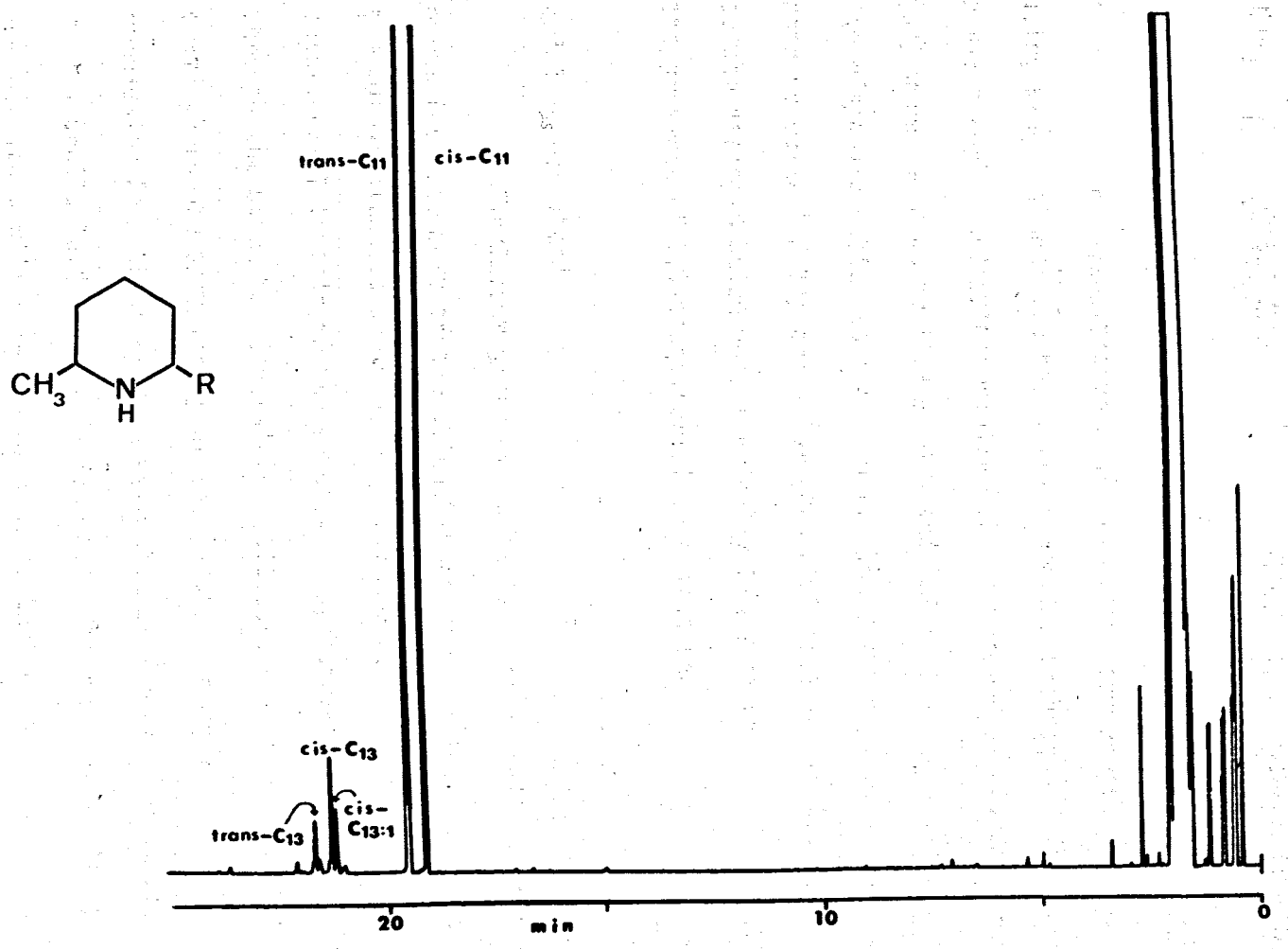
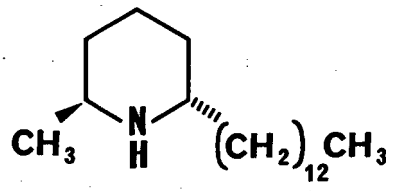
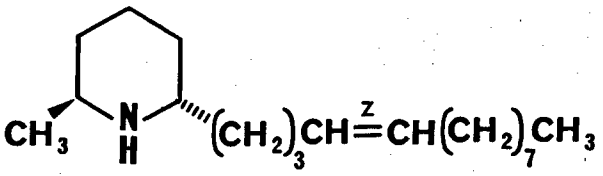


FIGURE 25. A gas chromatogram of an extract of the poison gland of Solenopsis geminata. One poison gland was extracted with dichloromethane (5 µl) and the solution was chromatographed (0.5 µl) on the OV-1 capillary column with the oven temperature programmed from 40 to 300 °C at 10 °C/min (attenuation x 128).



[57]



[58]

The quantitative analysis of the components were carried out using single glands via the solid injection technique. The amounts of *cis*-2-methyl-6-undecylpiperidine [53] and *trans*-2-methyl-6-undecylpiperidine [54] were found to be 11.8 ± 0.8 and 6.9 ± 0.5 µg/worker ant respectively. The *cis/trans* ratio was 1.7 ± 0.1. The *cis* isomer always eluted first on both nonpolar (SE 30) and polar (PEG 20M) stationary phases. This may be due to the nitrogen being more sterically hindered in the *cis* isomer because of the presence of 2,6-diequitorial substitution.

2.3.3 Dufour gland

The Dufour gland of *S. geminata* is a small elongated sac that opens into the bulb of the sting (Figure 22). Wilson[355] first established the Dufour gland as the source of the trail pheromone of *S. invicta*. The actual trail is laid on the substratum by streaking the Dufour gland contents through the sting[98]. Several investigators have conducted studies on the trail pheromone specificity of the genus *Solenopsis*[18,98,142]. The general conclusion from these studies were that *S. invicta* and *S. richteri* will follow trails laid separately from each other's Dufour glands and *S. geminata* and *S. xyloni* will make a similar pair that respond to each other's Dufour gland extracts.

A controversy seem to exist about the composition of the trail pheromone of *S. invicta*. According to Vander Meer *et al.*[120] it is a multicomponent pheromone and four of the components were reported as

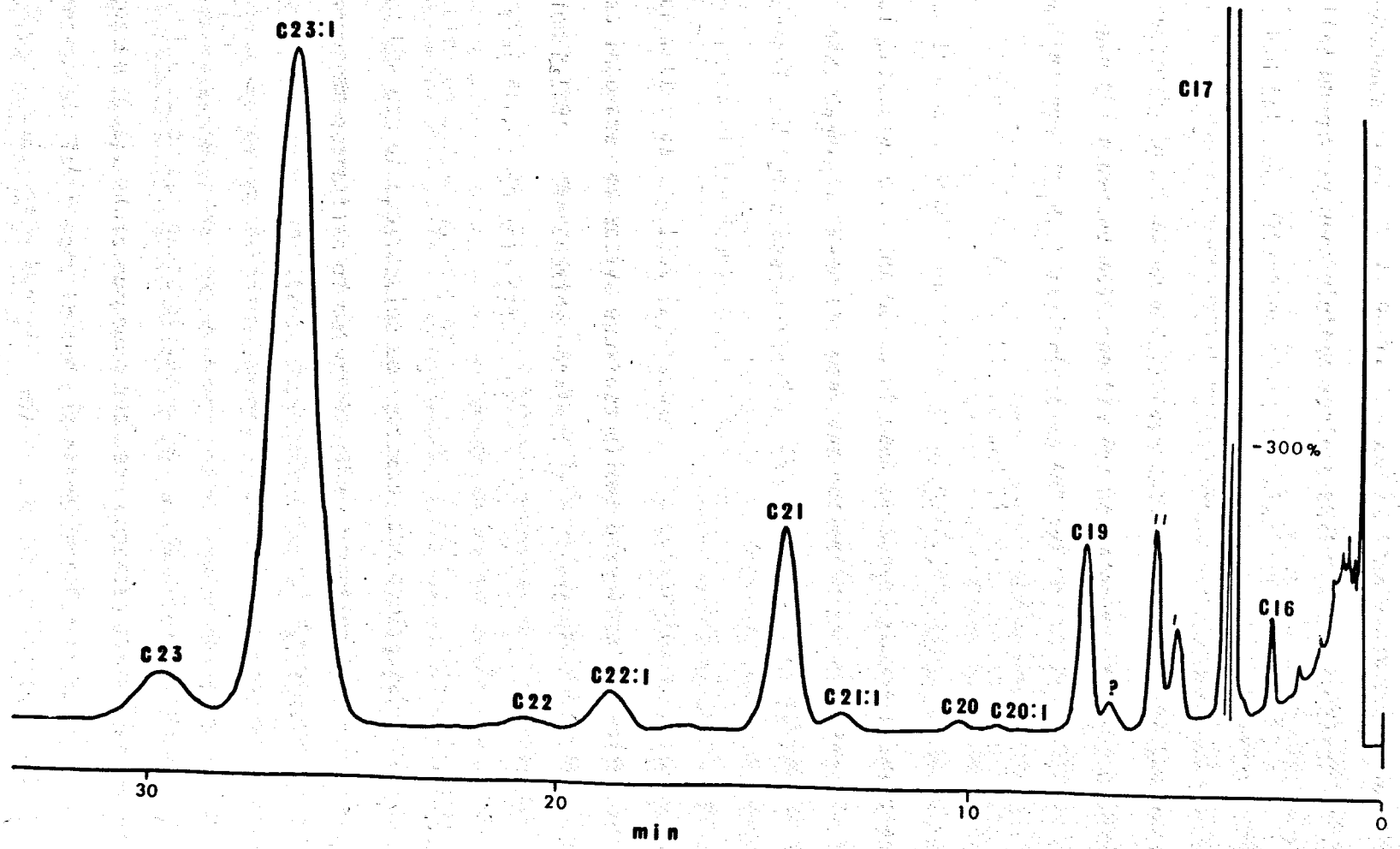
(Z,E)-α-farnesene [**17**], (E,E)-α-farnesene [**18**], Z,Z-homofarnesene [**19**] and (Z,E)-homofarnesene [**20**]. Williams *et al.*[118,119] have reported (Z,Z,Z)-allofarnesene [**16**] as the trail pheromone of *S. invicta*. Further investigations are required to find an answer to these discrepancies.

A preliminary survey of the Dufour gland contents of *S. geminata* has been performed by Barlin *et al.*[18]. Heptadecane and nonadecane were the only components that were identified, although a peak in the GC profile had been assignd as the trail pheromone. Furthermore, Barlin *et al.*[18] have suggested $C_{17}H_{28}$ as the molecular formula of the trail pheromone on the basis of comparative GC retention data. A complete analysis of Dufour gland contents of *S geminata* was considered important and it was undertaken in the present study.

The main difficulty in examining the Dufour gland contents of fire ants by GC, is the presence of large quantities of alkaloids in the neighbouring poison gland. The alkaloidal content of the poison gland was about 19 μg/ant whereas the Dufour gland contained only about 200 ng/ant of volatile material. Therefore utmost care was needed to dissect the Dufour glands cleanly in order to avoid any contamination with alkaloids. Even a small contamination from the sting base was able to mask some of the small peaks in the GC traces, arising from the Dufour gland contents. A pre-column of boric acid was found useful to remove these piperidine alkaloids from the GC traces. Although boric acid has been usually used only to subtract alcohols from the GC effluent[261,262], it is also effective for amines.

A typical GC trace obtained from Dufour gland contents on a SE-30 column containing a boric acid pre-column is shown in Figure 26. The identification of the peaks in the GC trace is summarized in Table 20. The average total amount of hydrocarbons present in the Dufour gland is about 200 ng/ant. The total amount of hydrocarbons present in a Dufour gland varied widely between individuals resulting in large standard deviations. However, the percentage composition of each component showed much less variability.

FIGURE 26. A gas chromatogram of the Dufour gland contents of Solenopsis geminata. Two Dufour glands were solid injected on a 1.5 m x 4 mm packed column of 5% SE-30 on Chromosorb W containing a pre-column of 20% boric acid (12 cm x 4 mm). The oven temperature was 190 °C (attenuation x 50).



The logarithms of the retention times from an isothermal GC run of the components, 1, 2, 3, 7, 9, 11, 13 and 15 (Table 20) lie on one straight line when they were plotted against their carbon numbers. These components had the same retention times on polar and nonpolar GC phases as those of the corresponding authentic n-alkanes. Furthermore, these peaks were unaffected by bromination and hydrogenation. The GC-MS of the peaks 3, 11 and 15 gave the expected mass spectra for heptadecane (M^+ 240, $C_{17}H_{36}$), heneicosane (M^+ 296, $C_{21}H_{44}$) and tricosane (M^+ 324, $C_{23}H_{48}$) respectively.

The peaks 4, 5, 6, 8, 10, 12 and 14 were completely eliminated from the GC traces upon micro-bromination or hydrogenation indicating the presence of unsaturation. The GC-MS of peak 14 showed it was a tricosene isomer (M^+ 322, $C_{23}H_{46}$). When the peak was collected and ozonolyzed, it yielded nonanal and tetradecanal locating the double bond at C9 (Figure 27). The configuration of the double bond of 9-tricosene was established by the epoxidation method as the 100% (Z)-isomer. The conversion of (E)- and (Z)-alkenes into the respective epoxides allows base line separation by GC (*cf.* Section 2.1.3). The epoxide obtained from 9-tricosene from the Dufour gland and that from authentic (Z)-9- tricosene, both showed the same retention time on chromatography on the PEG 20M column. This application exemplified the use of epoxidation to determine the configuration of double bonds in nanogram quantities of material. The conventional argentation-TLC method usually requires at least several micrograms for a similar determination.

The major component of the Dufour gland of *S. geminata* is (Z)-9-tricosene (46%). This compound has been reported from a number of insect sources. It is present in the sex attractant for the male house fly, *Musca domestica* and known as muscalure[357]. It has been identified in the cuticular waxes of *Apis mellifera*[270] and in the Dufour gland of some attine ants[333]. The same substance was found in the postpharyngeal gland in substantial quantities (*ca.* 5 µg/ant) as described in section 3.3.1. Vander

Table 20. Chemical composition of the Dufour glands of *Solenopsis geminata*

No[a]	Compound	Abbreviation	Mean composition[b] by weight (ng/ant ± S.D.)	Mean % by weight ± S.D.	Evidence
1	Pentadecene	C15	0.2 ± 0.1	0.1 ± 0.0	GC,Br-,H_2-
2	Hexadecane	C16	1.2 ± 0.3	0.6 ± 0.2	GC,Br-,H_2-
3	Heptadecane	C17	57.7 ± 16.8	27.2 ± 2.5	GC,Br-,H_2-,MS
4	Pheromone[c] I	I	4.0 ± 1.0	2.0 ± 0.5	Br+,H_2+
5	Pheromone[c] II	II	7.4 ± 1.2	3.6 ± 0.6	Br+,H_2+
6	Unidentified		1.3 ± 0.4	0.6 ± 0.1	GC,Br+,H_2+
7	Nonadecane	C19	9.0 ± 2.3	4.4 ± 1.0	GC,Br-,H_2-
8	Eicosene	C20:1	trace		GC,Br+,H_2+
9	Eicosane	C20	0.5 ± 0.1	0.2 ± 0.0	GC,Br-,H_2-
10	Heneicosene	C21:1	1.5 ± 0.3	0.7 ± 0.2	GC,Br+,H_2+
11	Heneicosane	C21	17.7 ± 3.5	8.6 ± 0.7	GC,Br-,H_2-,MS
12	Docosene	C22:1	4.0 ± 1.0	1.9 ± 0.3	GC,Br+,H_2+
13	Docosane	C22	1.7 ± 0.7	0.8 ± 0.4	GC,Br-,H_2-
14	(Z)-9-tricosene	C23:1	96.5 ± 21.5	46.3 ± 2.7	GC,Br-,H_2-,MS,OZ,EPO
15	Tricosane	C23	6.6 ± 2.1	3.1 ± 0.7	GC,Br+,H_2+,MS
	Total		209 ± 46	100	

[a] numbers refer to peaks of Figure 26.
[b] number of replicates=10
[c] peaks 4 and 5 appear to be pheromone components because they lie in the active region of the GC effluent.
GC : the substance has the same retention times as the assigned compound on polar and nonpolar GC phases.
Br-: component unaffected by bromine treatment.
Br+: component removed form GC profile on bromine treatment.
H_2-: component unaffected by hydrogenation.
H_2+: hydrogenation of the component yields the corresponding saturated analogue.
MS : identification confirmed by mass spectrum.
OZ : olefinic position determined by ozonolysis.
EPO: olefinic configuration determined by epoxidation.

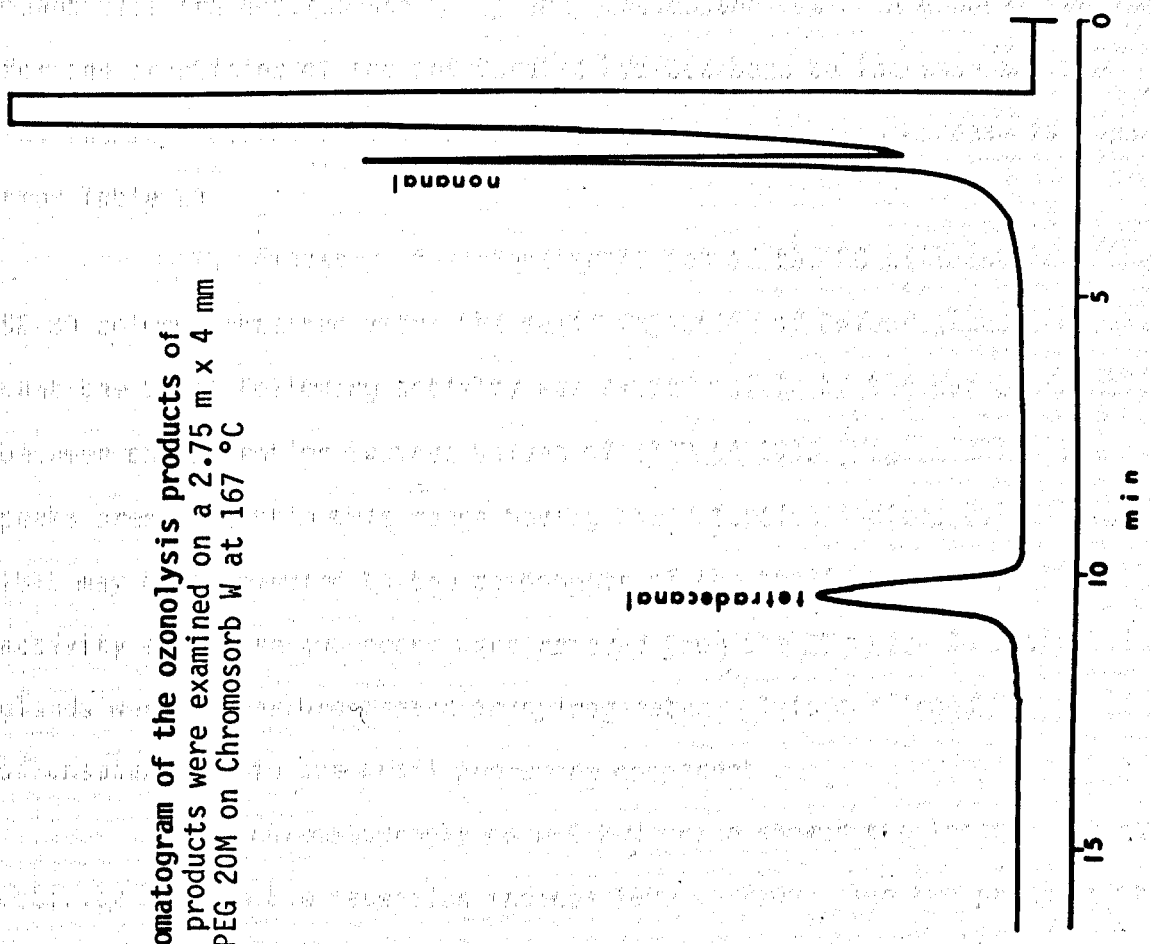


FIGURE 27. **A gas chromatogram of the ozonolysis products of (Z)-9-tricosene.** The products were examined on a 2.75 m x 4 mm packed column of 10% PEG 20M on Chromosorb W at 167 °C (attenuation x 200).

Meer[56] has reported that in *S. invicta* heptadecane, a Dufour gland component, when added to the pheromone mixture, could better duplicate the recruitment response of a Dufour gland extract. However, heptadecane itself was inactive to evoke any trail following activity.

The other hydrocarbons present in the Dufour gland in substantial quantities are heptadecane (27%) and heneicosane (9%). A general tendency for the quantities of the unsaturated hydrocarbons to increase and the saturated hydrocarbons to decrease as the carbon numbers increase is apparent from Table 20.

The bioassay of various fractions of the GC effluent from the SE-30 column, obtained after the solid injection of Dufour glands revealed that the trail following activity was evoked mainly by the narrow fraction between the retention indices values of 1700 to 1900 (Figure 28). The two peaks present within this range having the retention indices values 1789 and 1819 may be attributed to the components of the trail pheromone. The activity and these two peaks were removed from the GC trace when the Dufour glands were either brominated or hydrogenated. This confirmed the presence of unsaturation in the trail pheromone components.

The chromatography on PEG 20M phase showed the location of the activity between the retention indices 1900 to 2100. The two peaks in the pheromone region had values of 1958 and 2002.

The examination of Dufour gland extracts by TLC using hexane as the effluent demonstrated the activity to be concentrated in the two bands of R_f values between 0.3 - 0.4 and 0.4 - 0.5. Under the same conditions synthetic α-farnesene showed a R_f value of 0.4 indicating the trail pheromone components may have chemical properties similar to farnesene.

The main component of the trail pheromone of *S. invicta* is (Z,E)-α-farnesene [**17**][120]. The same compound is present in the Dufour gland of *M. scabrinodis*[180]. Therefore *S. invicta* can be expected to follow a Dufour gland extract of *M. scabrinodis*. On a cross-activity test *S. geminata*

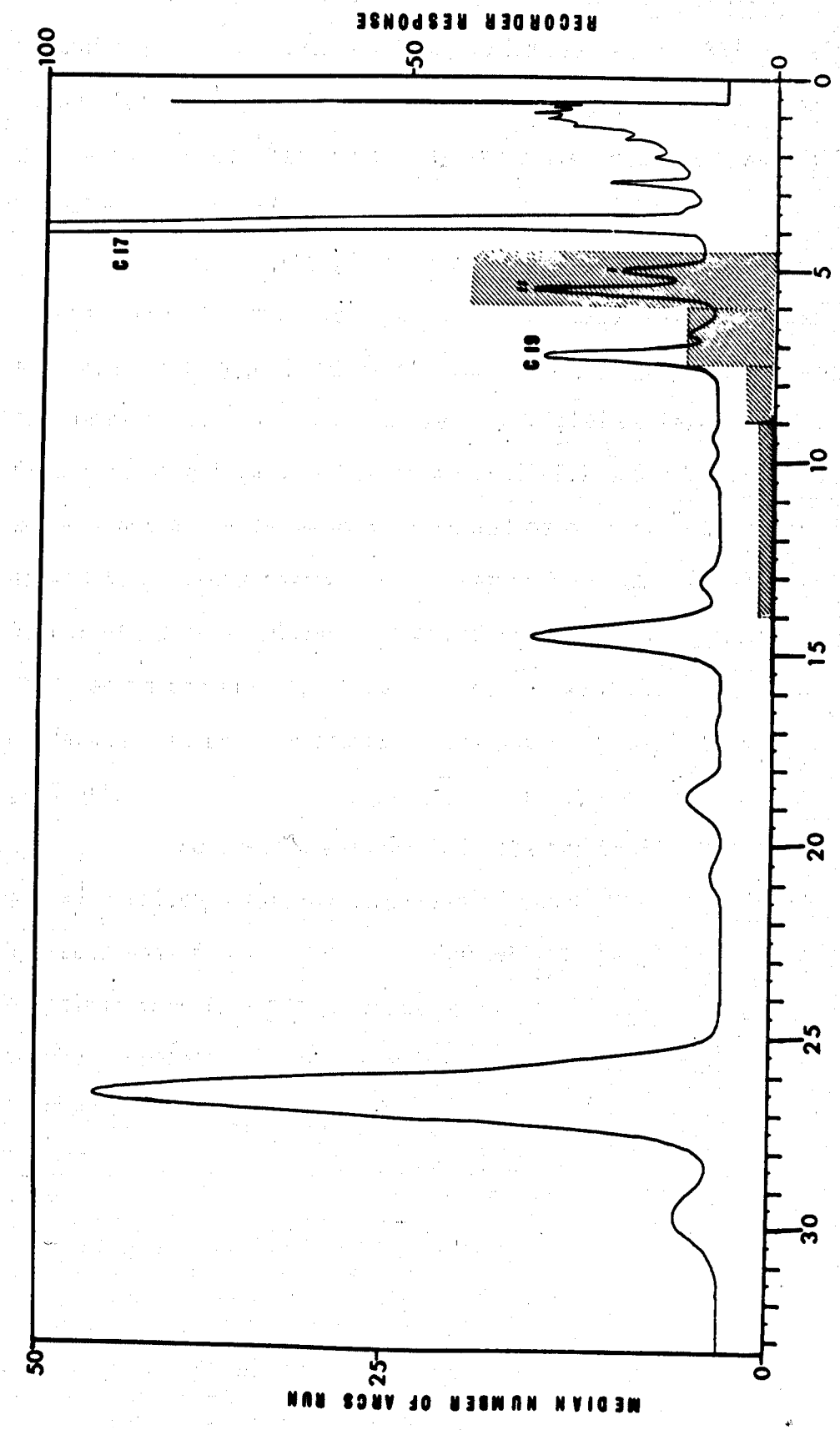


FIGURE 28. GC-bioassay of Dufour gland contents of Solenopsis geminata. Two Dufour glands were chromatographed on the 5% SE-30 column at 190 °C and venom fractions of the GC effluent were collected and bioassayed. The hatched bars represent the trail releasing activity of collected fractions.

workers failed to follow the Dufour gland extracts of *M. scabrinodis*. The Dufour gland of *M. scabrinodis* has a homofarnesene [41] and a bishomofarnesene [42] in addition to (Z,E)-α-farnesene. Therefore it can be concluded none of these compounds is active to evoke trail following behaviour in *S. geminata*.

The results of the cross-activity test is summarized in Table 21. It was surprising to find that *S. geminata* and *Monomorium pharaonis* followed each other's Dufour gland extracts. The major component of the trail pheromone of *M. pharaonis* has been identified as faranal [15]. A trail laid from synthetic faranal could evoke trail following behaviour on *M. pharaonis* workers but *S. geminata* workers showed no positive response. This clearly showed *S. geminata* follows some other trace constituent(s) present in the Dufour gland of *M. pharaonis* but not faranal. This phenomenon has been observed in the transposition studies on the attine ants. The retention time of faranal did not correspond to the suspected trail pheromone peaks of *S. geminata*.

The present results indicate the trail pheromone of *S. geminata* may be multicomponent and comprises of unsaturated terpenoid type hydrocarbons of C_{18}-C_{19} range. The activity passes through a boric acid precolumn therefore the presence of hydroxyl groups and nitrogen heteroatoms can be excluded. The complete identification of the pheromone requires further work.

Table 21. Responses of some myrmicine ants to artificial trails laid from their Dufour glands extracts and synthetic faranal

	Sources			
	Dufour gland extraction of			
Test species	*S. geminata*	*M. pharaonis*	*M. scabrinodis*	Synthetic faranal
Solenopsis geminata	+++	++	0	0
Monomorium pharaonis	++	+++	0	+++
Myrmica scabrinodis	0	0	0	0

+++ : high trail following activity

++ : moderate trail following activity

0 : no trail following activity

2.4 Dufour Gland Substances of the Genus *Myrmica*

Seven species of the genus *Myrmica* commonly found in the British Isles and *M. rugulosa* from the adjacent continent, use the same substance 2,5-dimethyl-3-ethylpyrazine as their trail pheromone and the workers of any of the above species will impartially follow an artificial trail made with the poison gland of its own or another species [109,110]. Dufour glands of five of the *myrmica* species investigated so far, contain a mixture of hydrocarbons characteristic of that species. The worker ants are able to recognize the contents of a Dufour gland as coming from their own or an alien species. This was demonstrated by measuring the speed of older pioneer workers on a foraging area marked with a hexane solution of the Dufour gland contents [71,358]. These Dufour gland secretions have also been shown from the work of Cammaerts[359] to be involved in the recruitment of the workers to foraging. It was also clearly evident from the five *Myrmica* species which had been studied, that the Dufour glands contain a mixture of hydrocarbons with qualitative and quantitative differences. Therefore it is possible with a little experience to recognize one species from another by examining the gas chromatographic trace produced from the Dufour gland. Hence the results are important also for the chemical taxonomy of the genus *Myrmica*. It was proposed from the previous studies of the Dufour gland contents of the five *Myrmica* species, that the genus can be divided into two distinct groups depending on the nature of hydrocarbons present in the Dufour gland [360]. If the Dufour gland contained predominently linear hydrocarbons as in *M. rubra* and *M. ruginodis* it was classed as "R type group of species". On the contrary the "S type group of species" had essentially branched, terpenoid type hydrocarbons in the Dufour gland and typical members of this group are *M. scabrinodis* and *M. sabuleti*.

The present work was undertaken to complete the chemical investigation of Dufour gland contents of *Myrmica* ants from the British Isles

and the adjacent continent, and it was achieved by studying *M. sulcinodis*, *M. rugulosa* and *M. schencki*. The aim of the investigation was to identify and quantify the chemical substances present in the glands in order to find to which "group of species" they belong.

A further species of doubtful taxonomy, which we shall here call *M. albuferensis*, from the highly specialized environment of a salt marsh on the mediterranean island of Mallorca, was also studied. According to one school of thought it is identical with *M. scabrinodis* and the others believe it may be *M. aloba*. The chemical investigation of the Dufour gland contents of *M. albuferensis* was undertaken as the comparison of its results with those of *M. scabrinodis* and *M. aloba* may answer the questions about the identity of *M. albuferensis*.

2.4.1. Dufour gland substances of *M. sulcinodis*

The Dufour gland of *M. sulcinodis* appears like an elongated pear-shaped sac when observed under the microscope. The approximate length of the Dufour gland is about 500 µm and the diameter at the widest point about 200 µm. The contents of the Dufour glands are lighter than water and insoluble in it. When the gland was pierced under water a colourless globule of liquid was released which floated on the surface of the water.

The very volatile portion of the contents on the Dufour gland of *M. sulcinodis* studied on the Porapak Q column showed two major components, which were identified as ethanal and propanone. The minor components identified were 2-methylpropanal, butanone and butenone (Figure 29). The identifications were based on the comparison of GC retention times with authentic samples and GC traces from *M. rubra*[361,362]. The GC traces obtained were very similar to those obtained from *M. rubra* [361,362].

The identification of various less volatile compounds are summarized in Table 22. The components were quantified by measuring the peak areas of the GC profiles obtained from 10% PEGA column (Table 28, column D).

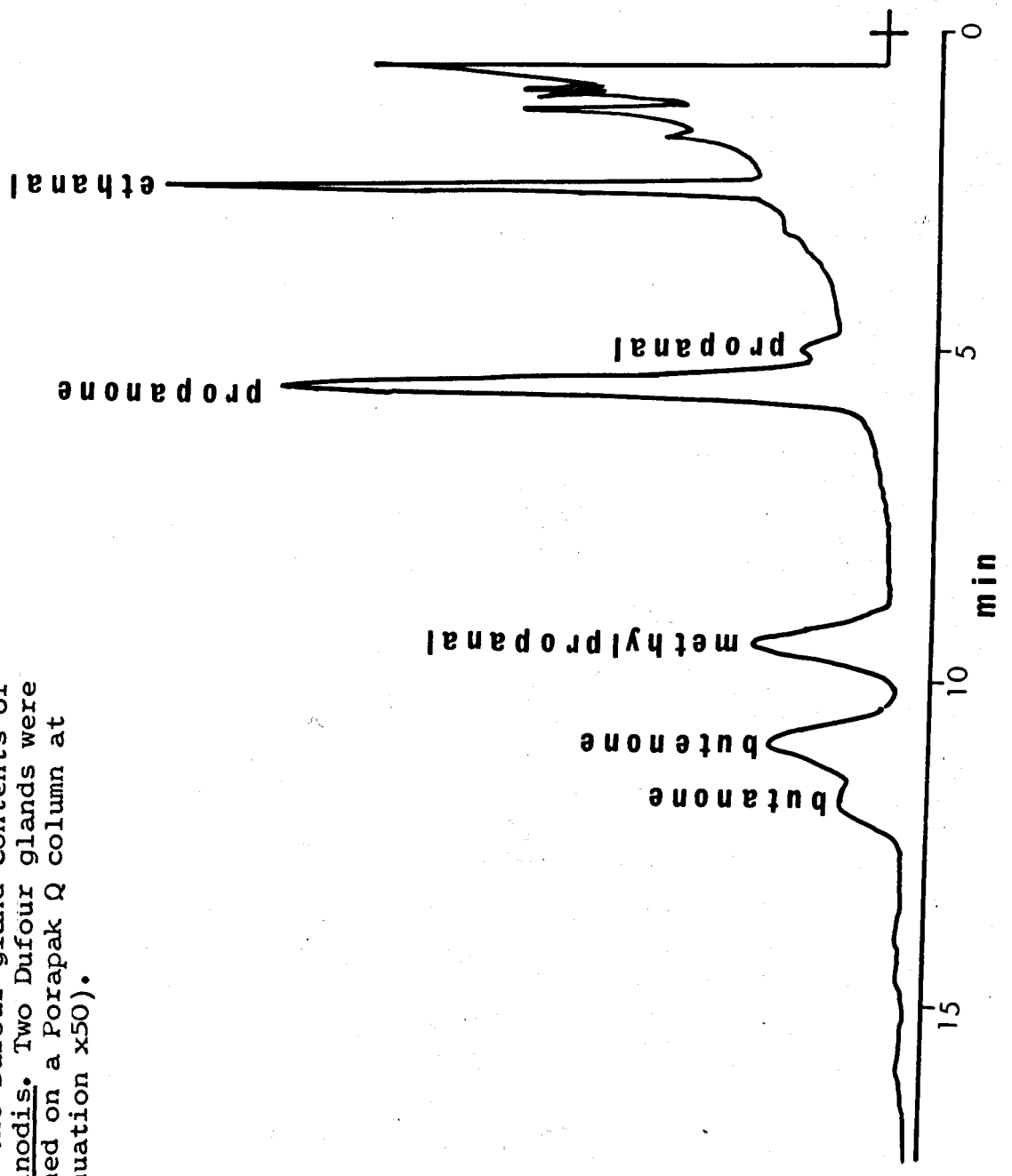


FIGURE 29. A gas chromatogram of very volatile components of the Dufour gland contents of Myrmica sulcinodis. Two Dufour glands were chromatographed on a Porapak Q column at 170 C (attenuation x50).

The temperature programme from 125-162 °C at 2 °C/min produced sharp peaks for convenient quantification but nonadecene and bishomofarnesene peaks were not well resolved (Figure 30). The resolution of nonadecene and bishomofarnesene was achieved by the 5% OV-101 silicone column (Table 28, column B) using a temperature programme of 140-192 °C at 4 °C/min. The total amount of material in a Dufour gland varies widely between individuals, therefore the percentage composition of the compounds for the each individual was calculated and then the mean and standard deviation of these percentages were calculated for the group. The standard deviation of the percentage of each component gives a measure of the variability or constancy of composition of the glandular secretion.

The plots of components 1, 3 and 4 (Table 22) of the less volatile portion of the contents of the Dufour glands, lie on one straight line when the logarithms of their retention times were plotted against their carbon numbers. These components had identical retention times to those of the corresponding n-alkanes. These peaks were not removed or shifted from the GC-profile on bromination or hydrogenation (Figure 30) indicating that those components were not unsaturated. The GC-MS of peak 4 gave the expected mass spectrum (Figure 31) for n-heptadecane (M^+ 240, $C_{17}H_{36}$) and was identical to that of authentic heptadecane.

Micro-bromination of the whole Dufour glands resulted in the elimination of the peaks 2, 5, 6, 7, 8, 9, and 10 indicating unsaturation (Table 22).

The hydrogenation of a whole Dufour gland resulted in the elimination of the peaks 2, 5, 6, 7, 8, 9 and 10 confirming the presence of unsaturation (Table 22, Figure 30). The size of the pentadecene and heptadecene peaks were increased after hydrogenation, confirming the presence of pentadecene and heptadecene in the Dufour gland contents. The size of the hexadecane peak was unaffected, hence no presence of hexadecene was indicated. New peaks for octadecane and nonadecane appeared as the reduction

Table 22. Chemical composition of the Dufour glands of *Myrmica sulcinodis*. The analytical evidence for structure assignment is summarized.

	Compounds	Abbreviation	Mean composition by weight (ng/ant±S.D.)	Mean % by weight ± S.D.	Evidence
1	Pentadecane	C15	9.0 ± 5.5	1.2 ± 0.7	GC,Br-,H_2-,
2	Pentadecene	C15:1	9.1 ± 3.5	1.2 ± 0.5	GC,Br+,H_2+,
3	Hexadecane	C16	5.3 ± 1.5	0.7 ± 0.2	GC,Br-,H_2-
4	Heptadecane	C17	77.3 ± 25.3	10.1 ± 3.4	GC,Br-,H_2-,MS
5	8-Heptadecene	C17:1	89.0 ± 32.9	11.5 ± 3.9	GC,Br+,H_2+,MS,OZ
6	α-Farnesene	F	10.6 ± 5.3	1.3 ± 0.5	GC,Br+,H_2+,
7	Homofarnesene	HF	157.5 ± 39.6	20.5 ± 4.0	GC,Br+,H_2+,MS
8	Octadecene	C18:1	12.8 ± 5.6	1.7 ± 0.8	GC,Br+,H_2+
9	Bishomofarnesene	BHF	318.1 ± 92.5	40.6 ± 5.5	GC,Br+,H_2+,MS
10	9-Nonadecene	C19:1	88.1 ± 25.6	11.2 ± 1.3	GC,Br+,H_2+,MS,OZ
	Total		777 ± 148	100	

Notes :

GC: the substance has the same retention times as the assigned compound on polar and nonpolar GC phases.

Br-: component unaffected by bromine treatment.

Br+: component removed from GC profile on bromine treatment.

H_2-: component unaffected by hydrogenation.

H_2+: hydrogenation of the component yields the corresponding saturated analogue.

MS: identification confirmed by mass spectrum.

OZ: olefinic position determined by ozonolysis.

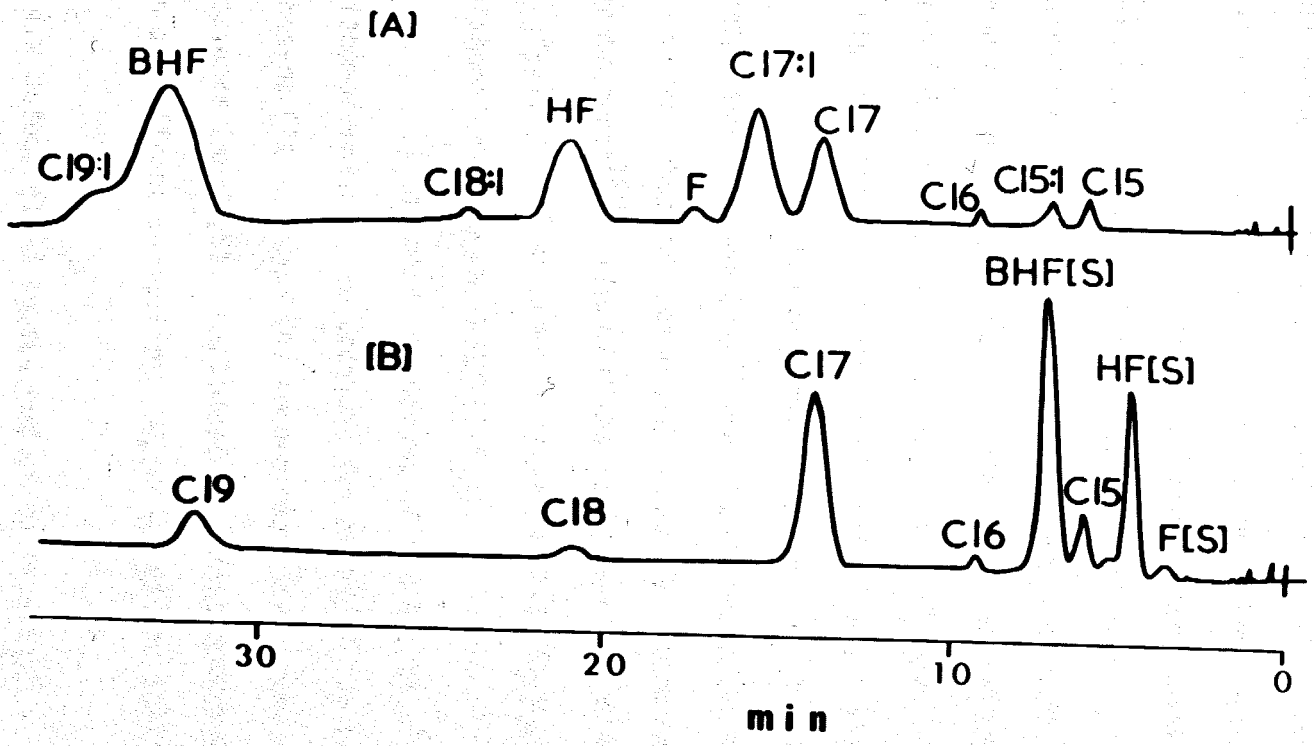


FIGURE 30. **A gas chromatogram of the contents of the Dufour gland of *Myrmica sulcinodis* [A] and the same mixture after hydrogenation is shown in [B].** A single gland was injected on a 2.75 m x 4 mm packed column of 10% PEGA on Chromosorb W, at 127 °C. [B] was obtained with a 6 cm 1% palldium catalyst as a pre-column and hydrogen as the carrier gas. The symbols F, HF and BHF represent farnesene, homofarnesene and bishomofarnesene and [S] for their saturated counterparts respectively. The straight chain hydrocarbons are represented as C17:1 for heptadecene, C15 for pentadecane etc.

products of octadecene and nonadecene in the hydrogenated GC trace. Three new peaks corresponding to farnesane, homofarnesane and bishomofarnesane appeared at shorter retention times. The amount of shift of retention times of farnesanes from their parent unsaturated analogues confirmed the presence of four carbon-carbon double bonds in the farnesenes.

The ozonolysis of heptadecene (peak 5, Table 22) collected from the Dufour gland, yielded octanal and nonanal, which confirmed the alkene position at C8. Similarly nonadecene (peak 10, Table 22) on ozonolysis gave nonanal and decanal to indicate the unsaturation at C9. The GC-MS examination of peaks 5 and 10 (Table 22) gave the expected mass spectra (Figure 31) for heptadecene (M^+ 238, $C_{17}H_{34}$) and nonadecene (M^+ 266, $C_{19}H_{38}$).

The Dufour gland components 6, 7 and 9 (Table 22) had the same retention times on OV-101 and PEGA columns, as the peaks identified as farnesene, homofarnesene and bishomofarnesene from the Dufour gland of M. scabrinodis[180]. Linked GC-MS confirmed peak 6 as α-farnesene (M^+204, $C_{15}H_{24}$), peak 7 as homofarnesene (M^+ 218, $C_{16}H_{26}$) and peak 9 as bishomofarnesene (M^+ 232, $C_{17}H_{28}$).

The major component of the Dufour gland of *M. sulcinodis* is bishomofarnesene, it accounts for 40.6% of the total quantity of the material present (Table 22). The second major component is homofarnesene and it accounts for 20.5%. Heptadecane, 8-heptadecene and 9-nonadecene are present in significant quantities, 10.1%, 11.5% and 11.2% respectively.

2.4.2 Dufour gland substances of *M. rugulosa*

The external appearance of the Dufour gland of *M. rugulosa* is similar to *M. sulcinodis* but relatively large and contains more material. The workers of *M. rugulosa* are rather small compared to those of *M. rubra* but their Dufour gland is large in proportion and has about 75% of the volume of an *M. rubra* Dufour gland. The Dufour gland contents of *M. rugulosa* are also immiscible in water.

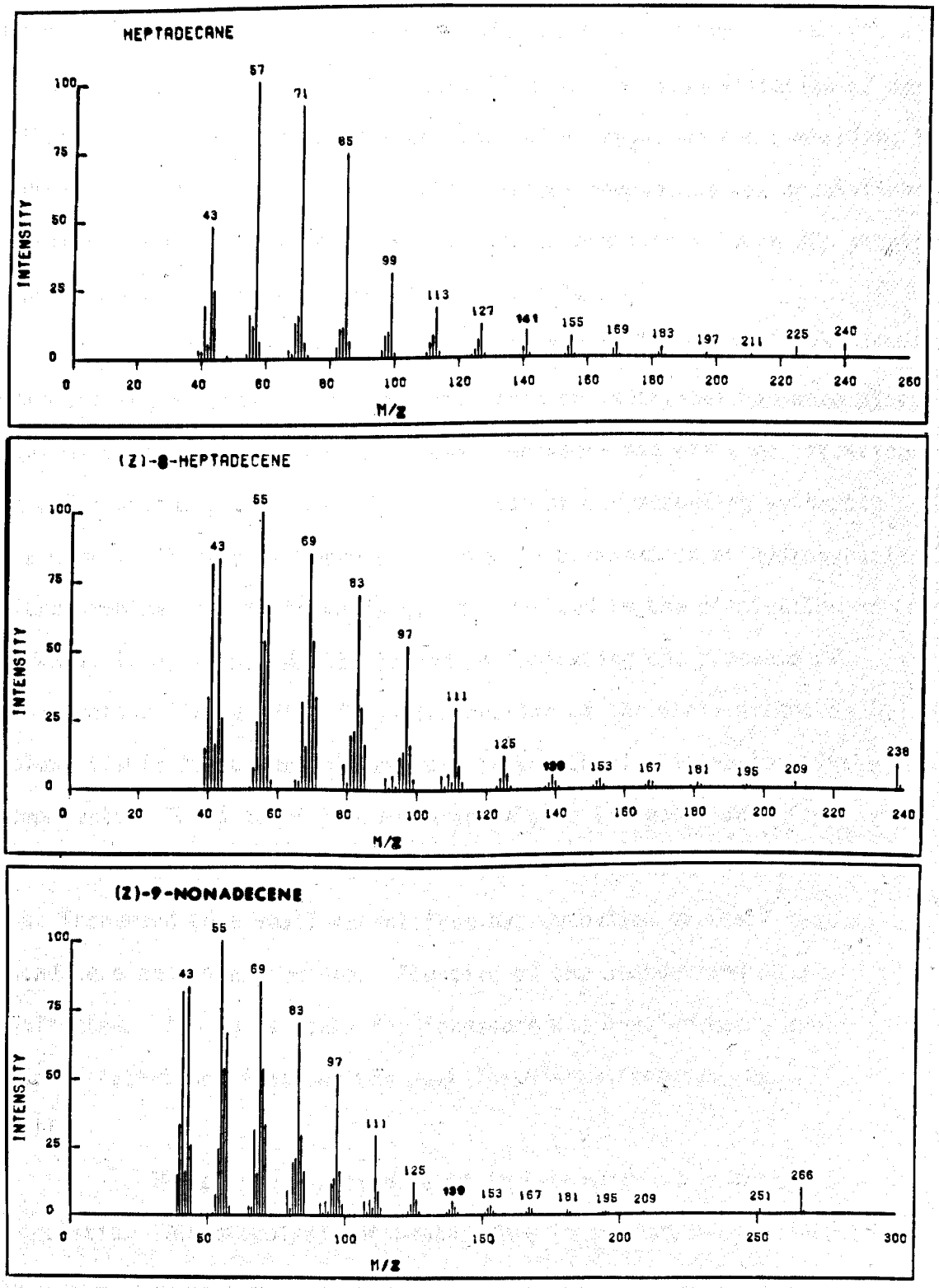


FIGURE 31. Mass spectra of heptadecane, (Z)-8-heptadecene and (Z)-9-nonadecene.

The very volatile portion of the Dufour gland contents of *M. rugulosa* was studied on the Porapak Q column (Table 28, column E), and found to be very similar to that of *M. sulcinodis* and *M. rubra*.

The results of the identification and quantification of less volatile components of the Dufour gland of *M. rugulosa* are summarized in Table 23. The quantification of the various components was accomplished by the measurement of the peak areas of the GC profiles (Figure 32) obtained on the 5% OV-101 silicone column (Table 28, column B).

The components 1, 2, 5, 8, 11 and 15 lie on one straight line when the log of their retention times from an isothermal run were plotted against their carbon numbers. These components had the same retention times on polar and nonpolar GC phases, as those of corresponding authentic n-alkanes. These peaks were unaffected by bromination or hydrogenation. Micro-bromination of the whole glands resulted in the elimination of the peaks 3, 4, 6, 7, 9, 10, 12, 13 and 14 indicating the presence of unsaturation (Table 23). The hydrogenation of the whole glands on 10% PEGA column (Table 28, column D) resulted in the total elimination of the same components. The size of the heptadecane peak increased on hydrogenation due to the hydrogenation of large quantities of heptadecene. The nonadecane peak also increased to a small extent from hydrogenation of small quantities of nonadecene and nonadecadiene. The size of the pentadecane peak was unaffected. Two large peaks for farnesane and homofarnesane appeared at shorter retention times and the peak for bishomofarnesane was relatively small.

The alkene position at C8 in heptadecene was determined by ozonolysis. The ozonolysis of heptadecene in solvent produced octanal and nonanal as the only products. The GC-MS of peak 5 (Table 23) gave the expected mass spectrum for pentadecane (M^+ 212, $C_{15}H_{32}$). The peaks 10 and 11 had the identical mass spectra, compared to those of 8-heptadecene (Figure 31) and heptadecane (Figure 31) from *M. sulcinodis* respectively. The Dufour

Table 23. Chemical composition of the Dufour glands of *Myrmica rugulosa*. The analytical evidence for structure assignment is summarized.

	Compounds	Abbreviation	Mean composition by weight (ng/ant±S.D.)	Mean % by weight ± S.D.	Evidence
1	Tridecane	C13	0.6 ± 0.5	0.03 ± 0.02	GC,Br-,H_2-
2	Tetradecane	C14	1.1 ± 0.7	0.06 ± 0.04	GC,Br-,H_2-
3	Unknown I		0.4 ± 0.5	0.03 ± 0.02	Br+,H_2+
4	α-Farnesene	F	196 ± 73	12.76 ± 1.37	GC,Br+,MS,H_2+
5	Pentadecane	C15	164 ± 58	10.88 ± 1.69	GC,Br-,MS,H_2-
6	Unknown II		59 ± 32	3.68 ± 0.89	Br+,H_2+
7	Homofarnesene	HF	309 ± 127	20.00 ± 2.85	GC,Br+,MS,H_2+
8	Hexadecane	C16	17 ± 10	1.10 ± 0.40	GC,Br-,H_2-
9	Bishomofarnesene	BHF	115 ± 60	7.26 ± 2.25	GC,Br+,MS,H_2+
10	Heptadecene	C17:1	489 ± 168	32.57 ± 4.02	GC,Br+,MS,OZ,H_2+
11	Heptadecane	C17	145 ± 52	9.64 ± 1.12	GC,Br-,MS,H_2-
12	Trishomofarnesene	THF	16 ± 6	1.10 ± 0.24	GC,Br+,H_2+
13	Nonadecadiene	C19:2	3.1 ± 1.9	0.21 ± 0.13	Br+,H_2+
14	Nonadecene	C19:1	6.9 ± 1.3	0.50 ± 0.34	Br+,H_2+
15	Nonadecane	C19	1.5 ± 1.5	0.15 ± 0.08	Br-,H_2-
	Total		1524 ± 604	100	

Notes :

GC : the substance has the same retention times as the assigned compound on polar and non-polar GC phases.
Br- : component unaffected by bromine treatment.
Br+ : component removed from GC profile on bromine treatment.
H_2- : component unaffected by hydrogenation
H_2+ : component shifted by hydrogenation
MS : identification confirmed by mass spectrum
OZ : alkene position determined by ozonolysis

gland components 4, 7 and 9 had the corresponding identical retentions times on polar and nonpolar columns, to those of farnesene, homofarnesene and bishomofarnesene from *M. scabrinodis*[180]. The confirmation of peak 4 as farnesene (M^+ 204, $C_{15}H_{24}$), peak 7 as homofarnesene (M^+ 218, $C_{16}H_{26}$) and peak 9 as bishomofarnesene (M^+ 232, $C_{17}H_{28}$) was accomplished by GC-MS. The mass spectra of components 1, 2, 3, 6, 8, 12, 13, 14 and 15 were too weak for complete identification but on the strength of other evidences the structures were assigned as, peak 1 tridecane, peak 2 tetradecane, peak 8 hexadecane, peak 12 trishomofarnesene, peak 13 nonadecadiene, peak 14 nonadecene and peak 15 nonadecane. The other minor components remain unidentified.

The major component in the Dufour gland of *M. rugulosa* was 8-heptadecene, it accounted for about 32% of the total quantity of the material present (Table 23). Pentadecane and heptadecane were present in significant proportions, 10.8% and 9.6% respectively.

2.4.3. Dufour gland substances of *M.schencki*

The Dufour gland of *M. schencki* is the smallest out of all the myrmicine ants investigated so far but still it is similar to the others in external shape. The very volatile fraction of the Dufour gland contents of *M. schencki* was found to be very similar to that of other myrmicine ants so far investigated. The various less volatile compounds present in the Dufour gland of *M. schencki* were identified and quantified. The results are summarized in Table 24. The quantification was performed by peak area measurement of the GC profiles (Figure 32) obtained on the 5% OV-101 (Table 28, column B).

The components 2, 4, 8 and 11 had the same retention times on polar and nonpolar GC phases, compared to those of authentic n-alkanes. They lie on one straight line when the logarithms of retention times from an isothermal run were plotted against their carbon numbers. The peaks were identified as pentadecane, hexadecane, heptadecane and nonadecane

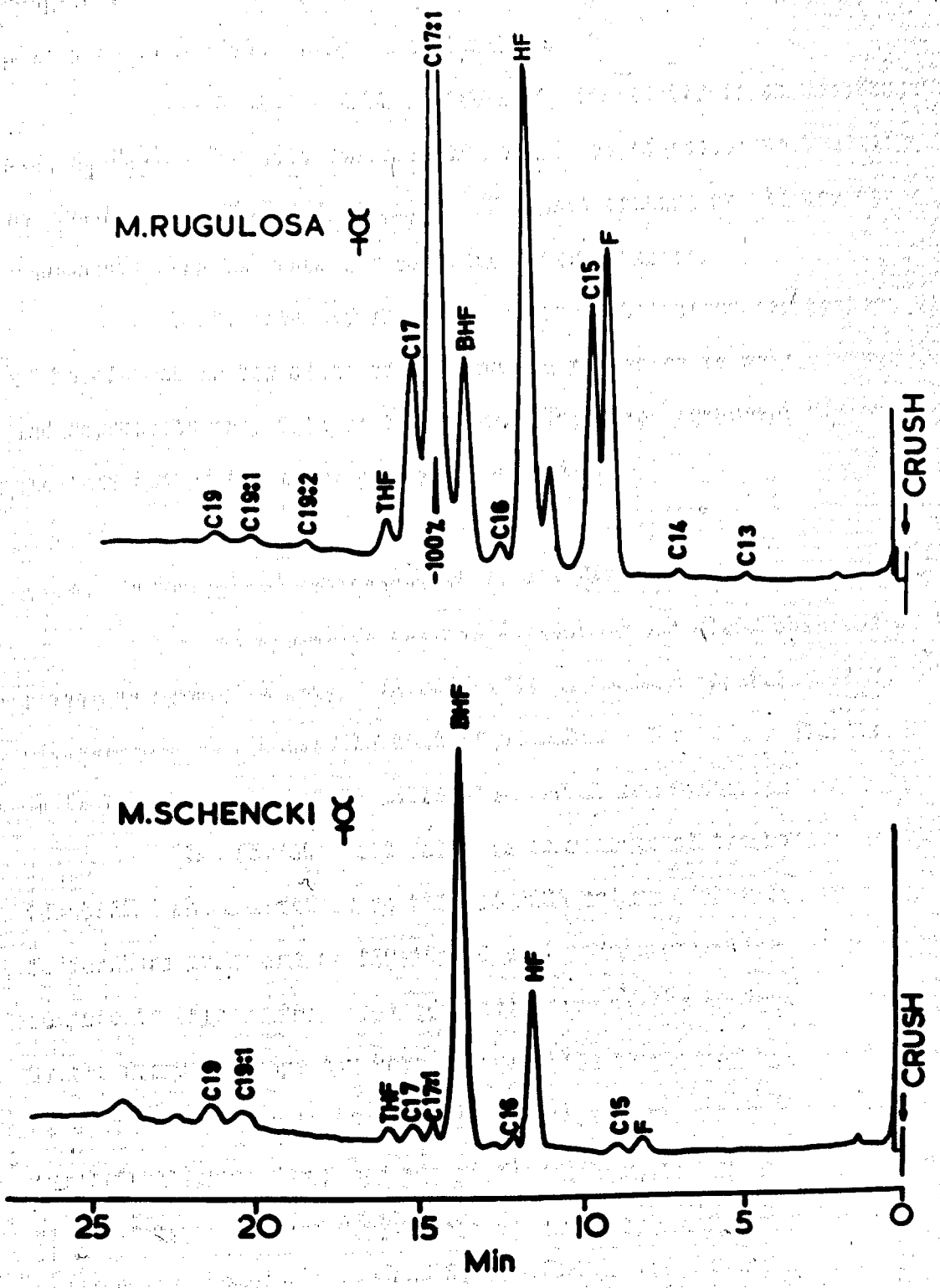


FIGURE 32. Gas chromatograms of Dufour gland contents of Myrmica rugulosa and M. schencki. The Dufour glands were chromatographed on a 1.5 m x 4 mm packed column of 5% OV-101 on Diatomite M with the oven temperature programmed from 130 to 192 °C at 4 °C/min. The symbols F, HF, BHF and THF represent farnesene, homofarnesene, bishomofarnesene and trishomofarnesene respectively.

respectively. The results from bromination and hydrogenation of whole Dufour glands confirmed the above identification.

The GC-MS of peak 3 (Table 24) identified it as homofarnesene (M^+ 218, $C_{16}H_{26}$). The mass spectrum of peak 6 was identical to that of bishomofarnesene (M^+ 232, $C_{17}H_{28}$). The mass spectra of all the other components were too weak for complete interpretation.

In *M. schencki* about 80% of the total glandular content consists of homofarnesene and bishomofarnesene. α-farnesene is only a minor component and represents only 0.1% of the total. The major component bishomofarnesene provides 63% of the total glandular material.

2.4.4. Dufour gland substances of *M. albuferensis*

M. albuferensis has the largest Dufour gland observed so far among the myrmicine ants. In Figure 33 the poison apparatus of *M. albuferensis* is compared to that of *M. rubra*. The Dufour gland is about 600 µm in length and about 250 µm in diameter at the widest point.

The various less volatile components of the Dufour gland were identified and quantified on the 10% PEGA column (Table 28, column D). The temperature programme of 120-160 °C at 3 °C/min effectively separated the components (Figure 34). The quantification of the components was carried out by the Pye Unicam:Spectra Physics computing integrator using a standard hexadecane solution in hexane (770 ng/µl) as the external standard. The identification of the peaks was carried out by similar methods as to those used for other species of *Myrmica*. The peaks 6, 7 and 9 were confirmed as α-farnesene, homofarnesene and bishomofarnesene by their mass spectra. They also had the same retention times as the corresponding peaks from *M. scabrinodis* on polar and nonpolar GC phases.

The farnesenes account for 92% of the total hydrocarbon content of the Dufour gland and α-farnesene (43%) and homofarnesene (38%) are the major components. The results of the quantitative study is presented in

Table 24. Chemical composition of the Dufour glands of *Myrmica schencki*. The analytical evidence for structure assignment is summarized.

Compounds	Abbreviation	Mean composition by weight (ng/ant±S.D.)	Mean % by weight ± S.D.	Evidence
1 α-Farnesene	F	0.5 ± 0.2	0.12 ± 0.02	GC,Br+,H_2+
2 Pentadecane	C15	0.4 ± 0.9	0.15 ± 0.07	GC,Br-,H_2-
3 Homofarnesene	HF	73 ± 25	18.13 ± 3.22	GC,Br+,H_2+
4 Hexadecane	C16	3.8 ± 1.5	0.92 ± 0.32	GC,Br-,H_2-
5 Unknown I		2.1 ± 1.9	0.79 ± 0.33	Br+
6 Bishomofarnesene	BHF	258 ± 91	62.89 ± 7.93	GC,Br+,H_2+,MS
7 Heptadecene	C17:1	6.3 ± 5.7	1.79 ± 0.99	GC,Br+,H_2+
8 Heptadecane	C17	9.9 ± 3.9	2.60 ± 1.34	GC,Br-,H_2-
9 Trishomofarnesene	THF	6.2 ± 3.5	1.58 ± 0.92	GC,Br+,H_2+
10 Nonadecene	C19:1	8.0 ± 6.8	2.45 ± 1.79	GC,Br+,H_2+
11 Nonadecane	C19	9.5 ± 9.1	2.79 ± 2.32	GC,Br-,H_2-
12 Unknown II		11.3 ± 6.2	2.68 ± 0.98	Br+
13 Unknown III		13.0 ± 6.2	3.21 ± 1.11	Br-
Total		402 ± 194	100	

Notes :

GC : the substance has the same retention times as the assigned compound on polar and non-polar GC phases.
Br- : component unaffected by bromine treatment.
Br+ : component removed from GC profile on bromine treatment.
H_2- : component unaffected by hydrogenation
H_2+ : component shifted by hydrogenation
MS : identification confirmed by mass spectrum
OZ : alkene position determined by ozonolysis

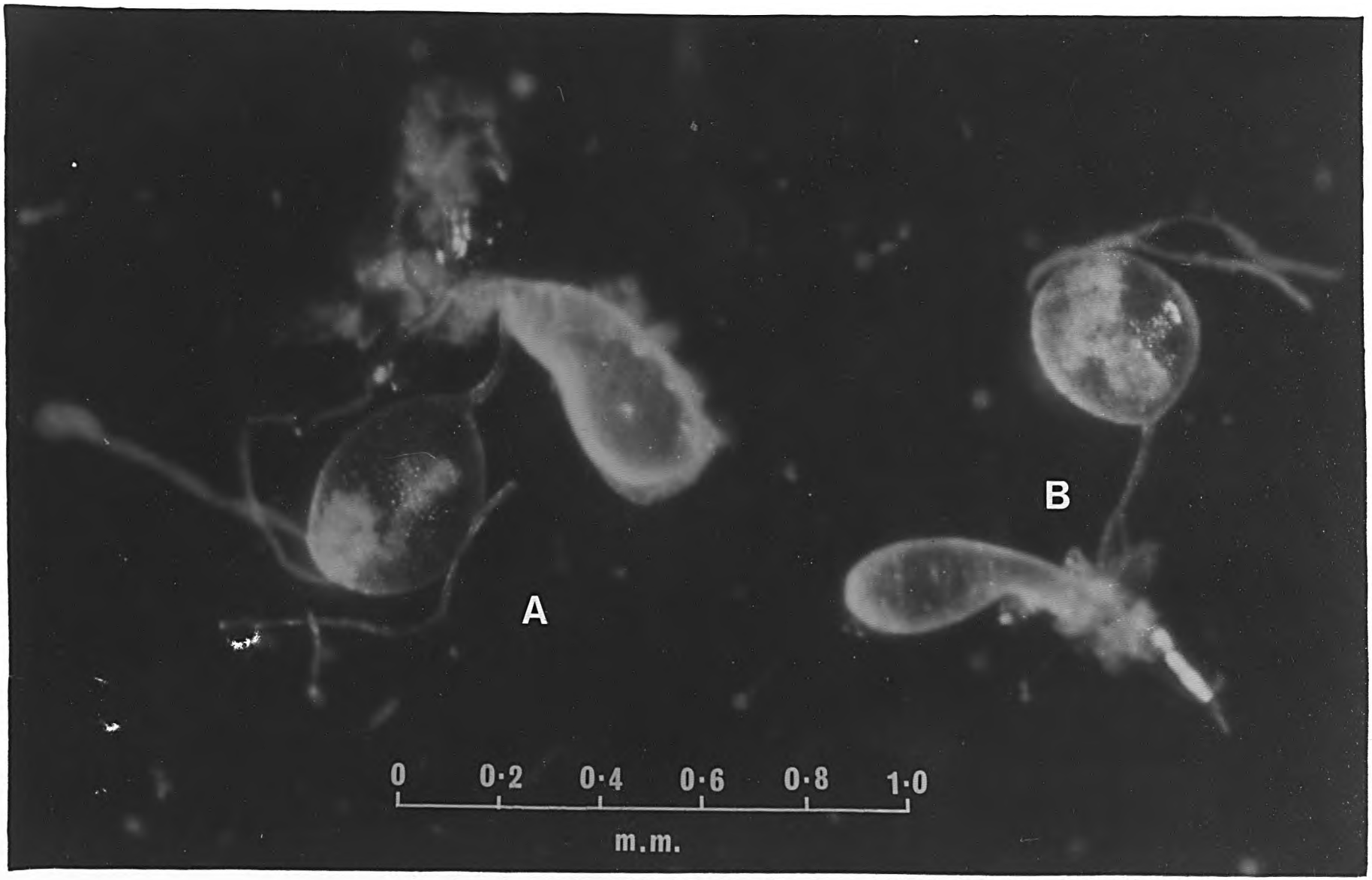


FIGURE 33. Venom apparatii of M. albuferensis (A) and M. rubra (B)

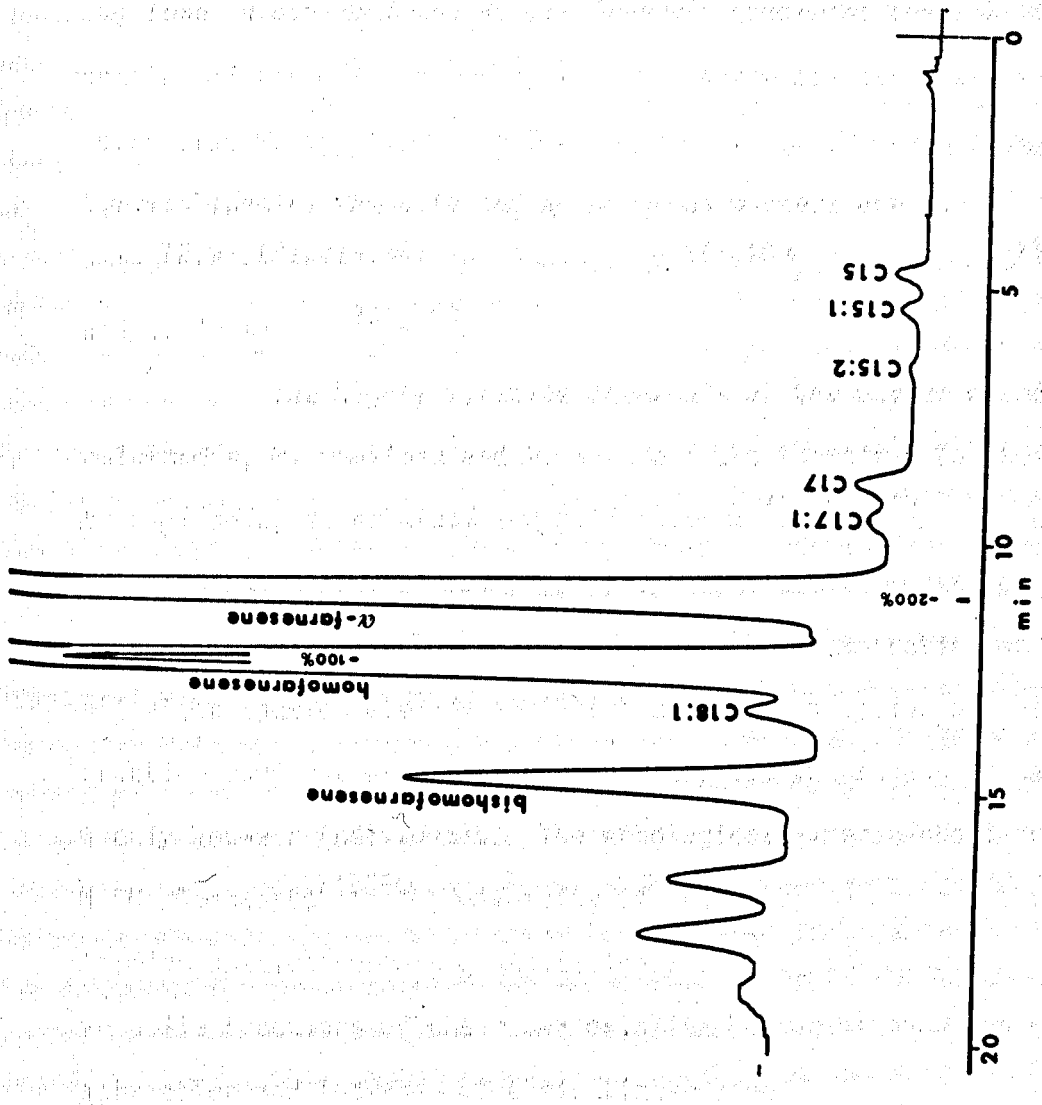


FIGURE 34. A gas chromatogram of Dufour gland contents of Mymica albuferensis. One Dufour gland was chromatographed on a 2.75 x 4 mm packed column of a 10% PEGA on Chromosorb W. The oven temperature was programmed from 120 to 160 °C at 3 °C/min (attenuation x 1000).

Table 25.

2.4.5 Comparative survey of Dufour gland substances of myrmicine ants

The present study of the Dufour gland contents *M. sulcinodis*, *M. rugulosa*, *M.schencki* and *M. albuferensis* completes the investigation of nine species of the genus *Myrmica*, *i.e.* the seven species found in the British Isles plus *M. rugulosa* from Belgium and *M. albuferensis* from Mallorca. The *Myrmica* species investigated by previous workers are *M. rubra*[72,91,181,219,361], *M. scabrinodis*[171,180], *M. ruginodis*[71], *M. sabuleti*[71] and *M. lobicornis*[334,363].

The highly volatile chemicals of the Dufour gland of *M. sulcinodis*, *M. rugulosa* and *M. schencki* are identical to those of *M. rubra*, *M. ruginodis*, *M. sabuleti* and *M. lobicornis*[71,180,362]. The two main components are ethanal and propanone, with lesser amounts of 2-methylpropanal, butanone and butenone. The examination of the results of all eight species clearly exhibit that the composition of this very volatile fraction does not vary with the species, but the amount of material can vary widely between individuals. The ethological experiments carried out by M. C. Cammaerts[71,350] (University of Brussels) shows that the workers of each *Myrmica* species respond quantitatively in the same manner to the very volatile fractions of their own or alien Dufour gland. The response being, attraction and increase of their linear speed.

The Dufour glands of *M. sulcinodis*, *M. rugulosa* and *M. schencki* emit less volatile compounds which differ qualitatively and quantitatively from each other species. Similar results had been observed for other species of Myrmica by previous workers. The examination of the results of the eight species of *Myrmica* clearly indicates how the genus can be subdivided into "R type" and "S type" groups (Figure 35). *M. rubra* and *M. ruginodis* are typical members of "R type", as their Dufour glands contain above 90% of linear hydrocarbons[71]. *M. rugulosa* can be considered to belong to the "R type", but

Table 25. Chemical composition of the Dufour glands of *Myrmica albuferensis*

Compounds	Abbreviation	Mean composition by weight (ng/ant±S.D.)	Mean % by weight ±S.D.
1 Pentadecane	C15	25 ± 6	0.51 ± 0.05
2 Pectadecene	C15:1	20 ± 3	0.39 ± 0.04
3 Pentadecadiene	C15:2	trace	-
4 Heptadecane	C17	20 ± 4	0.40 ± 0.04
5 Heptadecene	C17:1	10 ± 2	0.23 ± 0.10
6 α-Farnesene	F	2031 ± 384	43.35 ± 0.79
7 Homofarnesene	HF	1848 ± 358	38.42 ± 1.34
8 Octadecene	C18:1	36 ± 13	0.64 ± 0.15
9 Bishomofarnesene	BHF	506 ± 116	10.47 ± 1.15
10 Unknown I		131 ± 13	2.05 ± 0.76
11 Unknown II		150 ± 32	3.08 ± 1.19
12 Unknown III		22 ± 8	0.05 ± 0.31
Total		4792 ± 868	100

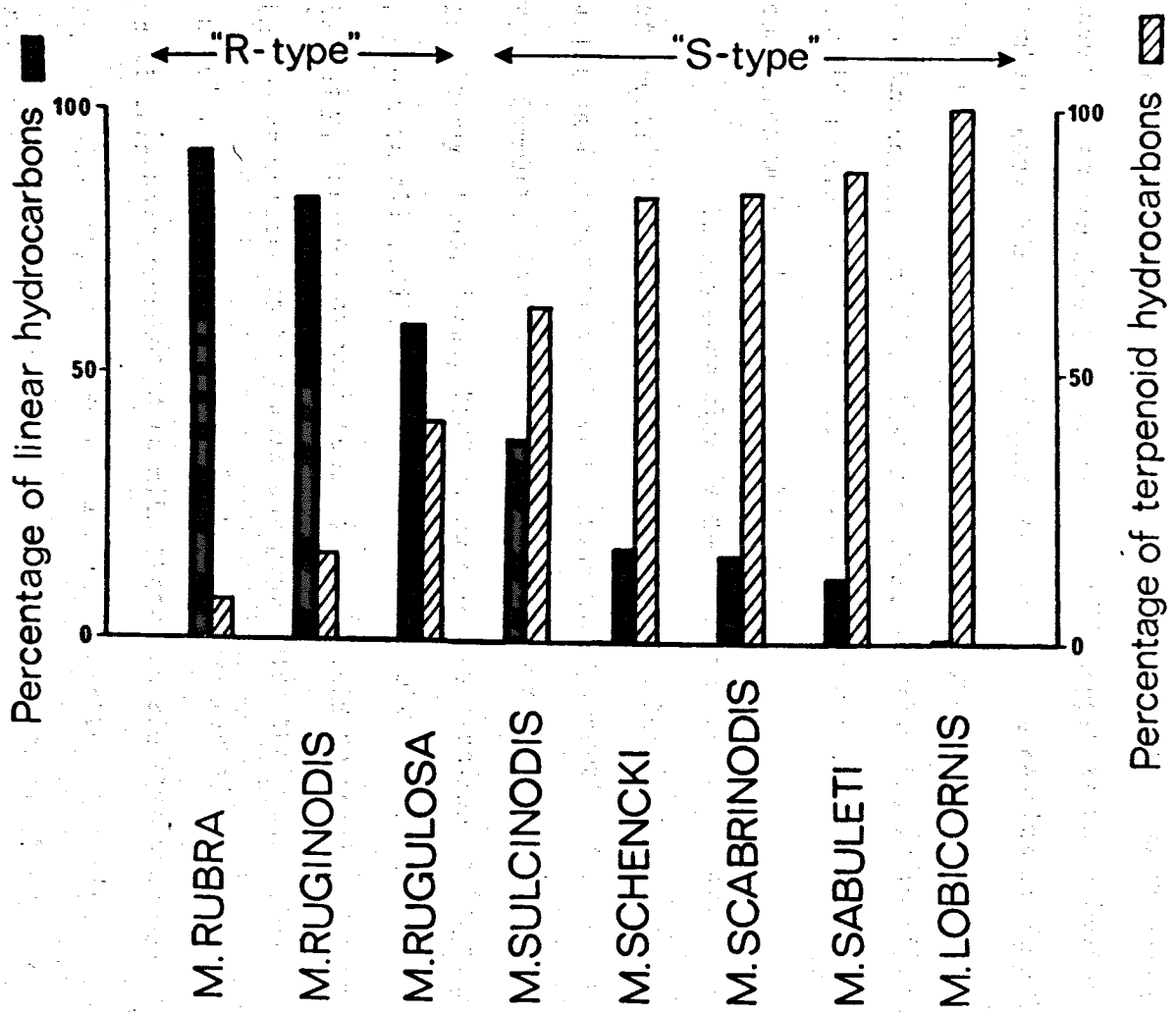


FIGURE 35. A graphical illustration of mean percentages of linear and terpenoid hydrocarbons in eight species of Myrmica.

with some closeness to "S type". *M. sulcinodis* is clearly an intermediate species between the two groups as it contains about 60% terpenoid hydrocarbons and the rest being linear hydrocarbons. *M. lobicornis* can be regarded as a typical member of the "S type"[334,363] and it is unique as it contains 100% branched terpenoid hydrocarbons only. *M. sabuleti* and *M. scabrinodis* are also typical members of "S type" as their Dufour glands contain above 80% branched hydrocarbons[71]. The results of *M. schencki* shows that it is also a typical member of "S group". The position of various *myrmica* species in this classification, are illustrated in Figure 35. It should however be noted that this diagram hides the differences in individual compounds which also separate the species.

It has been clearly demonstrated by M. C. Cammaerts (University of Brussels) that the ants move rapidly on a territory marked with the less volatile fraction of their own Dufour gland secretion[350]. The results of M. C. Cammaerts for *M. rugulosa* and *M. schencki* are illustrated in Figure 36. It clearly shows, first, that *M. rugulosa* workers run faster than *M. schencki* workers in all conditions although *M. schencki* individuals are larger. Secondly, foragers of both species move slowly on control papers at first and then move quickly as they mark the paper with their own Dufour secretion. Thirdly, they move even more slowly on paper marked with an alien secretion until they have overmarked it with their own and then they move quickly on it. Finally they both move rapidly on a territory already marked with their own secretion, and further marking by the second, or third, etc. forager does not result in any increase of speed over that of the first forager. All eight Myrmica species mentioned possess the same trail pheromone in the poison gland[109,110], therefore it can be presumed that in natural conditions it is the quantitative and qualitative differences in the Dufour gland secretions which confer a specific label to their trails when those *Myrmica* ants share the same environment.

The analysis of Dufour gland contents of *M. albuferensis* and its

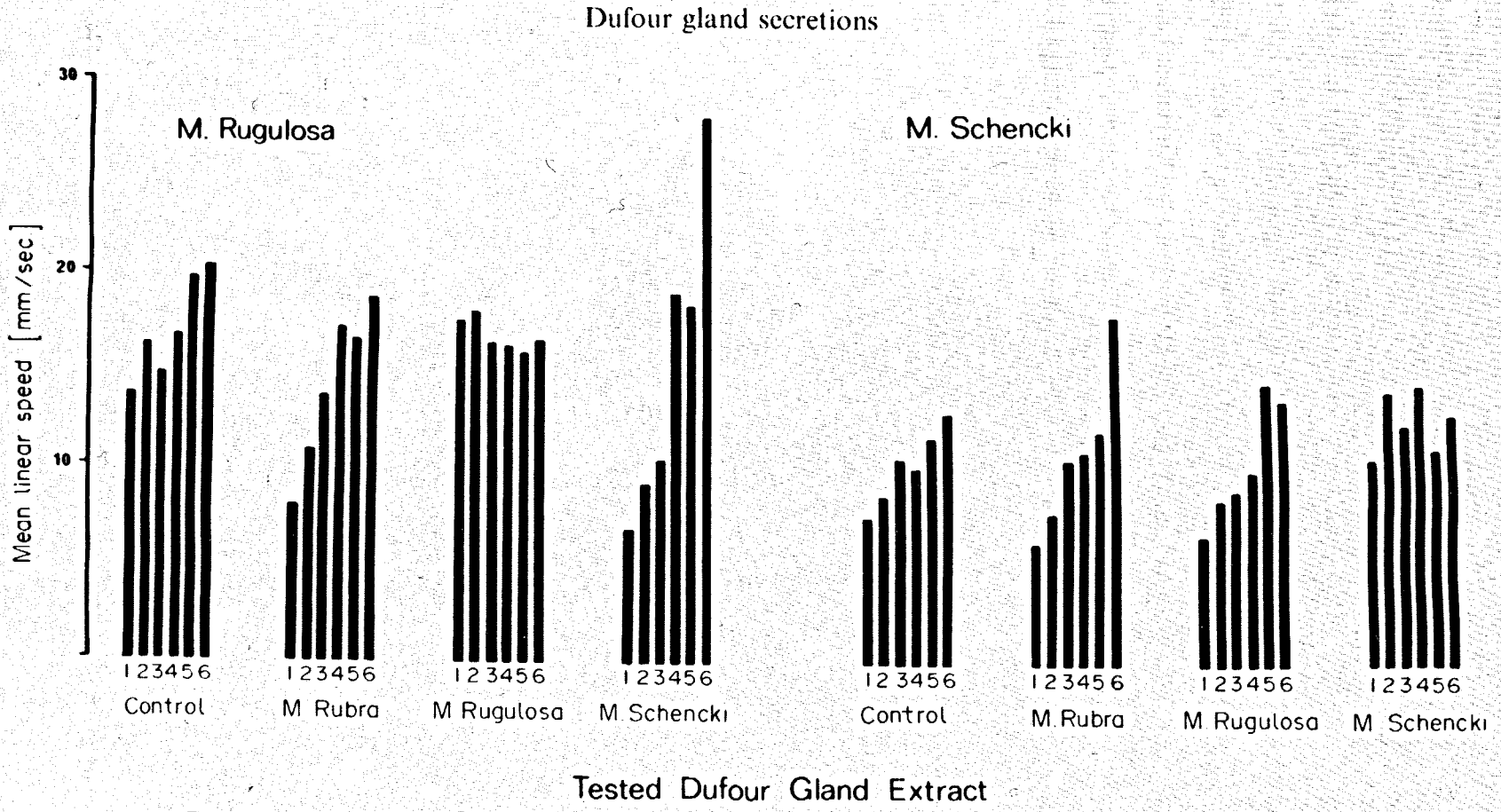
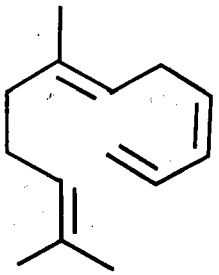


FIGURE 36. Graphical representation of mean linear speed (mm/sec) of first six foragers of *M. rugulosa* or *M. schencki*, reaching areas either untreated (control experiment) or impregnated separately with a hexane extract of Dufour gland of *M. rubra*, *M. rugulosa* and *M. schencki*, respectively. Each value is a mean of six separate determinations.

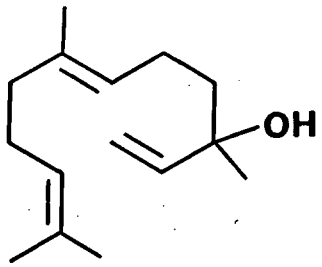
comparison with those of other eight species of *Myrmica* clearly shows that it is more close to *M. scabrinodis* than any other species. Nevertheless there are significant differences to make it different from *M. scabrinodis*. The size and the total contents are about twice that of *M. scabrinodis*. In *M. scabrinodis* the major components are homofarnesene (41%) and bishomofarnesene (25%), and α-farnesene (15%) is the third most abundent[71]. In *M. albuferensis* the largest component is α-farnesene (43%). In fact it has the largest amount of α-farnesene among the *Myrmica* species studied. The amount of bishomofarnesene is comparatively small (10%).

2.5 Structures of Farnesenes

In an investigation of the Dufour gland of the ant *Myrmica rubra*, Morgan and Wadhams found small quantities (*i.e.* nanogram per ant) of farnesene and two related compounds[181]. The latter were seen to differ from farnesene by a CH_2 and C_2H_4 increment respectively, and were identified by their mass spectra and named homofarnesene and bishomofarnesene. The farnesene isomer from the ants was identified by K. Parry[182] as (*Z*,*E*)-α-farnesene, *i.e.* (*Z*,*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene [**17**] by the comparison of its mass spectrum and GC retention times on different phases with those of a mixture of isomers prepared from nerolidol [**40**] by dehydration[180], and published data[364].

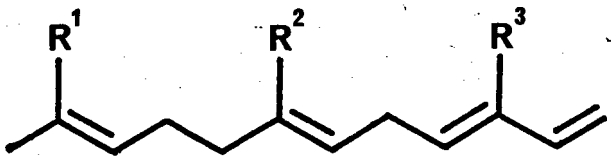


[**17**]



[**40**]

Recently Lorna Thompson (University of Keele) has confirmed this structure by total synthesis[183]. The structures [**41**] and [**42**] had been proposed by Morgan and Wadhams[181] for homofarnesene and bishomofarnesene from the ants of the genus *Myrmica* on the basis of their mass spectra.

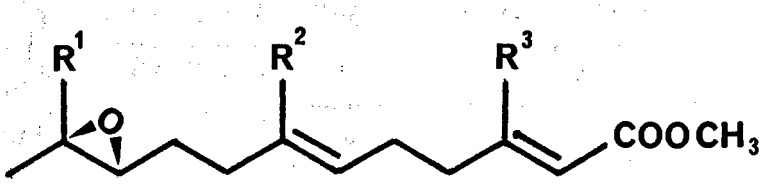


[**41**] R^1=R^3=Me, R^2=Et

[**42**] R^1=R^2=Et, R^3=Me

At about the same time, and subsequently, a series of

biologically important sesquiterpenoid compounds of similar structure called the juvenile hormones (JH 0-III, structures [59-62], respectively) have been isolated from a number of insect species.



[59] $R^1=R^2=R^3=Et$ JH 0

[60] $R^1=R^2=Et$, $R^3=Me$ JH I

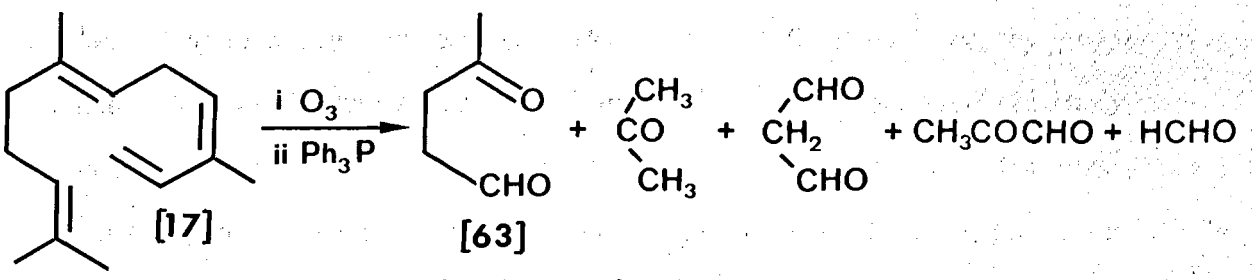
[61] $R^1=Et$, $R^2=R^3=Me$ JH II

[62] $R^1=R^2=R^3=Me$ JH III

Faranal [15], a bishomofarnesene compound, has been shown to be the trail pheromone of the ant *Monomorium pharaonis*[90]. Two different homofarnesene isomers, (Z,Z)- and (Z,E)-3,4,7,11-tetramethyl-1,3,6,10-dodecatetraene [19, 20] have been reported recently as trail pheromone components in *Solenopsis invicta*[120]. Because the positions of the extra methylene units in *Myrmica* homofarnesene are different from those in other reported homoterpenoids, it was considered important to provide unequivocal proof of the structure for homo and bishomofarnesene.

The amounts of natural farnesene and its homologues available (*ca.* 0.5 µg/ant) precludes the application of the usual spectral techniques such as NMR, IR and UV. The only spectral technique generally applicable to nanogram quantities of compounds in mass spectrometry. The mass spectral information of the farneseneswas already available[181]. To confirm the mass spectral evidence and to prove beyond doubt that the ethyl branches were on C-7 in homofarnesene and on C-7 and C-11 in bishomofarnesene, recourse was made to micro-degradation and reaction gas chromatography. The selected degradative method was ozonolysis. The reliability and feasibility of the method were established by using a synthetic sample of mixture of (Z,E)-and

(Z,Z)-α-farnesene, available through the courtesy of Miss Lorna Thompson (University of Keele).



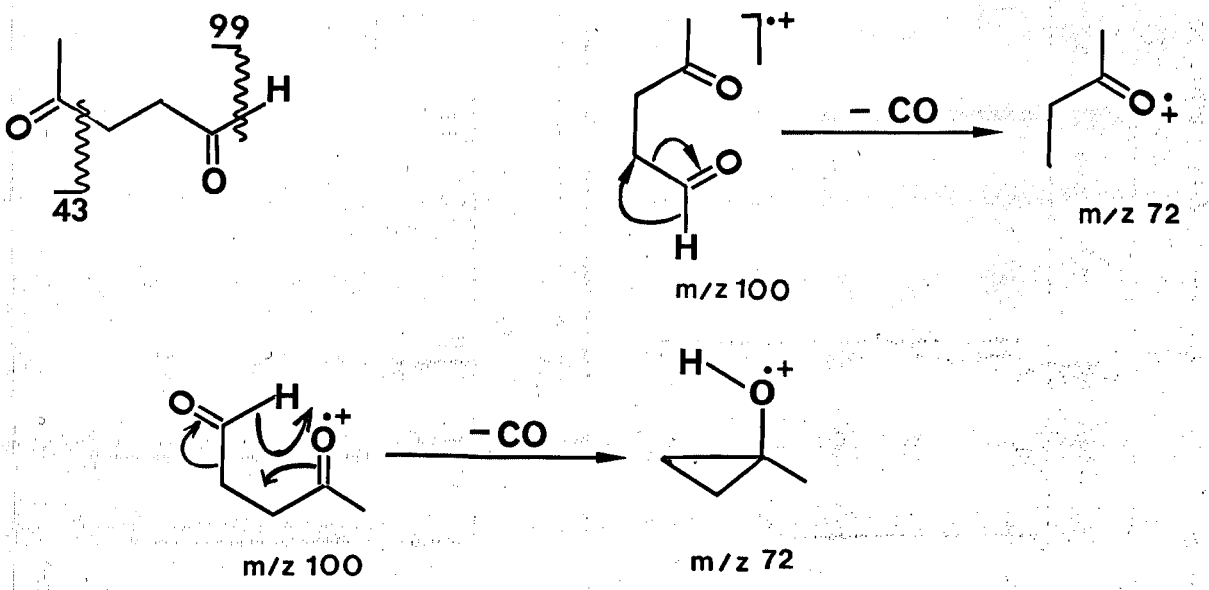
[17] [63]

The synthetic (Z,E)- and (Z,Z)-α-farnesene mixture, when ozonolyzed in solvent gave the expected 4-oxopentanal (levulinaldehyde)[63] among other products. A mixture of (Z)-and (E)-nerolidol [40] or any other terpene with similar skeleton, gave the same product 4-oxopentanal [63], identified by the retention time on a 10% PEG 20M column and its mass spectrum. The examination of ozonolysis products for small molecules on the Chromosorb 102 column, gave a peak corresponding to propanone to confirm the presence of an isopropylidene group in synthetic α-farnesene and nerolidol. A suitable solvent was required for ozonolysis and examination of small molecular products on the Chromosorb 102 column. A number of solvents, such as methanol, ethylacetate, hexanol were tried but carbon tetrachloride was found to be the most suitable. The small molecules up to six carbon atoms can be examined on the Chromosorb 102 column before the appearence of the carbon tetrachloride solvent peak. The other advantageous of carbon tetrachloride are its availability in high purity, ozonation takes place readily without the solvent being ozonized, and its moderate volatility which allows ozone to be bubbled through it without much solvent evaporation.

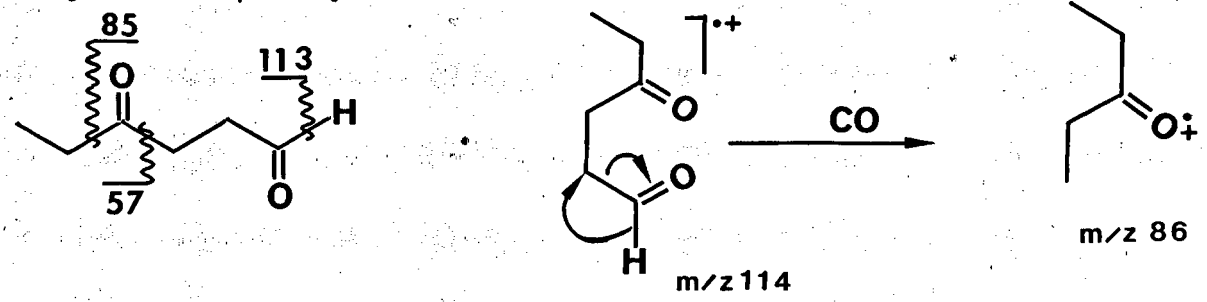
The other ozonolysis products expected from the synthetic farnesene mixture, propanedial, 2-oxopropanal and methanal were not observed in the gas chromatograms due to either their poor stability or low response factor towards the flame-ionization detector. Beroza and Bierl[286] have also reported the same problem with propanedial.

The results obtained from the ozonolysis of synthetic α-farnesene and nerolidol demonstrated that the method can be applied reliably to study

the nanogram quantities of farnesene and its two higher homologues, available from the ants of the genus *Myrmica*. The three compounds were therefore collected separately from whole gasters of myrmicine workers, by preparative GC and ozonolyzed separately in solvent. The ant farnesene [17] gave the expected 4-oxopentanal[63] and propanone. The mass spectra of 4-oxopentanal (Figure 37) obtained by GC-MS were identical irrespective of the source. The molecular ion is present at m/z 100 (3%, M^+). The base peak is at m/z 43 for the $MeCO^+$ ion. The fragmentation pathways and possible structuresof the various ions are illustrated below.



The analysis of ozonolysis products of ant homofarnesene and bishomofarnesene on the 10% PEG 20M column did not show a peak corresponding to 4-oxopentanal. A peak with higher retention time corresponding to a compound with one extra carbon atom than 4-oxopentanal was produced instead (Figure 38). This peak was identified as 4-oxohexanal [64] by its mass spectrum (Figure 37). The molecular ion is seen at *m/z* 114 (2%, M^+) and the base peak is at *m/z* 57 for the $EtCO^+$ ion. The possible mass spectral fragmentation pathways are illustrated below.



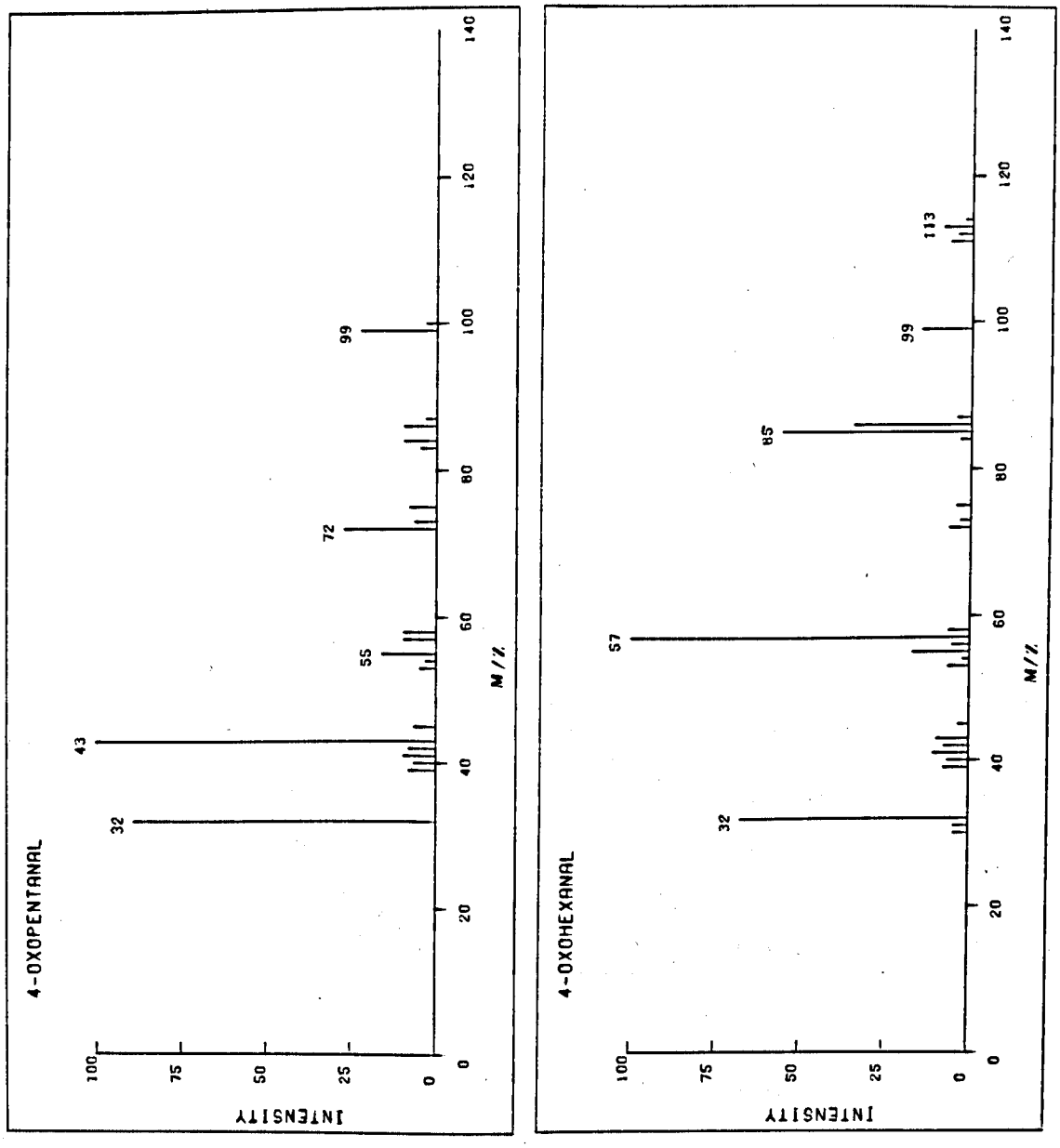


FIGURE 37. Mass spectra of 4-oxopentanal and 4-oxohexanal

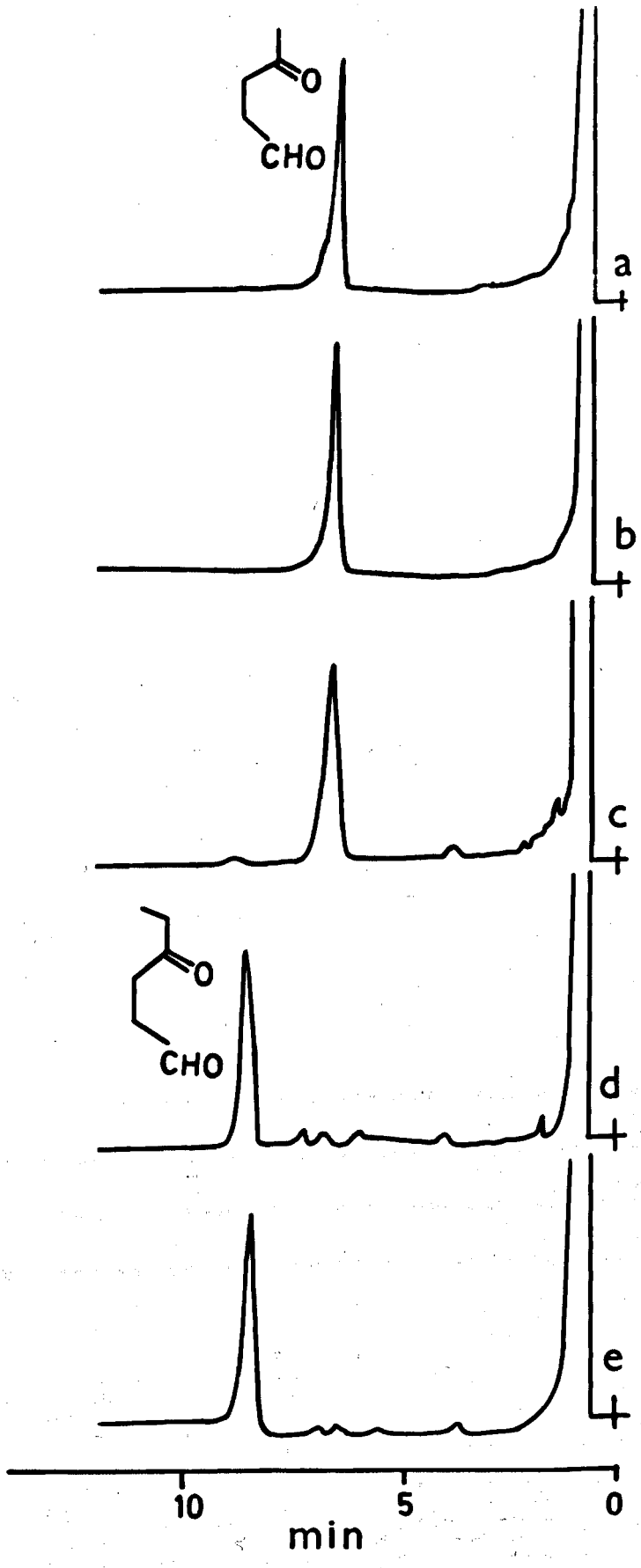
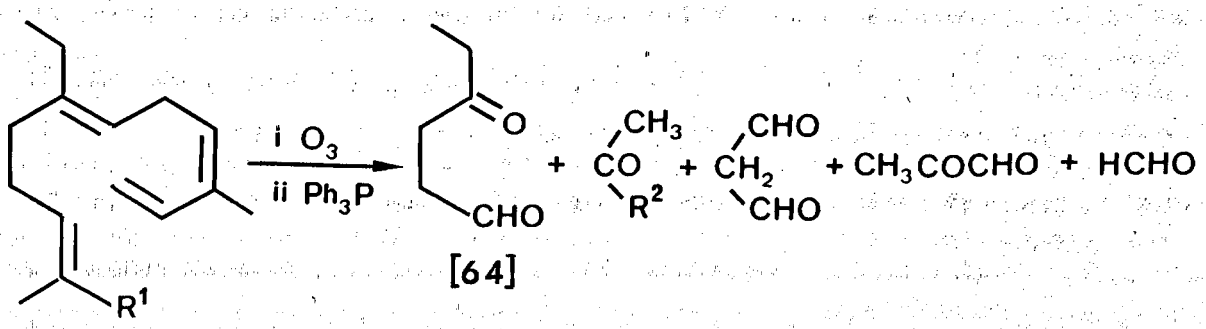


FIGURE 38. Gas chromatograms of ozonolysis products of farnesenes. (a) ant farnesene, (b) synthetic farnesene, (c) nerolidol, (d) ant homofarnesene and (e) ant bishomofarnesene were ozonolyzed. The products were chromatographed on a 2.75 m x 4 mm column of 10% PEG 20M at 140°C. (attenuation x200)

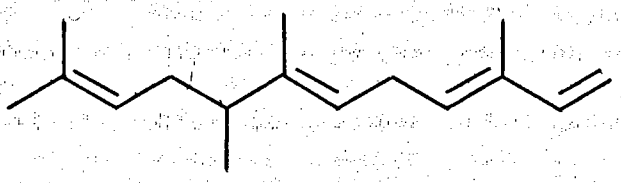
The examination of ozonolysis products of ant homofarnesene and bishomofarnesene for small molecules on the Chromosorb 102 column revealed their differences in structure. The ant homofarnesene gave propanone to show the presence of an isopropylidene group similar to α-farnesene or nerolidol. Bishomofarnesene did not give a peak for propanone (therefore an isopropylidene group is absent) but a peak corresponding to butanone was identified by its retention time and mass spectrum. The production of butanone from bishomofarnesene demonstrated the presence of an isobutylidene group in its structure. The fragments observed on degradation can be assembled together, to confirm the structures for homo and bishomofarnesene.



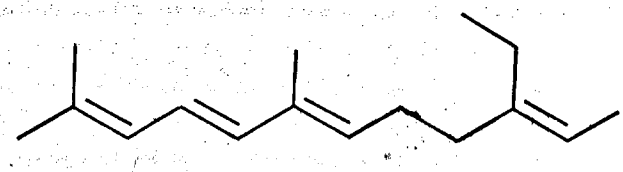
[41] R^1 = Me

[42] R^1 = Et

The production of 4-oxohexanal from homofarnesene from ants of the genus *Myrmica*, shows that it is structurally different from the homofarnesene isomers reported from *Solenopsis invicta*[120] [19,20]. Similarly a structure like [65] can also be eliminated as it will



[65]

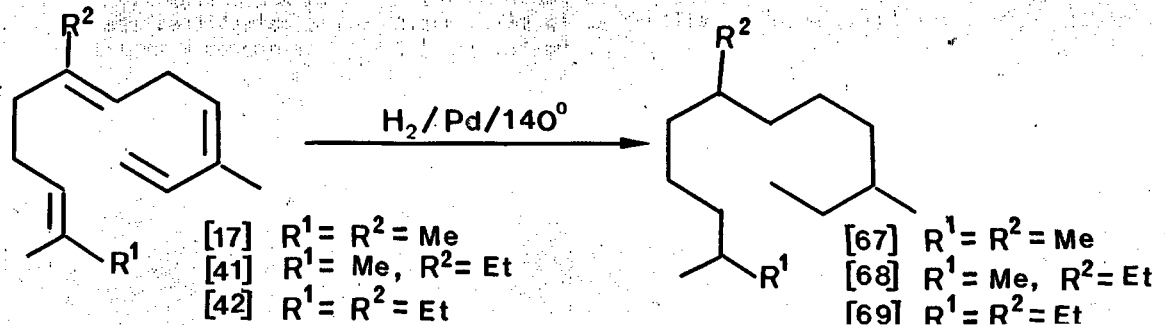


[66]

not produce 4-oxohexanal. A structure such as [66] can be eliminated as no ethanal was observed as an ozonolysis product.

The ozonolysis of α-farnesene and its homologues in solvent required the material from a minimum of 20 ants. The technique was refined by the development of the solventless ozonolysis in glass capillaries. Hence the amount of substance from only one *M. scabrinodis* ant was sufficient for the analysis. Figure 39 shows the GC profiles on Chromosorb 102 , of the solventless ozonolysis products of farnesene, homofarnesene and bishomofarnesene collected separately from one ant. The elegance of this technique is that the GC can be operated at its maximum possible sensitivity as there is no solvent peak and no impurities are introduced by the solvent. The method is described in detail in the experimental section.

The retention times on different GC phases and the mass spectra of farnesene, homofarnesene and bishomofarnesene obtained from all ants of the genus *Myrmica* studied so far, are identical. The mass spectra of the farnesenes (Figure 40) were obtained by GC-MS using 5 whole gasters of workers of *M. rugulosa*. L.J. Wadhams[365] interpreted the mass spectral fragmentation patterns of the farnesenes and proposed the structures for homofarnesene and bishomofarnesene[181]. He had suggested that the C-7 methyl group of farnesene appears to be replaced by an ethyl group in homofarnesene. He also suggested ethyl groups at C-7 and C-11 in bishomofarnesene. It is difficult to confirm structures of complicated molecules such as homofarnesene and bishomofarnesene on their mass spectrum alone. As a branched saturated hydrocarbon gives a mass spectrum which can be interpreted more clearly to locate the branching positions, than its unsaturated analogue it was decided to hydrogenate the farnesenes and examine the farnesanes by GC-MS, as further confirmation of the structures assigned.



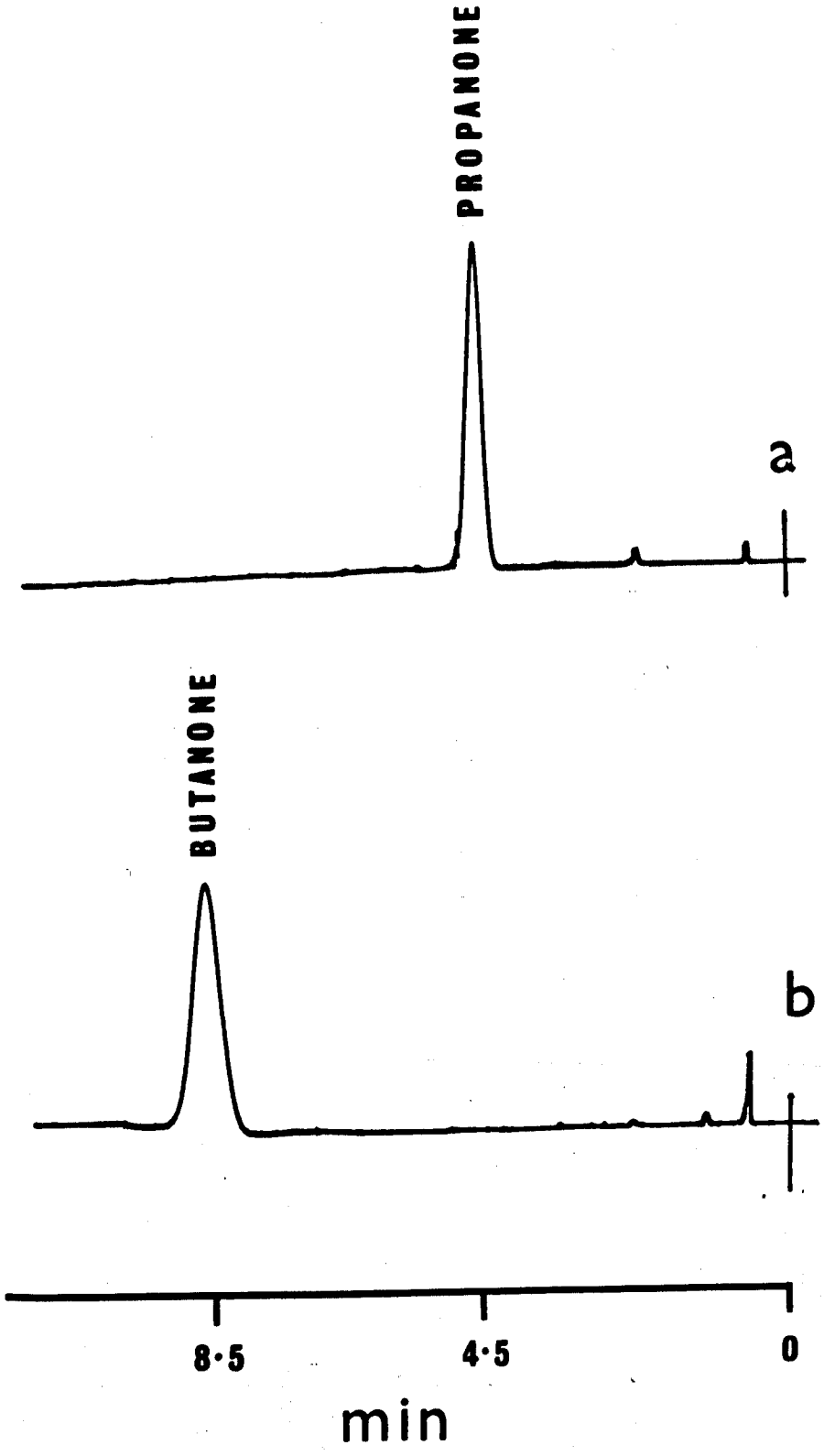


FIGURE 39. Gas chromatograms of solventless ozonolysis of the farnesenes. (a) homofarnesene and (b) bishomofarnesene, 200 ng each were collected separately in glass capillaries and ozonized. The glass capillaries were solid injected on a Porapak Q column at 160 °C (attenuation x 200).

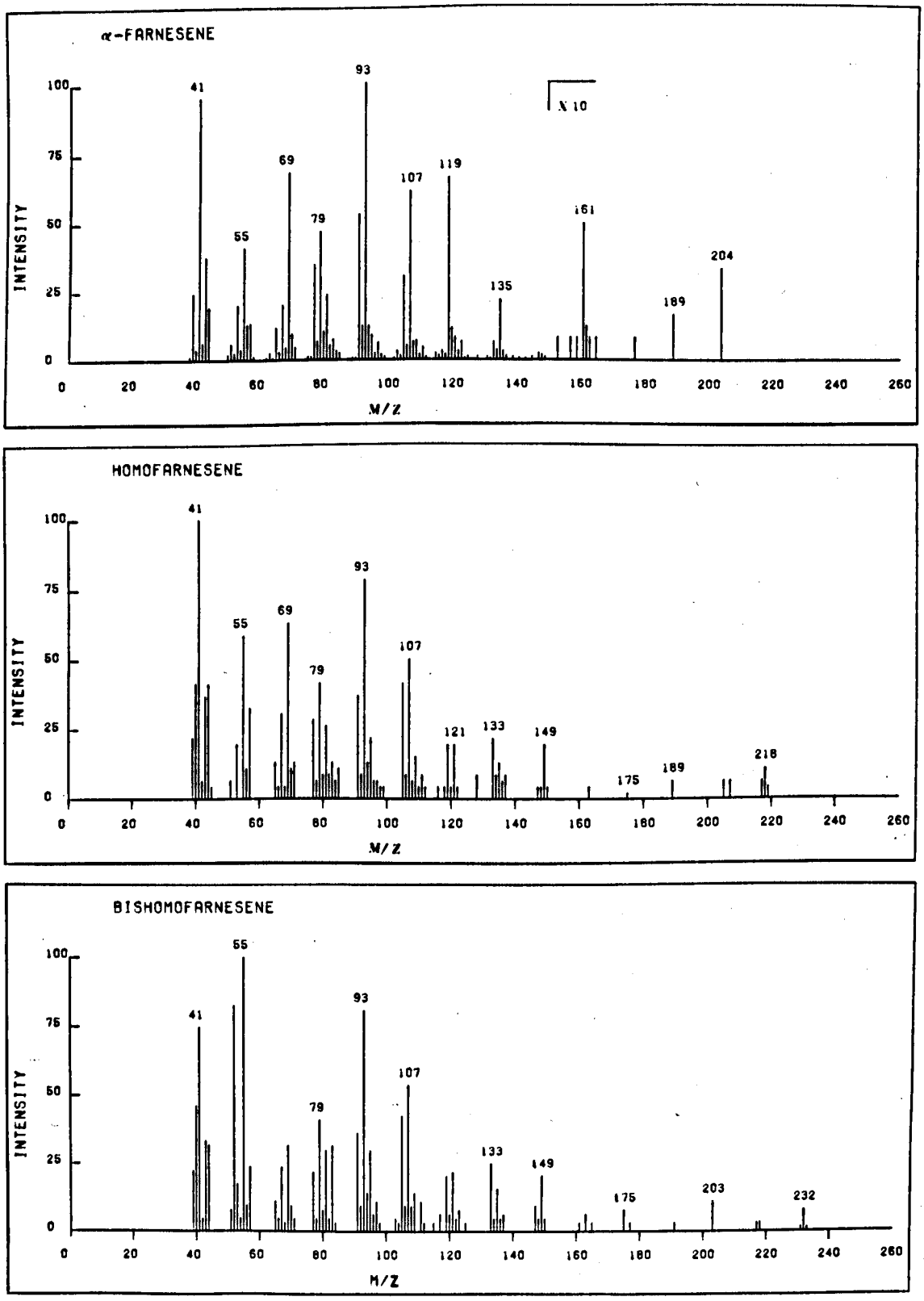


FIGURE 40. Mass spectra of farnesenes

On column hydrogenation can be performed directly by using a pre-column of 1% Pd catalyst [254] and employing hydrogen as the carrier gas [257]. The farnesenes are more difficult to reduce than monounsaturated alkenes but six cm of pre-column catalyst and a hydrogen flow rate of 40 ml/min, completely hydrogenated microgram quantities of farnesenes instantaneously. Figure 41 illustrates hydrogenation of total material from one Dufour gland of *M. scabrinodis*, on the 10% PEGA column. The amount of shift of retention times on hydrogenation corresponds to the presence of four double bonds in the farnesenes. The farnesanes produced by the reduction of corresponding farnesenes, from all the ants of the genus *Myrmica* studied so far had the identical corresponding retention times. The single peak produced by the reduction of the mixture of synthetic (*Z*,*E*)-and (*Z*,*Z*)-α-farnesene also had the identical retention time as the farnesane derived from the ants.

The mass spectra of the farnesanes obtained by GC-MS are illustrated in Figure 42. The addition of eight hydrogen atoms per molecule of farnesene (M^+ 204, $C_{15}H_{24}$), homofarnesene (M^+ 218, $C_{16}H_{26}$) and bishomofarnesene (M^+ 232, $C_{17}H_{28}$) to generate farnesane (M^+ 212, $C_{15}H_{32}$) [67], homofarnesane (M^+ 226, $C_{16}H_{34}$) [68] and bishomofarnesane (M^+ 240, $C_{17}H_{36}$) [69] respectively, confirmed the presence of four double bonds and the absence of any carbocyclic rings in the farnesenes. The molecular ions are present in all the spectra of farnesenes and farnesanes. The M^+-29 peak is not very significant in the mass spectra of either farnesene [17] or farnesane [67]. This can be expected as farnesene has no ethyl group and farnesane has only a terminal ethyl group. The M^+-29 peak can be clearly seen in the mass spectra of compounds containing non-terminal ethyl branching. In the mass spectrum of homofarnesane [68] the *m/z* 141, peak is weaker than the *m/z* 127 peak. However in the case of bishomofarnesane [69] the peak at *m/z* 141 is stronger than that at *m/z* 127 because the ions produced by the cleavage of bonds on either side of C-7 are of equal mass.

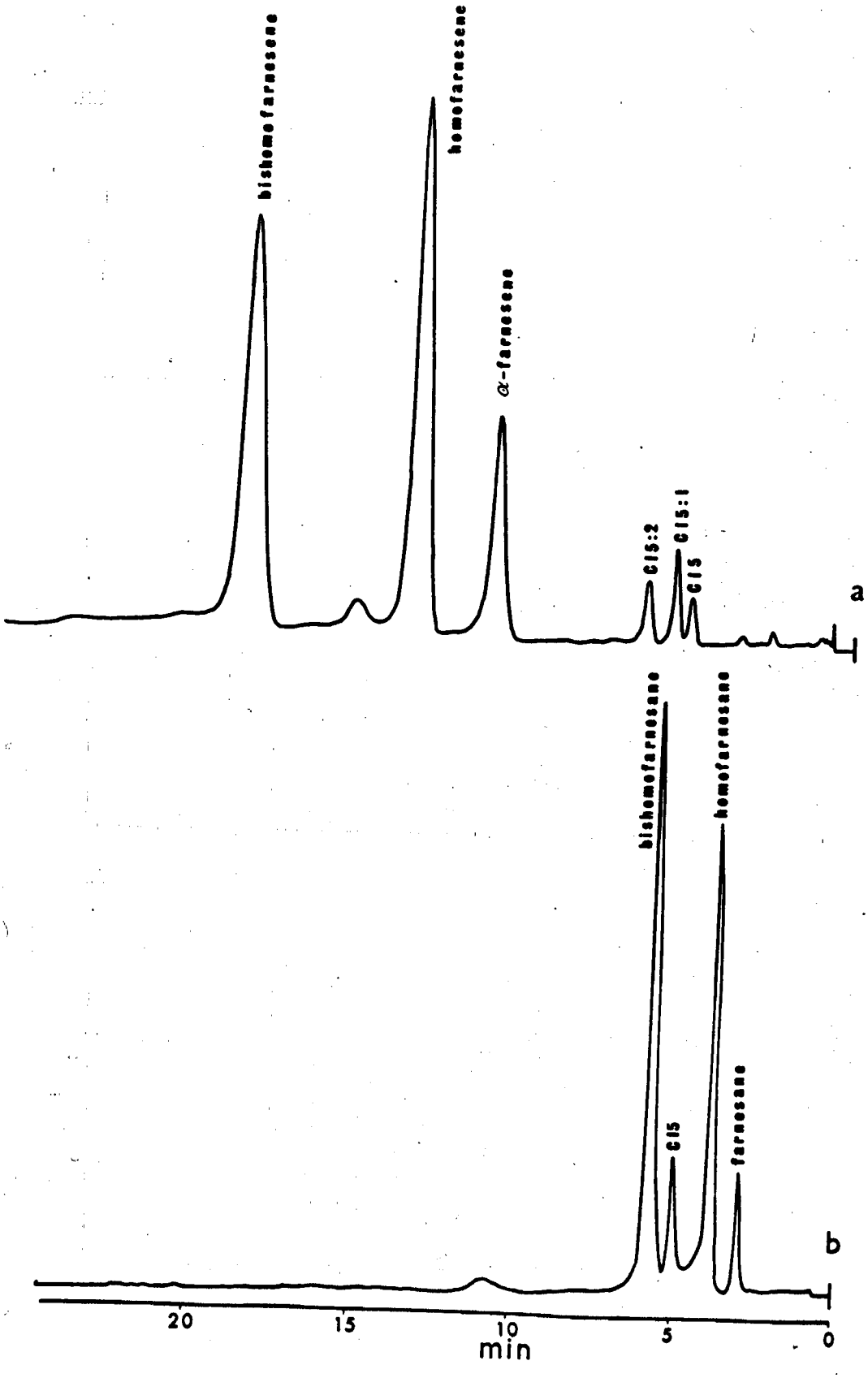


FIGURE 41. **Gas chromatographic reduction of farnesenes.** (a) one Dufour gland of Myrmica scabrinodis chromatographed on a 2.75 m x 4 mm packed column of 10% PEGA on Chromosorb W, at 160 °C (attenuation x 500). (b) The same mixture was hydrogenated using a 6 cm pre-column of 1% palladium catalyst on top of the PEGA column and hydrogen was used as the carrier gas at 40 ml/min.

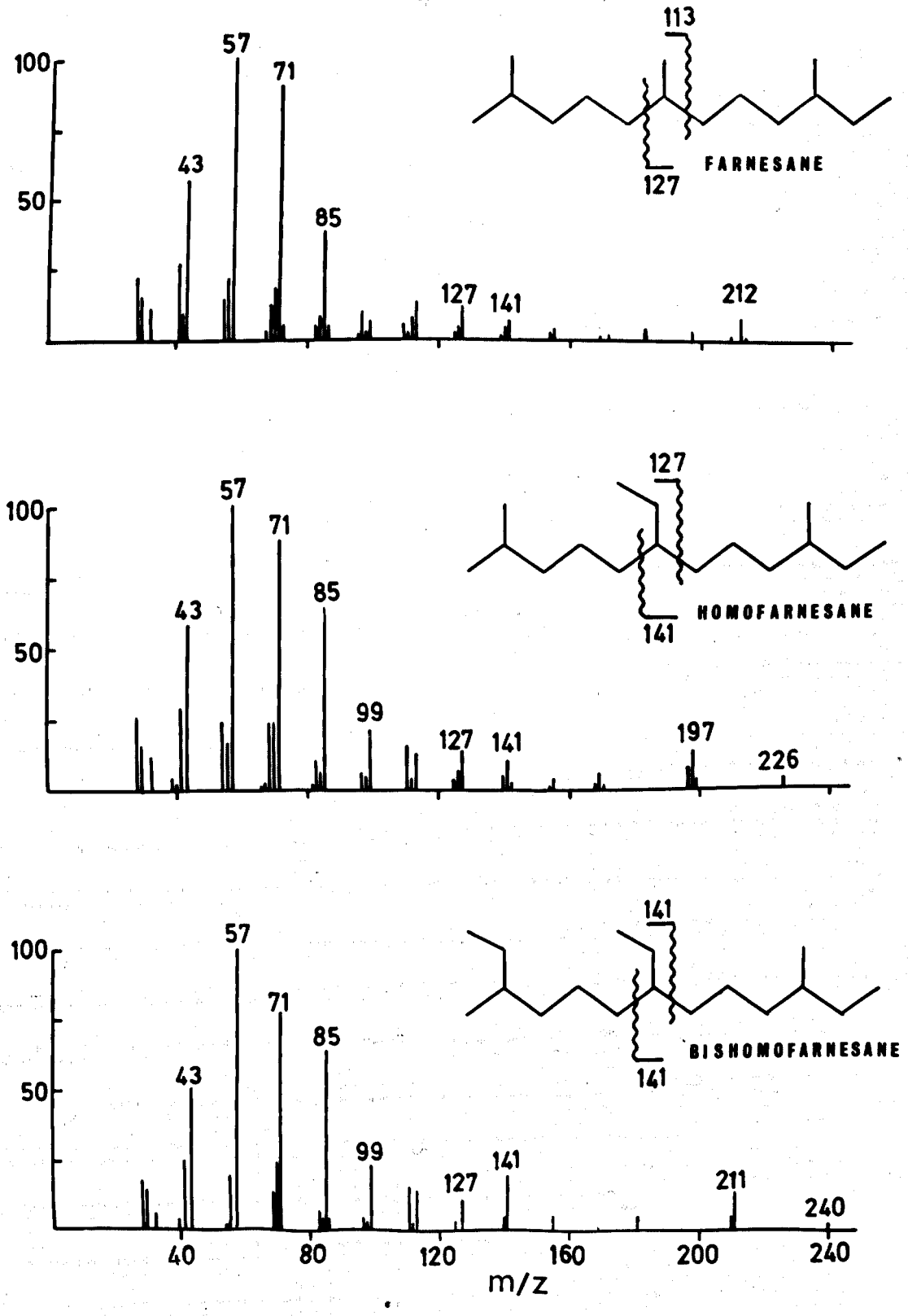
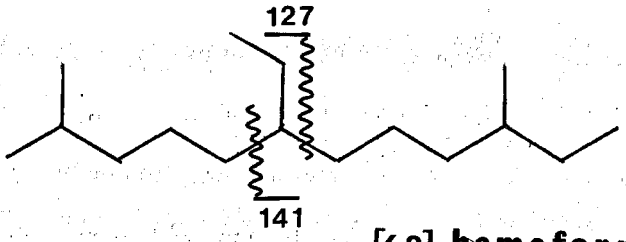
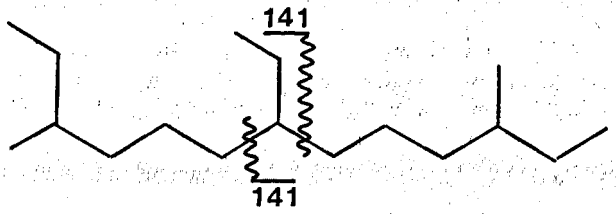


FIGURE 42. Mass spectra of farnesanes



[68] homofarnesane



[69] bishomofarnesane

Therefore it may be presumed that bishomofarnesane [69] is symmetrical around C-7. Such a structure for bishomofarnesane is possible only if an ethyl group is present at C-11 in its precursor, bishomofarnesene. Homofarnesane and bishomofarnesane are novel compounds, not reported previously.

On the strength of the evidence presented so far, homofarnesene and bishomofarnesene from ants of the genus *Myrmica*, are identified as 7-ethyl-3,11-dimethyldodeca-1,3,6,10-tetraene [41] and 7-ethyl-3,11-dimethyltrideca-1,3,6,10-tetraene [42], respectively. The determination of the geometry of 3,4-and 6,7- double bonds of homofarnesene, and 3,4-, 6,7- and 10,11- double bonds of bishomofarnesene awaits stereospecific synthesis of homofarnesene and bishomofarnesene. By analogy with (*Z*,*E*)-α-farnesene, the 3,4-and 6,7- double bonds in each of the higher homologues may be expected to (*Z*) and (*E*) respectively. Although no definite information is available yet on the additional isomers possible at 10,11-double bond of bishomofarnesene, it might be expected to be (*Z*) by comparison of its structure with juvenile hormone I [60]. Similarly the trishomofarnesene isomer identified from *M. scabrinodis*[180] may be expected to have the extra methylene unit at C-4, as in faranal [15], or at C-3 as an

ethyl group as found in juvenile hormone 0 [59].

It can now be established that the location of the extra methylene group in *Myrmica* homofarnesene is at C-7 as an ethyl group therefore it is structurally different from the homofarnesene [19,20] reported from *Solenopsis invicta* where the extra methylene group is present at C-4. It is also different structurally from juvenile hormone II [61] which has the extra methylene group at the end of the carbon chain. The bishomofarnesene [42] from the genus *Myrmica*, has the same basic carbon skeleton as the juvenile hormone I [60] but it is different from faranal [15], the trail pheromone of *Monomorium pharaonis*.

2.6 Mandibular Gland Substances of the Genus *Myrmica*

The mandibular gland secretions of eight species of *Myrmica* have been investigated previously and their qualitative and quantitative chemical compositions are documented[171,334,360,366,367]. As the composition of the mandibular gland has been found to be species-specific through these studies, it could be of use to the chemical taxonomist as a means of distinguishing morphologically closely related species from one another. The study of mandibular glands of *M. albuferensis* was undertaken as the results may complement those obtained from their Dufour glands, to solve the controversy about its identity. The major components of the mandibular glands of myrmicine workers are commonly 3-octanone and 3-octanol. Ethological tests have been carried out by M. C. Cammaerts (University of Brussels) to find out the responses of myrmicine workers to their mandibular gland substances[366]. The queens of myrmicine ants also have the same 3-octanone and 3-octanol as the major components. The analysis of large no of heads of *Myrmica rubra* queens sent by M. C. Cammaerts, was undertaken to enable her to find out whether there is a relationship between the aggregative power of the queens and the 3-octanol content. 3-octanol acts alone or synergistically with 3-octanone, to attract worker ants, increase their linear speed and decrease their sinuosity of movement[360,366]. 3-Octanol has a chiral centre at C-3 and it was considered to be important to find the enantiomeric composition of 3-octanol from myrmicine ants because "odour" receptors in insects can discriminate between enantiomers.

2.6.1 Mandibular gland substances of *M. albuferensis*

Mandibular glands are associated with the mesal side of the base of the mandible. In the study of *M. rubra*, Wadhams[365] found that the GC profiles obtained from excised mandibular glands were the same as those of their individual heads. The major components of the mandibular glands of

myrmicine ants were initially found by Crewe and Blum to be 3-octanone and 3-octanol[368]. A complete analysis of the mandibular glands of *M. scabrinodis* by Morgan *et al.*[367] revealed the presence of a homologous series of 3-alkanones and 3-alkanols in the C_6 to C_{11} chain length range. Similar qualitative results were obtained when *M. albuferensis* was studied. A typical GC profile obtained by the analysis of a worker's head is shown in Figure 43. The identifications were done mainly by the comparison of GC retention times with authentic samples and with published data[360]. The linear 3-alkanones and 3-alkanols lie on two separate straight lines parallel to each other, when the logarithms of their retention times were plotted against their carbon numbers. The branched chain alkanones and alkanols do not line on these straight lines. The 3-octanone peak, when trapped and reduced with $NaBH_4$ produced 3-octanol. The results of quantitative analysis of various components are summarised in Table 26. Conclusions similar to those made by Dufour gland analysis can be made, when the results of mandibular gland analysis of *M. albuferensis* are compared to those published for *M. scabrinodis*. The major difference between the two species is seen in the 3-octanone to 3-octanol ratio. In *M. scabrinodis* 49% of the total glandular contents is 3-octanone and 3-octanol represents only 17.8%[367]. But in *M. albuferensis* 3-octanol is found in more significant quantities and represent 31% of the glandular contents. The amount of 3-octanone is significantly less than in *M. scabrinodis* and represents only 37%. The comparison of the results of *M. albuferensis* with those found in literature for other myrmicine ants clearly shows that it is more close to *M. scabrinodis* than any other species studied so far[360,361], but the differences between them are significant enough to classify them as two separate species. This conclusion is supported by the results of the Dufour gland analysis discussed before (2.4.4). The fact that *M. albuferensis* originates from an extremely specialised habitat of salt marshes of Albufera can not be ignored and the differences observed may be purely environmental. A chemical

Table 26. Chemical composition of the mandibular glands of *Myrmica albuferensis*

Compounds	Mean composition by weight (ng/ant±S.D.)	Mean % by weight ±S.D.
1 Ethanal	150 ± 38	4.32 ± 0.91
2 Propanone	115 ± 29	3.31 ± 0.74
3 Methylpropanal	265 ± 84	7.37 ± 1.81
4 3-Hexanone	43 ± 19	1.25 ± 0.47
5 3-Pentanol	24 ± 7	0.70 ± 0.17
6 3-Heptanone	35 ± 16	1.00 ± 0.36
7 3-Hexanol	55 ± 21	1.56 ± 0.47
8 3-Octanone	1278 ± 106	37.07 ± 1.58
9 3-Heptanol	47 ± 7	1.39 ± 0.23
10 6-Methyl-3-octanone	76 ± 10	2.09 ± 0.16
11 3-Nonanone	42 ± 8	1.22 ± 0.14
12 3-Octanol	1057 ± 122	30.93 ± 4.77
13 6-Methyl-3-octanol	trace	-
14 3-Decanone	243 ± 65	6.95 ± 1.27
15 3-Nonanol	16 ± 3	0.48 ± 0.08
16 Methyl undecanone	trace	-
17 3-Undecanone	13 ± 2	0.38 ± 0.05
Total	3560 ± 280	100

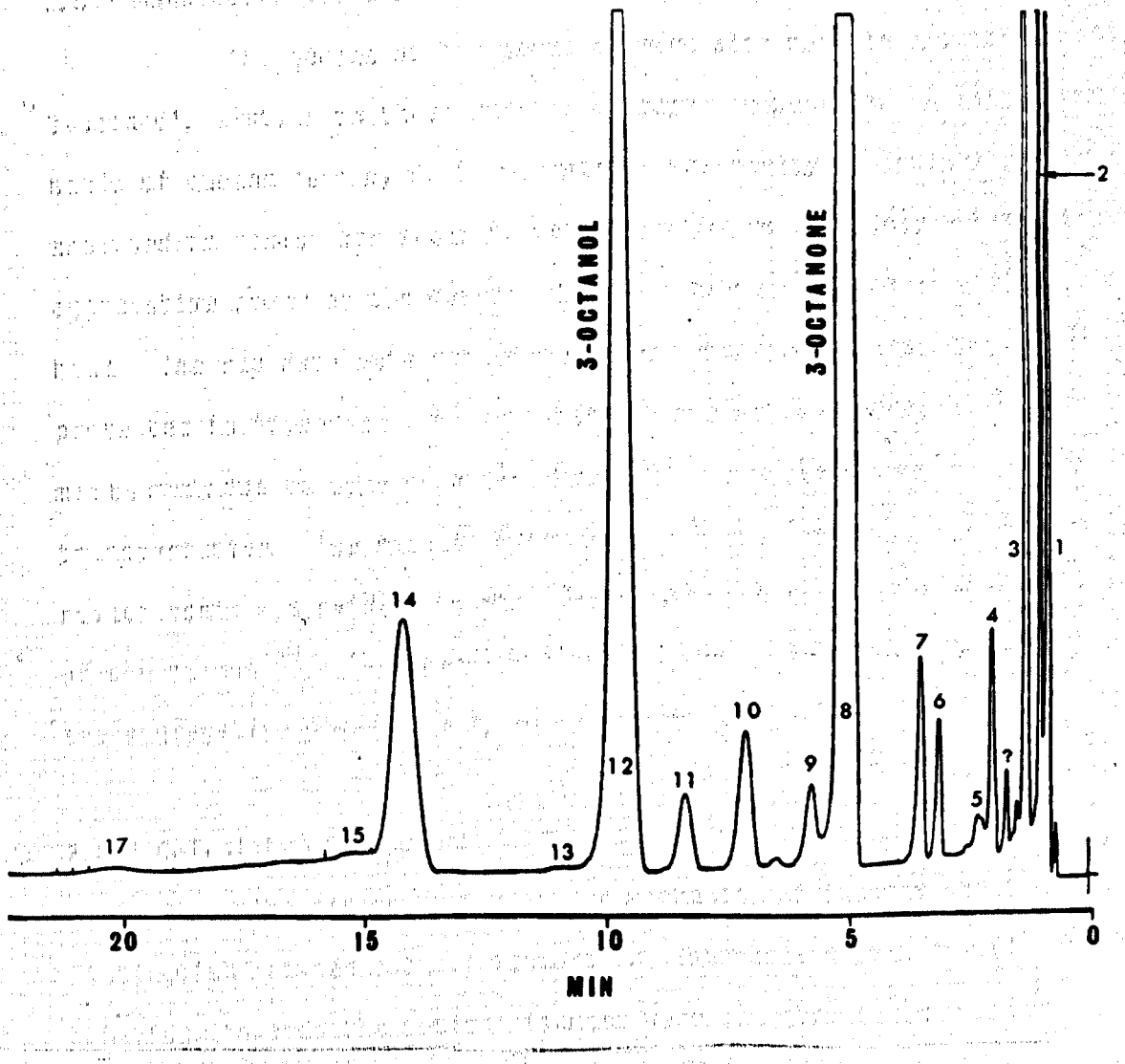


FIGURE 43. A gas chromatogram of mandibular gland contents of Myrmica albuferensis. An individual head was chromatographed on a 2.75 m x 4 mm column of 10% PEG 20M on Chromosorb W at 130 °C (attenuation x 500). 1= ethanal; 2= propanone; 3= methylpropanal; 4= 3-hexanone; 5= 3-pentanol; 6= 3-heptanone; 7= 3-hexanol; 8= 3-octanone; 9= 3-heptanol; 10= 6-methyl-3-octanone; 11= 3-nonanone; 12= 3-octanol; 13= 6-methyl-3-octanol; 14= 3-decanone; 15= 3-nonanol; 17= 3-undecanone.

investigation of *M. aloba* is required to determine how it is related to *M. albuferensis* before any final conclusions can be made.

2.6.2 Mandibular gland substances of *M. rubra* queens

The queens of the genus *Myrmica* also contain 3-octanone and 3-octanol, similar to their workers as major components. A large number of heads of queens sent by M. C. Cammaerts (University of Brussels) were analysed to assist her study to determine the relationship between the aggregative power of the queens and the amount of 3-octanol present in the head. The raw data obtained from over one hundred queens from 5 nests are presented in Appendix 1. A very wide range of values were obtained and this may be partly due to some volatiles been lost in certain samples during transportation. The results were sent to M. C. Cammaerts but no clear relationship was evident between 3-octanol content and the aggregative power of the queens[369]. They believe that the age is an important factor to decide the aggregative power of a myrmicine queen.

2.6.3 Chirality of 3-octanol

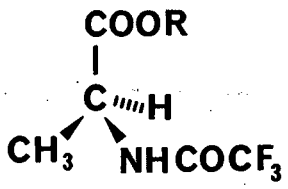
The olfactory perception mechanism of insects can often distinguish between optical isomers[29]. The various possibilities in insect behaviour towards the optical isomers were categorized in section 1.3.2. Mori[370] has recently divided or categorized the chiral pheromones on the basis of the relationship between their physiological activity and stereochemical structure. 3-octanol has been reported from a number of species of *myrmica* ants as a component of the alarm pheromone[360,368]. Depending upon the species, 3-octanol can act alone or synergistically with 3-octanone, to attract worker ants, increase their linear speed and decrease their sinuosity of movement[360,366]. As no information was available on the enantiomeric composition of 3-octanol from *Myrmica* ants, it was examined in the present study.

The isolation of sufficient amounts of pure pheromone components to perform an accurate determination of optical rotation by conventional methods is difficult or impossible. A number of alternative methods are available to examine the chirality of minute quantities of material. NMR spectroscopy has been used to determine optical isomeric composition of chiral pheromones either directly by the use of a chiral shift reagent[303-305] or indirectly by the preparation of a diastereomeric derivative and subsequent examination with an achiral shift reagent[303,305]. However, this method required at least 500 µg of pure material and highly sophisticated instrumentation. The chromatographic technique are preferred for enantiomer composition studies as they are more sensitive, needs less sophisticated instrumentation and can be applied even to impure biological samples. The direct resolution of enantiomers has been achieved by HPLC using either using a chiral stationary phase[243-247] or an optically active reagent in the mobile phase[248,249]. A recent example has used a chiral absorbent in TLC to separate enantiomers[251].

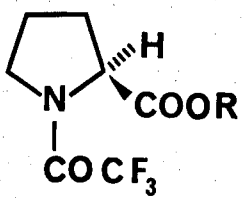
The direct resolution of enantiomers by GC has been achieved using optically active stationary phases[239]. An alternative to the direct separation of enantiomers, is the separation of a diastereomeric derivative formed with an optically pure derivatizing agent, on achiral stationary phases, which are less expensive and more widely available. Although a number of derivatives have been used for alcohols, only a few comparative data of the various methods that can be utilized were available. In this investigation three candidate methods were explored to find the most efficient and sensitive method to determine chiral composition of nanogram to microgram quantities of alcohols available from insects.

The three diastereomeric derivatives of 3-(±)-octanol prepared were the N-TFA-(S)-(+)-alanyl ester, the N-TFA-(S)-(-)-prolyl ester and the (+)-trans-chrysanthemoyl ester. Of these the N-TFA-(S)-prolyl ester was the most conveniently prepared as it was readily formed from highly optically

pure, commercially available N-TFA-(S)-(-)-prolyl chloride. A long time was required (3 days at room temperature) for the formation of the N-TFA-(S)-(+)-alanyl ester [70], which lessened its usefulness and a higher temperature resulted in partial recemization. The other two derivatives were formed much more readily. The N-TFA-(S)-(-)-prolyl ester [71] was produced in sufficient amounts for analysis in 10 min at 90 °C and (+)-trans-chrysanthemoyl ester [72] in 2 h at 40 °C.

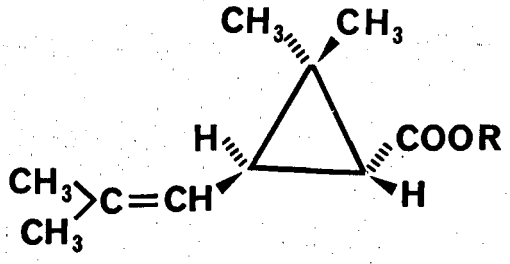


[70]



R = 3-octyl

[71]



[72]

The gas chromatography of the three derivatives of 3-(±)-octanol on the OV-1 capillary column (Table 28, Column G) resulted in baseline separation of the diastereomeric pairs with separation factors (α) of 1.03 or more (Table 27). The oil of Japanese peppermint (*Menthae japonicae*) was known to contain (S)-(+)-3-octanol enantiomer as a minor constituent[371,372].

Table 27. Comparison of GC properties of 3-(±)-octanol derivatized with different chiral resolving agents on an apolar (OV-1) capillary column.

Derivative	OV-1[a]					
	Retention time/min		α	R	Elution order	
	1st isomer	2nd isomer			1st isomer	2nd isomer
N-TFA-(S)-(+)-alanyl ester	2.7	2.8	1.03	1.2	R	S
N-TFA-(S)-(-)-prolyl ester	8.6	8.9	1.04	1.8	R	S
(+)-trans-chrysanthemate ester	8.3	8.6	1.03	1.9	S	R

[a] Analyses performed isothermally at 150 °C (OV-1).

A sample of (S)-(+)-3-octanol was isolated from the Japanese peppermint oil by preparative GC on the 10% PEG 20M column (Figure 44). The (S)-(+)-3-octanol thus obtained was used to determine the elution orders of the three racemic derivatives (Table 27). Gas chromatography showed that the 3-octanol from Japanese peppermint oil consisted of 100% (S)-enantiomer. This fact also showed that no recemization occurred during the derivatization.

The EI-MS of neither the N-TFA-alanyl nor prolyl esters of 3-octanol gave observable molecular ions. However, the EI-MS of 3-octyl chrysanthemate exhibited a clear molecular ion (m/z 280, 1%). The presence of the molecular ion in the chrysanthemate spectrum is particularly advantageous in the analysis ofenantiomers present in complex mixtures where assignment of diastereomers from their retention indices on chromatography is sometimes difficult. Therefore, (+)-*trans*-chrysanthemic acid was chosen as the most useful derivatizing reagent to study the naturally occurring 3-octanol from *Myrmica* ants.

Comparison of the gas chromatogram of 3-(S)-octyl chrysanthemate prepared from 3-(S)-octanol from oil of *Menthae japonicae* with that prepared from 3-(±)-octanol established the elution order as *S* followed by *R* on the OV-1 stationary phase. This result was consistent with the elution order of the chrysanthemates of 2-octanol on SE-30 stationary phase observed by other workers[293].

Co-chromatography of the 3-octanol from the *Myrmica* ants and the recemate as their chrysanthemate esters revealed that the ant alcohol was essentially the later-eluting *R* enantiomer. The Figure 45 illustrates the reconstructed ion chromatograms of octyl chrysanthemates. The mass spectra of the chrysanthemate esters of 3-octanol from all three source were identical [m/z 280 (M^+, 1%), m/z 168 ($C_{10}H_{16}O_2^+$, 5%), m/z 151 ($C_{10}H_{15}O^+$, 13%), m/z 123 ($C_9H_{15}^+$, 100%), m/z 107 (9%), m/z 93 (7%), m/z 81 (32%), m/z 71 (40%), m/z 69 (24%), m/z 57 (63%), m/z 55 (26%)].

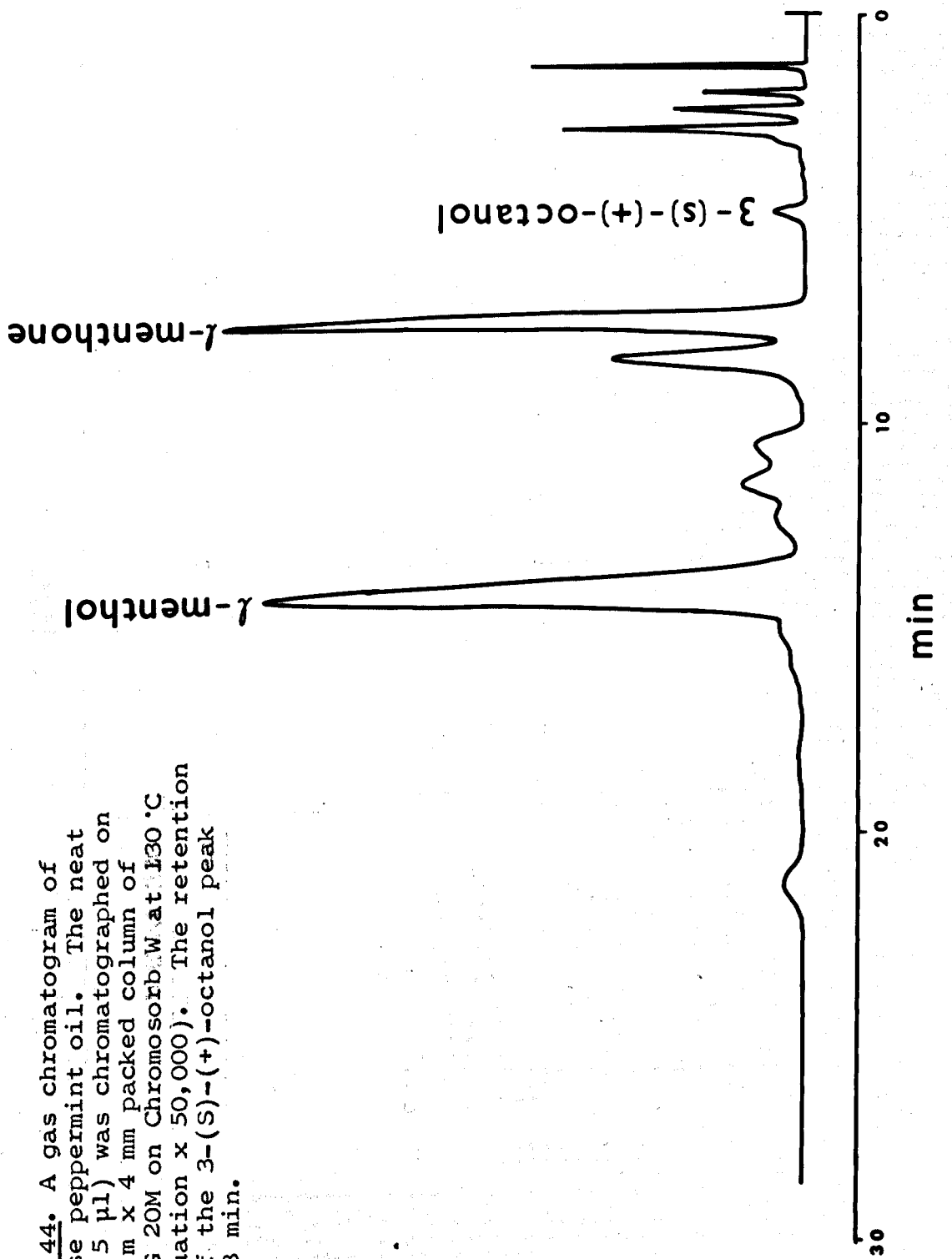


FIGURE 44. A gas chromatogram of Japanese peppermint oil. The neat oil (0.5 µl) was chromatographed on a 2.75 m x 4 mm packed column of 10% PEG 20M on Chromosorb W at 130 °C (attenuation x 50,000). The retention time of the 3-(S)-(+)-octanol peak was 4.8 min.

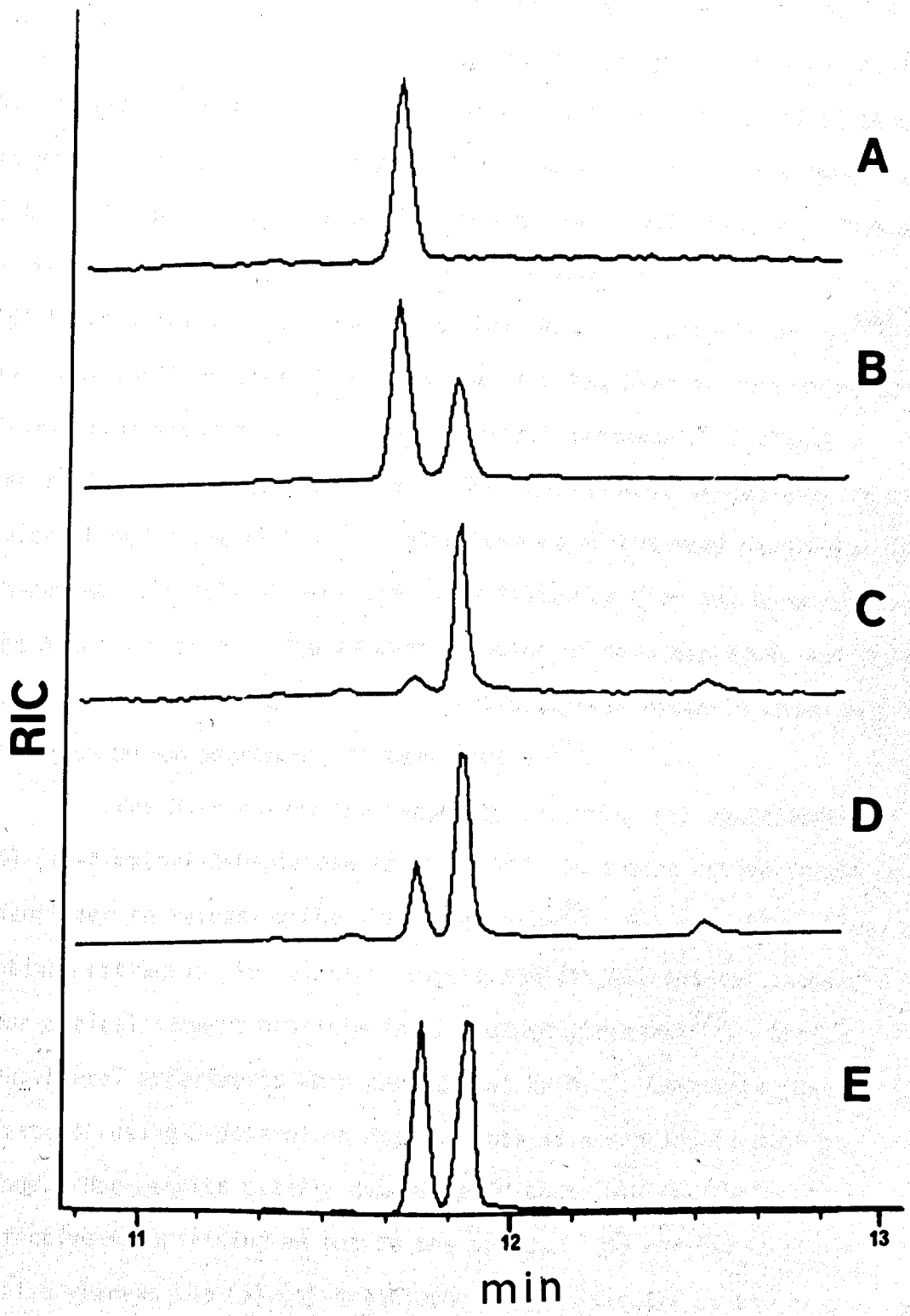


FIGURE 45. Reconstructed ion chromatograms (RIC) of 3-octyl-(+)-trans-chrysanthemates of 3-octanol. 3-Octanol from (A) oil of Menthae japonicae, (B) oil of menthae japonicae with commercial recemate, (C) Myrmica ants, (D) Myrmica ants with commercial recemate, and (E) commercial recemic mixture. Analysis was performed on a 20 m x 0.3 mm OV-1 capillary column at 150 °C.

The (S)-(+)-3-octanol had been reported as early as 1943[371], but this is the first report of (R)-(-)-3-octanol from a biological source. Silverstein[373] has reviewed the chiral insect pheromones and in those example so far reported, nearly always only one optical isomer or a specific blend of optical isomers occurs naturally, rarely the racemic mixture. The mandibular alarm pheromones of *Atta texana* and *A. cephalotes* in (S)-(+)-4-methyl-3-heptanone enantiomer only[29]. Pasteels *et al.*[198] found the absolute configuration of a pheromone from the head of another myrmicine ant *Tetramorium impurum* to be (3R,4S)-4-methyl-3-hexanol. Although a generalization on the fragments of information must be avoided, it will be interesting to see if the C-3 chiral centre of the many mandibular gland pheromonal alcohols of myrmicine ants listed by Blum and Hermann[70] are all of the *R* configuration. The *4S* configuration of *Atta* heptanone and *Tetramorium* hexanol (above) is also found in the 4-methyl-3-heptanols which are found in the aggregation pheromones of bark beetles[374].

For *Atta texana* the naturally occurring (S)-enantiomer of (S)-(+)-4-methyl-3-heptanone is about 100 times more active than the (R)-enantiomer in releasing the alarm behaviour[29]. For male flour beetles the optimal attraction is released only by the (4R,8R)-optical isomer, out of the four optical isomers possible for 4,8-dimethyldecanal[375]. Similar behavioural experiments were carried out by M. C. Cammaerts (University of Brussels) using 3-octanol on *Myrmica* ants as a continuation of the present study. Her results clearly demonstrated that (R)-(-)-3-octanol is highly effective in orienting an ant to the source. The racemate was partially active whereas the (S)-(+)-enantiomer was as inactive as the hexane control. The (R)-(-)-2-octanol also showed no significant activity in orienting the worker ants. The results are illustrated in Figure 46. Therefore it can be concluded that the correct configuration at the C-3 chiral centre and the position of the hydroxyl group alone the that (R)-(-)-3-octanol is highly effective in orienting an ant to the source. The recemate was partially

active whereas the (S)-(+)-enantiomer was as inactive as the hexane control. The (R)-(-)-2-octanol also showed no significant activity in orienting the worker ants. The results are illustrated in Figure 46. Therefore it can be concluded that the correct configuration at the C-3 chiral centre and the position of the hydroxyl group alone the chain are important for biological activity.

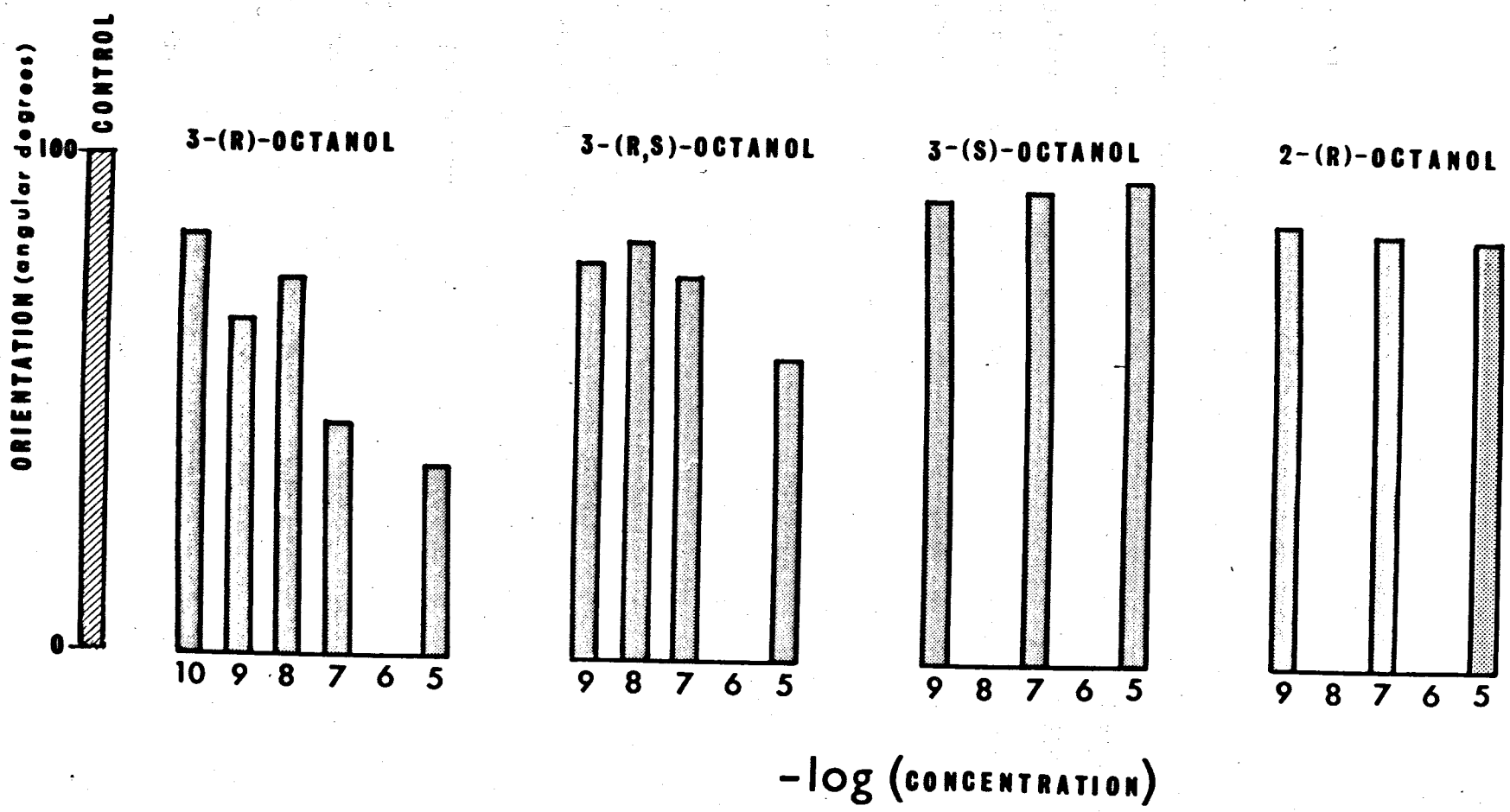


FIGURE 46. The orientation of ants towards a source of octanol. The orientation of Myrmica scabrinodis workers towards different concentrations of octanols were measured (angular degrees) by presenting the substances on a piece of filter paper (1 cm x 1 cm). An untreated paper was the control. The median values of orientation of 30 ants for each substance are represented in the vertical axis.

EXPERIMENTAL

3.1 General Procedures

3.1.1. Sources and collection of ant colonies

a. *Tetramorium caespitum*

Three large colonies (approximately 10,000 individuals/colony) were collected from Heartland moor in Dorset during August 1982. They were transported to the laboratory, separated from the soil using a pooter and transferred to artificial nests.

b. *Solenopsis geminata*

The ants were collected from Colombo, Sri Lanka and placed in a plastic bottle half filled with moist soil. The inner walls of the bottle were coated with polytetrafluoroethylene to prevent ants from escaping and the opening was closed with a piece of fine nylon netting. The colonies were transported within a few days by air as cabin luggage or air cargo.

c. *Myrmica rubra*

d. *Myrmica scabrinodis*

e. *Myrmica ruginodis*

These ants were collected from Chesterton, North Staffordshire. The nests were dug up and transported with the soil in polythene bags to the laboratory. In the laboratory the ants and brood were separated from the soil and placed in artificial nests.

f. *Myrmica sulcinodis*

A small colony was supplied by M. V. Brian and Mrs. J. C. Wardlaw from Furzebrook, Dorset.

g. *Myrmica rugulosa*

Two colonies were supplied by C. A. Collingwood (Leeds) and R. Cammaerts (Brussels). The Belgian colony originated from Brabant and Hainaut.

h. *Myrmica schencki*

A colony was supplied by M. Nielsen (Aarhus, Denmark).

i. *Myrmica albuferensis*

A small colony was collected by C. A. Collingwood from salt marshes of Albufera, Mallorca, Balearic Islands.

j. *Monomorium pharaonis*

A small colony was supplied by J. Edwards, Ministry of Agriculture, Slough.

3.1.2. Maintenance of ant colonies

a. *Tetramorium caespitum*

The ants were maintained in the laboratory at room temperature, in a wooden box (25 cm x 20 cm x 4 cm) with a sliding glass top, filled with moist soil and peat. The glass top was kept covered with a piece of black paper and the peat was moistened once a fortnight. Four holes (1 cm diameter) in the longest sides of the nest box provided exits and entrances for the foraging workers. The box was placed in an aluminium tray, serving as a foraging enclosure (60 cm x 45 cm x 7.5 cm) with its vertical walls coated with polytetrafluoroethylene (ICI, 'FLUON') to prevent ants from escaping.

The ants were fed on a diet of cheese, dessicated coconut, maggots, meal worm larvae (*Tenebrio molitor*), aqueous sugar solution (10% w/v) and Banks diet[376]. The Banks diet consited of 100 g of fried ground

beef, 3 whole eggs, 1 ml multiple vitamins, and 500 ml of fly pupae pureed in a blender and mixed with hot water (400 ml) and agar (15 g). This mixture was then poured into pans, cooled and cut into blocks for feeding. The mixture can be stored up to six months at -20 °C.

b. *Solenopsis geminata*

The ants were maintained in the animal house in a dark plastic bottle (7 cm o.d.) half filled with moist soil and peat. A glass tube (4 mm i.d.) pierced into the wall provided the exit. The lid was opened and the peat was moisturized once a fortnight. The nest was placed in plastic washing up bowl with its vertical wall coated with polytetrafluoroethylene. The plastic bowl was kept in an aluminium tray filled with water. The colony was maintained at 28-30 °C and 50-60% relative humidity. The ants were fed with a diet of brood from other ants, dessicated coconut, young locusts and 10% sugar solution.

c. *Myrmica species*

The *Myrmica* ants were maintained in the laboratory at room temperature in conical flasks (250 ml) filled with 1 cm of moistened plaster of Paris. Seven short lengths of glass tubing were connected to the bottom of the flask, four of which were connected to test tubes (10 mm x 80 mm) covered so as to provide darkened brood chambers. Two of the tubes served as exits from the nest whilst the other which opened below the level of the plaster provided a means of keeping the nest moist by adding water from time to time. The top of the nest was closed with a rubber bung. The ants were fed with a diet of meal worm larvae, maggots, Banks diet[376] and 10% sugar solution.

d. *Monomorium pharaonis*

The ants were maintained similar to *M. geminata* but kept at room

temperature in the laboratory.

3.1.3. Identification of ants

The species *M. rubra*, *M. scabrinodis* and *M. ruginodis* collected by A. B. A. were identified in the laboratory using the *Hand book for the Identification of British Insects*[377], by microscopic examination of the workers' antennal scapes, epinotal spines and petiole. S. geminata was tentatively identified by A. B. A. using the classification by Bingham[378], and C. A. Collingwood made the specific generic confirmation. The other ants in the study were identified by respective collectors and the identifications were confirmed by C. A. Collingwood to whom specimens were sent.

3.1.4. Gas liquid chromatography

a. *Instrumentation*

The analyses of the volatile constituents of the glands were carried out using the following gas chromatographs.

(i). Pye 104 series gas chromatograph fitted with dual flame ionization detectors (FID).

(ii). Pye Unicam PU 4500 chromatograph fitted with dual flame ionization detectors (FID).

(iii). Carlo-Erba HRGC Fractovap 4160 series gas chromatograph with split-splitless and on-column cold injection systems, and equipped with interchangeable flame ionization detector (FID-20) and nitrogen phosphorus detector (NPD-40).

b. *Columns*

The columns used in the investigations are listed in Table 28 together with their specifications.

Table 28. Specification of the columns employed in the GC analyses

Column	Material	Liquid phase	%Loading	Support	Mesh-range	Support treatment	Column dimensions length(m) x i.d.(mm)	
A	glass	SE-30	5	Chromosorb W	100-120	AW-HMDS	1.5	4
B	glass	OV-101	5	Diatomite M	100-120	AW-HMDS	1.5	4
C	glass	PEG 20M	10	Chromosorb W	100-120	AW	2.75	4
D	glass	PEGA	10	Chromosorb W	100-120	AW	2.75	4
E	glass	PORAPAK Q			100-150		1.5	4
F	glass	CHROMOSORB 102			100-120		1.5	4
G	fused silica	OV-1 [bounded]	**	***			25	0.32
H	glass	FFAP	15	Chromosorb W	100-120	AW-DMCS	1	4

** 0.4 μm thickness

*** capillary

c. *Preparation of column packings*

(i) Conventional packings

A known amount of liquid phase was dissolved in the solvent recommended by the manufacturers, in a ribbed "rotavapor"(Buchi, Switzerland) flask (500 ml). The solid support (approx. 10 g/m) of the stated mesh size was added slowly to the solution to form a slurry. The solvent was removed slowly under vacuum using a rotary evaporator. Hand turning of the flask was employed to ensure an even coating of the support with the stationary phase and to avoid bumping and excessive movement which would have caused the fragile support to fragment. Once dry and free-flowing, it was transferred to a fluidised bed drier and a steady stream of nitrogen was passed through the packing for six hours whilst maintaining the bed at approximately 100 °C. This completed the drying and freed the packing from any very fine particles. The inner surface of the glass column was deactivated by washing with either hexamethyldisilazane or dimethyldichlorosilane. The excess liquid was emptied and the column was dried at 120 °C for one hour. Columns were packed by applying a vacuum at one end through a column fitting, a silanized glass wool plug at this end ensured the retention of the particulate packing. The column was packed tightly by the application of minimum amount vibration. A 7 cm gap was left between the top of the column and the 1 cm silanized glass wool plug to accommodate the solid injector.

All the columns were conditioned for 12 h at 10 °C below the maximum operating temperature recommended for the particular phase, using a nitrogen carrier gas flow of 50 ml/min.

(ii) Porapak Q and Chromosorb 102 columns

Both Porapak Q (Waters Associates, Milford, Mass. U.S.A.) and Chromosorb 102 (Phase Separations Ltd., Clwyd, U.K.) columns were prepared by simply filling the deactivated glass columns with the powder polymer by the technique described in section (i).

(iii) Capillary columns

A "Crossbond" fused silica capillary column (25 mm x 0.32 mm) with OV-1 stationary phase of 0.4-0.45 mm film thickness was purchased from Carlo Erba Ltd. (Milan, Italy).

3.1.5. Dissection of glands and sample preparation for solid injection

The ants held with a pair of soft feather light tweezers (290 mm, Gallenkamp) and killed by momentarily immersing them in liquid nitrogen. The Dufour gland and poison vesicle with its two glandular filaments were removed by dissecting the ants in distilled water, under a Vickers Zoomax binocular microscope (magnification x 35). The ant was held by the petiole with one pair of fine forceps (Idealtek No.4, Trady's, Switzerland) and with another pair of forceps the final tergite and sternite were pulled apart to widen the opening for the sting. Finally the anterior portion of the gaster was pulled out by the final sternite. The hindgut, malpighian tubules, fat bodies and other muscular tissue were removed carefully until only the Dufour gland and the poison vesicle remained attached to the sting. One of the glands was removed carefully depending on which one was under study (even the sting lance was carefully removed when poison vesicles were studied), mounted on a small piece of glass and blotted dry with tissue paper. The small piece of glass containing the gland was pushed into the bottom of the small glass tube (25 mm x 1.8 mm) closed at one end and the open end was sealed in a micro-flame. As an alternative, if the glands are large enough, the glandular liquid can be drawn into a fine capillary (50-70 μm) by capillary action by piercing it and the capillary can be sealed in the larger glass tube.

3.1.6 Solid injection technique

The method described by Morgan and Wadhams [229] was employed. The sample to be analysed, such as insect tissue, dissected glands, glandular

contents extracted with a glass capillary (50-70 µm), whole heads or gasters, were sealed in a thin walled 35 mm x 1.8 mm glass tube. The glass tube was introduced into the solid injector (Figure 47), mounted in the modified injection port of either Pye 104 or Pye Unicam PU 4500 GC and allowed to heat to approximately 200 °C for 3 min. The tube was then crushed in the carrier gas stream by depressing the plunger in order to release the volatiles onto the packed GC column.

3.1.7 Trapping and rechromatography of GC effluent

An effluent splitter[228] was made from thick walled glass capillary tubing (6 mm o.d. and 0.4 mm i.d.), with a 6 mm metal screw sealed to the outlet (collector) end and a metal capillary fitted with a restrictor, sealed to the end for connection to the detector (Figure 48). The restrictor was a piece of fine wire inside the metal capillary, of such a diameter and length to give a 95:5 (outlet:FID) split ratio. The splitter was connected by a Swagelok union to the end of GC column. The outlet heater was usually maintained at 200 °C and the outlet kept closed with 6 mm Pye hexagonal coupling nut with a silicone rubber septum. The following methods were employed to trap and study the GC effluent.

a. *Trapping of microgram quantities*

Approximately 5 s before the desired GC peak or region under study emerged from the column, the outlet was opened and a new nut with a 25 cm x 1.5 mm o.d. (1 mm i.d.) U-shaped stainless steel tubing going through the silicone rubber septum was screwed in and the U-tube was cooled with a ethylacetate liquid nitrogen bath in a small Dewar flask. The trapped material was washed with a suitable solvent into a Reacti-vial for subsequent analysis by bioassay or reaction gas chromatography.

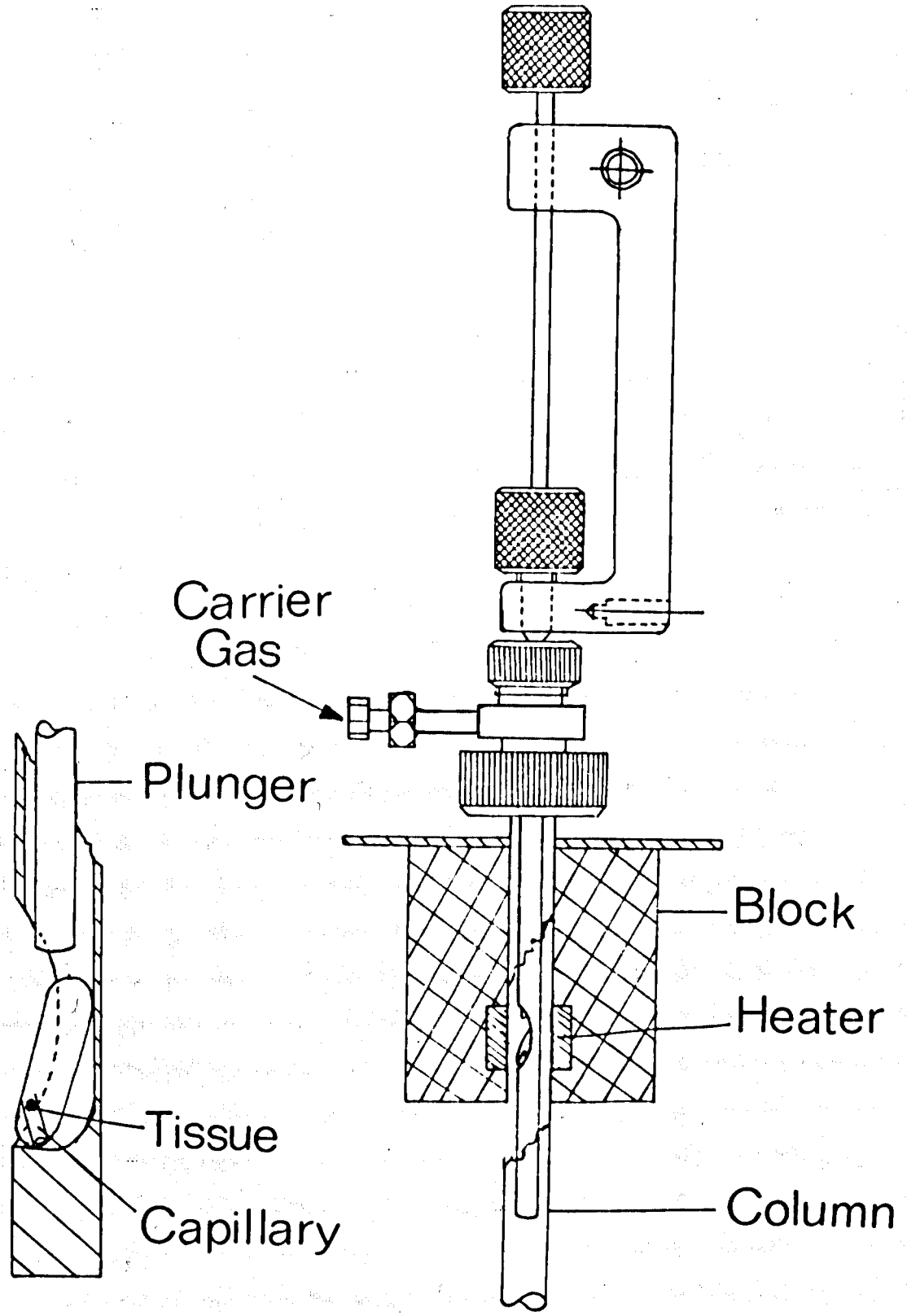


FIGURE 47. Solid sample injector (after Wadhams[365]).

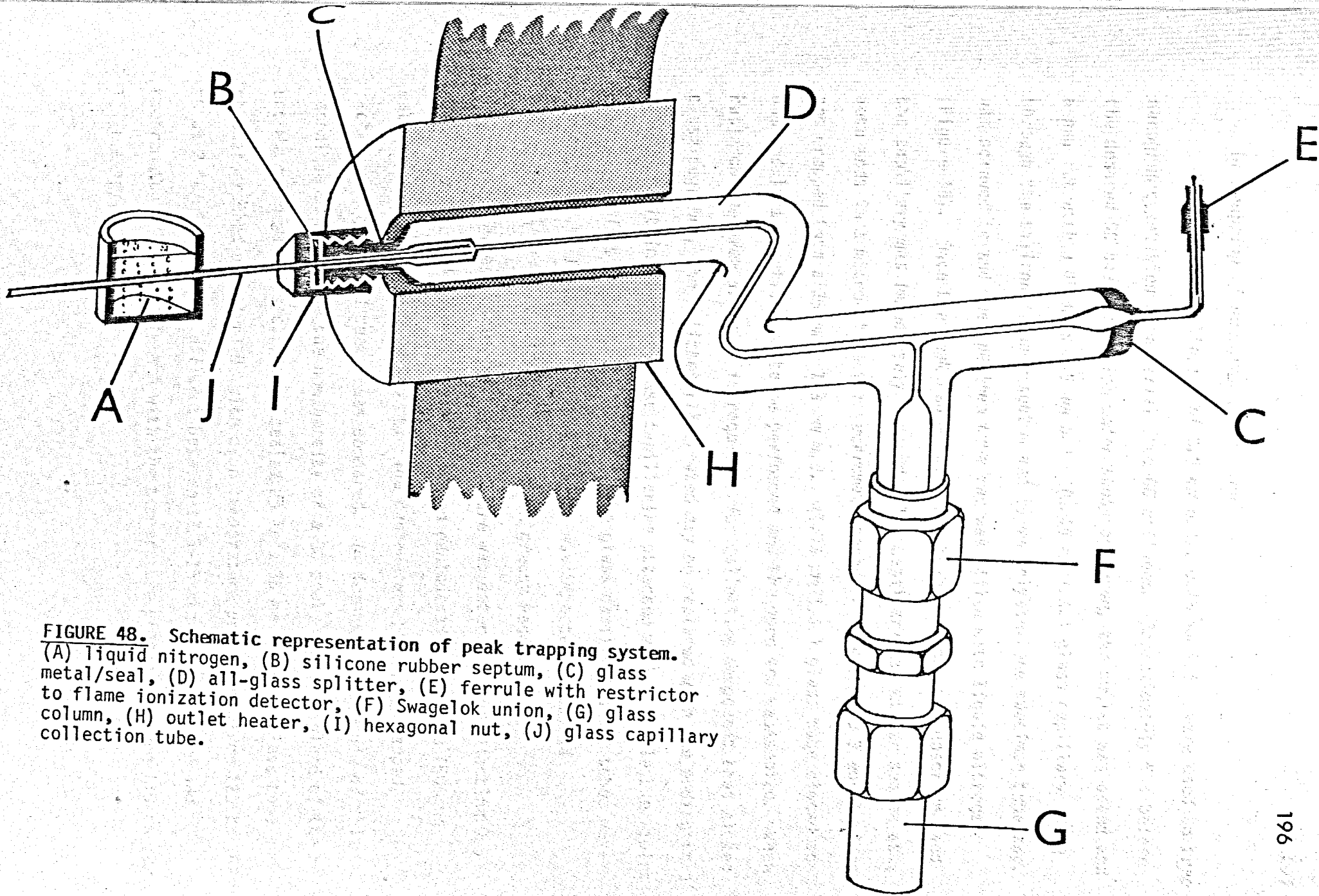


FIGURE 48. Schematic representation of peak trapping system. (A) liquid nitrogen, (B) silicone rubber septum, (C) glass metal/seal, (D) all-glass splitter, (E) ferrule with restrictor to flame ionization detector, (F) Swagelok union, (G) glass column, (H) outlet heater, (I) hexagonal nut, (J) glass capillary collection tube.

b. *Trapping of nanogram quantities*

Glass capillaries of 0.5 mm o.d., 0.45 mm i.d. were used to trap nanogram quantities of material from GC effluent. Approximately 5 s before the desired GC peak or region under study appeared, the outlet was opend and a new nut with a 45 mm x 0.5 mm o.d. (0.45 mm i.d.) glass capillary going through the silicone rubber septum and 15 mm polyethylene specimen tube cap, was screwed in and the specimen tube cap was filled with liquid nitrogen (Figure 48). Once the peak had eluted, the hexagonal nut was unscrewed and the capillary was immediately removed. The capillary was either sealed at both ends in a micro-flame for rechromatography or broken into 3 equal pieces and dropped into a 35 mm x 1.8 mm o.d. (1.75 mm i.d.) glass tube closed at one end if a reaction had to be performed and the open end was sealed. When rapid change-over of traps is required, for collecting compounds that eluted close together, the splitter illustrated may be replaced with one ending in a male Luer fitting and the glass capillaries attached with a female Luer joint holding a soft siliconerubber septum. The glass capillaries and tubes were baked at 230 °C for 30 min before use. It was important to avoid contamination of the tube by handling with greasy hands.

The efficiency of the procedure was determined by injecting 0.5 µl of standard solution of tetradecane and pentadecane in hexane (500 ng each/µl), onto column A at 145 °C. The pentadecane peak was trapped, rechromatographed and peak areas compared. To demonstrate the use of this technique to determine the homogeneity of a GC peak, a Dufour gland of the ant *M. rubra* was dissected out, sealed up in a 35 mm x 1.8 mm glass tube and chromatographed on column D, with oven temperature programmed from 125 to 162 °C at 2 °C/min, and the effluent between 13.5-15 min was collected and rechromatographed on column A with the temperature programmed from 140 to 192 °C at 4 °C/min.

3.1.8 Quantitative analysis

The quantity of material represented by a GC peak was determined by using the same or a similar compound as an external standard. Quantification was done either by peak area determination by triangulation (i.e. peak height x peak width at half height) or using a computing integrator (DP 101, Pye Unicam:Spectra Physics). The determination were usually made on ten replicate analysis of both unknowns and the standards, and the mean values used.

3.1.9 Gas chromatography-mass spectrometry

A Pye 104 gas chromatograph linked through a glass jet separator to an AEI MS 12 mass spectrometer was used with the following conditions. Trap current 10 μA; Electron energy 70 eV; Accelerating voltage 8 kV; Multiplier voltage 1.5 x 10 kV; source temperature 140 °C. The effluent from the GC column, before entering the glass jet separator, was split 90:10 (Mass spectrometer:FID) using a glass splitter. The collimating slits were kept wide open when maximum sensitivity was required. The carrier gas used was helium at a flow rate of 20 ml/min. When the system was operated at its maximum sensitivity, background spectra were recorded and subtracted manually from the total spectrum. The peak heights and the m/z values were fed into the University central computer and computer plots of the mass spectra were obtained.

3.2 Micro-Reaction Techniques Used With GC

The following reaction gas chromatographic techniques were employed to characterize microgram to nanogram quantities of volatile chemical compounds.

3.2.1 Epoxidation

Microgram quantities of alkenes either synthetic or from ant glands were directly trapped from the GC effluent in a solution of m-chloroperbenzoic acid in hexane (100 µl, 0.05 M). The GC effluent was split using an all-glass splitter [228] (Figure 48) with the outlet modified to end in a male Luer fitting instead of the metal screw. The desired GC peak was trapped by passing the effluent through a Luer needle (7 cm, 1 mm i.d.)bent at right angles, into the m-chloroperbenzoic acid solution in a micro-test tube cooled in liquid nitrogen and ethyl acetate. The micro-test tube (5 cm) was made from a Quickfit 5/13 neck. The needle was rinsed with the reagent solution (10 µl). Alternatively, microgram quantities of alkenes were trapped in metal U-tubes as in Section 3.1.7 and washed into a micro-test tube with the reagent solution (100 µl). The reaction solution was kept at room temperature for 10 min before the products were examined on either 10% PEG 20M column or OV-1 capillary column. (Table 28, columns C and G respectively)

Nanogram quantities of alkenes were directly trapped in glass capillaries (45 mm x 0.5 mm) as described in Section 3.1.7. The reagent solution (1 µl, 0.05 M) was injected with a 5 µl syringe (Scientific Glass Engineering, London), fitted with a 75 mm x 0.23 mm (o.d.) needle suitable for on-column injection, onto the trapped material in the glass capillary. The solution in the capillary was moved up and down and withdrawn back into the syringe. The solution in the syringe was immediately injected on a cold (40 °C) OV-1 capillary column and the products were investigated with the GC

oven temperature programmed from 40 °C to 300 °C at 10 °C/min.

The efficiency of the procedure was determined using hexane solutions (350 ng/µl) of (Z)- and (E)-6-pentadecene, (Z)- and (E)-7-pentadecene (synthesized by Miss Maria Bohan), (Z)- and (E)-8-heptadecene, (Z)- and (E)-9-nonadecene (synthesized by R.P.Evershed) and (Z)-9-tricosene (Aldrich).

3.2.2 Cleavage of epoxides

The epoxides not commercially available were synthesized from the corresponding alkenes by reacting the alkene with m-chloroperbenzoic acid. The Juvenile hormones were obtained from Sigma Chemical Co. (St.Louis, MO, U.S.A.). Periodic acid, purchased from Fluka (Switzerland), supplied as H_5IO_6 was dried to a constant weight in an evacuated drying pistol at 100 °C and ground to a fine powder in a mortar.

The cleavage of epoxides to corresponding carbonyl compounds was performed by the following methods.

a. *Cleavage in glass capillaries*

The epoxides were trapped separately (200-300 ng) into glass capillaries [45 mm x 0.5 mm (o.d.)] as described in Section 3.1.7, from the 10% PEG 20M column (Table 28 , column C). The trapping capillary was broken into 3 equal pieces and dropped into a 35 mm x 1.8 mm o.d. (1.75 mm i.d.) glass tube closed at one end which contained solid periodic acid (0.5 mg or less). The open end was sealed immediately and the tube was kept at room temperature for 3 min. This was reintroduced into the gas chromatograph with the solid injector and the products were identified either on 10% PEG 20M or Porapak Q column (Table 28, columns C and E respectively)

b. *Cleavage by pre-column packing*

A periodic acid pre-column packing (10% w/w) was prepared by

evaporating a solution of anhydrous periodic acid powder (100 mg) in absolute ethanol in contact with 5% OV-101 on 100-120 mesh Chromosorb W (1 g) in a rotating evaporator. When dry and free flowing it was packed in a glass tube between two silanized glass wool plugs and nitrogen (50 ml/min) was passed for 2 h at 200 °C for drying. Hexane (5 µl x 5) was injected onto it at 200 °C for conditioning. It was made into a pre-column packing (7 cm) between two silanized glass wool plugs on column C or E (Table 28). Epoxides trapped in glass capillaries (3.1.7), solutions, or crude reaction mixtures (alkene and m-chloroperbenzoic acid in dichloromethane) were injected onto the column and the carbonyl products formed were examined.

3.2.3 Ozonolysis

Ozone was generated to cleave the alkenes to corresponding carbonyl compounds using a micro-ozone generator[270,271] (Figure 49). Ozonolysis of the alkenes was performed either in solvent or in glass capillaries without the use of a solvent.

a. *Ozonolysis in solvent*

Microgram quantities of alkenes from the GC effluent were trapped separately into U-shaped metal tubing (3.1.7). The trapped material was washed out with carbon tetrachloride (x3, 30 µl) into a glass micro-test tube, cooled in ice. A fine stream of ozone (10 ml/min) through a fine glass capillary was passed directly into the solution for 3 min. (Figure 49). The presence of excess ozone was checked with a piece of wet starch iodide paper. The ozonides formed were reduced by the addition of a small crystal of triphenylphosphine. The products formed were analysed on 10% PEG 20M (Table 28, column C) or Chromosorb 102 (Table 28, column F) columns.

b. *Ozonolysis without solvent*

Nanogram quantities of alkenes were trapped in glass capillary

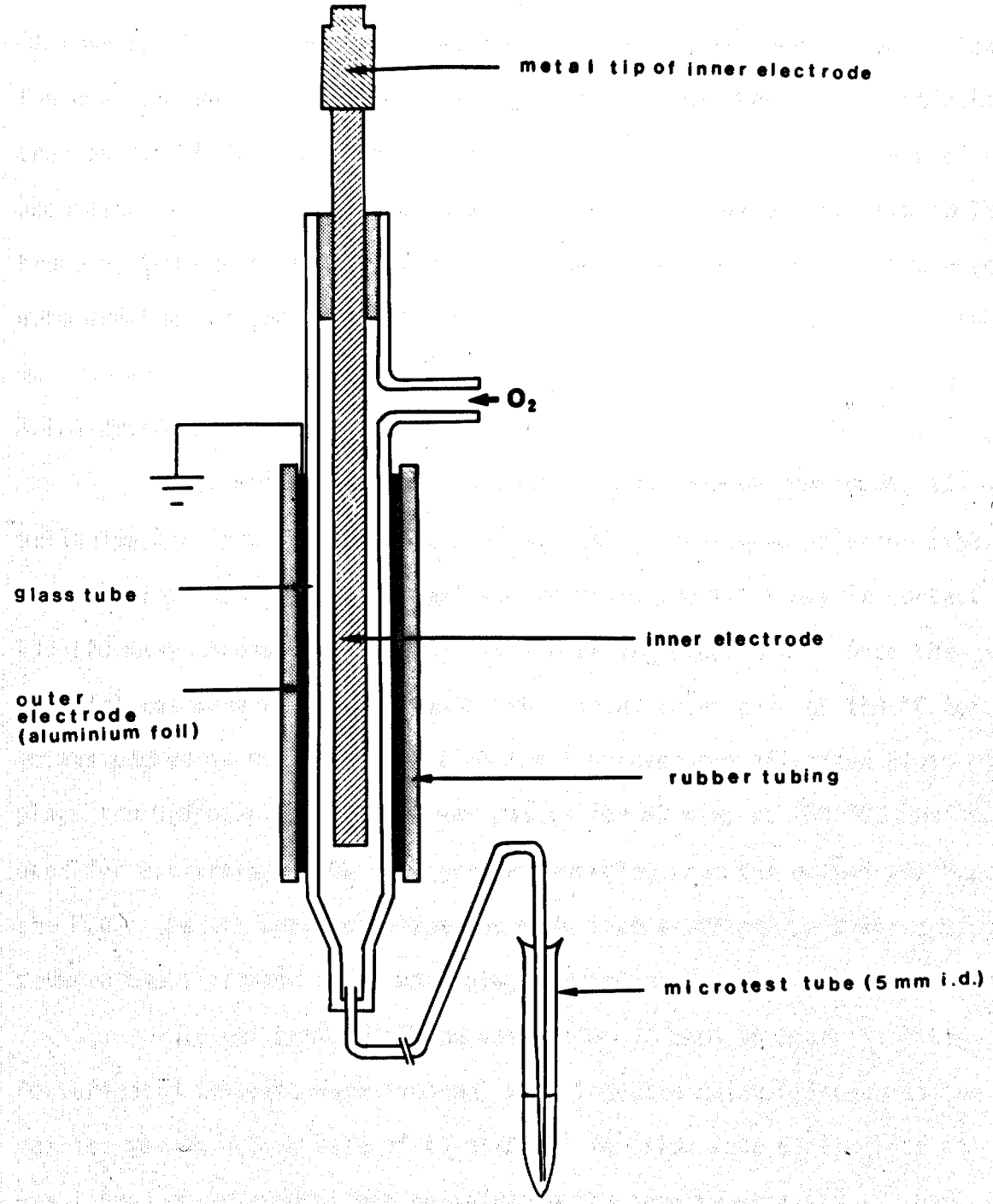


FIGURE 49. Ozonolysis Apparatus.
(after Beroza and Bierl[287])

tubing (3.1.7). The collection tube was broken into three equal pieces and dropped into a larger glass tube (35 mm x 1.8 mm) closed at one end. A fine stream of ozone (10 ml/min) was passed for 20 s, through a glass capillary (0.5 mm o.d.) extending to the bottom of larger glass tube cooled in ice. The open end was sealed immediately and the tube was kept in the injection area at 200 °C for 5 min before crushing for the pyrolytic cleavage of the ozonides. This was applied to a wide variety of known alkenes (Table 13) by trapping 200-300 ng samples from column C, and E (Table 28) and the products were examined on the same columns.

3.2.4 Hydrogenation

For microscale hydrogenation of unsaturated compounds, 1% palladium catalyst was prepared by evaporating an aqueous solution (150 ml) of palladium chloride (25 mg) and sodium hydroxide (11.2 mg) in contact with 100-120 mesh Chromosorb W (1.5 g) in a rotating evaporator. Once the material was free flowing it was further dried in an oven at 150 °C for 12 h. It was packed in a glass tube (4 mm i.d.) between two silanized glass wool plugs and hydrogen (40 ml/min) was passed for 60 min, at 200 °C, inside GC oven for activation. The hydrogen gas emerging from the column was burnt in the FID. The activated catalyst was made into a pre-column packing (6 cm) between two silanized glass wool plugs on column C or D (Table 28).

Either trapped alkene samples or alkenes in hexane solution (chlorinated solvents were avoided) were injected using hydrogen as the carrier gas at a flow rate of 40 ml/min. The flow rate of air into the FID was increased to 1200 ml/min to maintain the same sensitivity as when nitrogen was the carrier gas. The system was constantly checked for gas leaks. When hydrogenation was done on a micro-preparative scale for the subsequent analysis of the hydrogenated products by GC-MS, they were either trapped in metal U-tubes or in glass capillaries (3.1.7). The hydrogen from the end of the U-tube was vented.

3.2.5 Methoxymercuration-demercuration

Microgram quantities of alkenes trapped into metal U-tubes from the GC effluent (3.1.7) were washed separately with methanol (100 µl) into a special glass vial (Figure 10). Powdered mercuric acetate (0.1 mg) was added and the solution was shaken for 24 h in the dark. A minimum quantity of finely powdered sodium borohydride was added to the ice cooled solution until no more reaction was visible. The reaction mixture was made acidic with a few drops of glacial acetic acid followed by two drops of water. Ether (5 µl) was added and the mixture was thorougly shaken. The ethereal layer was pushed into the narrow neck region of the vial by adding water into the bottom chamber, using a syringe. The methoxy derivatives present in the ether layer were examined by GC on 10% PEG 20M column (Table 28, column C) and GC-MS.

3.2.6 Sodium borohydride reduction

Nanogram quantities of carbonyl compounds were trapped separately from the GC effluent from the 10% PEG 20M column (Table 28, column C) into glass capillaries (3.1.7). The collection tube was broken into three equal pieces and dropped into a glass tube (35 mm x 1.8 mm o.d.) closed at one end which contained finely powdered solid sodium borohydride (0.1 mg). The open end was sealed immediately in a micro-flame and the tube was kept for 15 min at room temperature. This was reintroduced into gas chromatograph kept for 5 min at 200 °C at the injection area before crushing the tube. The alcohol products formed were examined on the 10% PEG 20M column (Table 28, column C).

Alternatively, a solution of sodium borohydride in wet tetrahydrofuran (1 µl, 0.03 M) was injected onto the trapped carbonyl compounds in the glass capillary (3.1.7), using a capillary syringe (5 µl, Scientific Glass Engineering, London) fitted with a fine needle (75 mm x 0.23 mm o.d.). The capillary was kept for 10 min at room temperature. The syringe was rinsed several times with distilled water and the solution inside

the capillary tube was withdrawn back into the syringe and injected immediately on a cold (40 °C) OV-1 capillary column.

3.2.7 Bromination

Microscale bromination of glandular components was performed by adding bromine solution (0.5 µl, 1% v/v in carbon disulphide) to a cleanly dissected gland, mounted on a small piece of glass, placed inside a glass tube (35 mm x 1.8 mm o.d.) closed at one end. About 20 min time was allowed for the reaction to take place and excess bromine to evaporate. When there was no more visible colour of bromine present, the glass tube was sealed and introduced into the gas chromatograph.

Alternatively, nanogram quantities of material trapped from GC effluent into glass capillaries (3.1.7) were brominated by injecting a solution of bromine in hexane (1 µl, 5% v/v) onto the trapped material using a capillary syringe (5 µl, Scientific Glass Engineering, London) fitted with a fine needle (75 mm x 0.23 mm o.d.). The solution was withdrawn back into the syringe and injected immediately on a cold (40 °C) OV-1 capillary column. The gas chromatograms obtained were compared with those obtained without bromination.

3.2.8 Esterification with diazomethane

Microscale preparation of diazomethane was achieved by the method of Fales *et al.*[340]. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) (1 mg) was covered with water (100 µl) in a Reacti-vial (1 ml) cooled in an ice bath. Due care was taken in handing MNNG as it is a potent mutagen. The Reacti-vial was new and scratchless. Freshly distilled and anhydrous tetrahydrofuran (THF) (100 µl) was added on top of the aqueous layer and the vial was screw capped with a silicone rubber septum. A short piece of melting point tubing, flamed to smooth the ends was placed through the septum to prevent any possible build-up of back pressure. An aqueous KOH solution

(50%, 50 µl) was added with a syringe on MNNG. The yellow CH_2N_2 THF layer produced was withdrawn with a syringe fitted with a fine needle (0.23 mm o.d.).

The fatty acids were trapped in glass capillaries (3.1.7) from the 15% FFAP column (Table 28, column H). CH_2N_2/ in THF (1 µl) was injected onto the trapped material and the solution was withdrawn back into the syringe fitted with a fine needle (0.23 mm o.d.). The esters formed were examined by on-column injection on the OV-1 capillary column. The method was applied to investigate the esterfication of authentic samples (500 ng) of myristic, palmitic, stearic and benzoic acids.

3.3 Trail Pheromone of *Tetramorium caespitum*

3.3.1 Preparation of glandular extracts for bioassay

A tissue grinder was made from a Pyrex test tube (3 cm x 8 mm i.d.) and a piece of glass rod with a spherical knob at one end. The abrasive surfaces on the bottom of the test tube and on the spherical base of the rod were produced by grinding the two surfaces together with a slurry of carborundum and Teepol (detergent). The tissue grinder was baked in an oven at 230 °C for 2 h before use.

The ants were killed by exposing them to the cold vapour from liquid nitrogen. The poison glands and Dufour glands were dissected cleanly from the gasters as described in section 3.1.5. The glands were transferred with tweezers onto the wet surface of the base of the rod and macerated with a solvent such as hexane or acetone (100 µl). The extracts were kept ice-cold until further use.

3.3.2 Bioassay of trail following behaviour

The method of Pasteels and Verhaeghe[218] was employed to measure the trail following behaviour of ants towards the test solutions. A circle of 5 cm radius was drawn with a lead pencil on a piece of white paper (13 cm x 13 cm). The circumference of the circle was marked with arcs (1 cm). The solution under investigation (usually 25-100 µl) was injected into a Standardgraph (Blundel Harling, Dorset) 'S' funnel pen (0.8 mm) and a continuous streak was drawn on the circle. The solvent was allowed to evaporate for two min and the paper was placed in the foraging area of the ant nest. The number of arcs run along the trail by each individual worker ant was recorded for a specified period of time (usually 20 min). The median of the values thus obtained was used as a measure of activity.

The pens were thoroughly washed using a stream of acetone. This was done conveniently by placing the dismantled pen inside a syringe barrel

(5 ml) filled with acetone and applying low suction through the needle. A blank bioassay using solvent only was always performed before a test to ensure no residual activity was present in the pen.

3.3.3 Bioassay of glandular extracts

Two poison glandswere extracted into acetone (100 µl) as described in section 3.3.1. The trail bioassay was performed as section 3.3.2. The behaviour of the worker ants was observed for 20 min and the median of the number of arcs run was determined. A mean value was obtained by repeating the test three times. The activity of an extract of two Dufour glands was tested in a similar manner.

3.3.4 Thin layer chromatography

An extract of two cleanly dissected poison glands was made in distilled acetone (50 µl). The extract was applied to the origin of a silica gel layer (20 cm x 5 cm x 0.3 mm) on a glass plate and developed with hexane-acetone (60:40). The solvent front was allowed to run 15 cm. The plate was air dried and the silica was cut into ten bands (1.5 cm each). The bands were scraped separately into Pasteur pipettes plugged with glass wool. Each fraction of silica was extracted with acetone (100 µl) directly into a Standardgraph pen. The trail following activity evoked by each fraction was tested by the bioassay described in section 3.3.2. Blank bioassays using solvent only were performed before and in between each test to ensure no activity was present by contamination. The test was repeated in the same manner except only the region between 4.5-9 cm was scraped with a small spatula and width of each band was narrowed to 2 mm.

Similar experiments were performed to test for functional groups. Two poison glands were extracted separately in HCl in acetone (1%) and Br_2 in hexane (1%, v/v) respectively. The reaction mixtures were separated by TLC and the bioassays were carried out as before.

Samples of synthetic 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were chromatographed under the same conditions as above. The spots were visualized under the UV lamp and the R_f values were calculated.

3.3.5 Gas chromatography

Three poison glands were cleanly dissected without the Dufour gland and the sting and sealed in a glass tube (3.1.5). The contents of the tube was chromatographed via the solid injection method on the PEG 20M (Table 28, column C) at 130 °C. The ionization amplifier was used at attenuation x 50. A Dufour gland was chromatographed under the same conditions in order to distinguish any peaks that may arise as contaminants in the poison gland GC traces.

3.3.6 Trapping of GC effluent

Two poison glandswere injected onto the PEG 20M (Table 28, column C) at 130 °C. The effluent was split and collected in metal U-tubesas described in section 3.1.7. The collection tubes were changed at 1 min intervals. The trapped material was directly washed with acetone (50 µl) into Standardgraph pens and the activities of various fractions were bioassayed as before (3.3.2).

3.3.7 Gas chromatography-mass spectrometry

The poison glandsof 50 ants were cleanly dissected and sealed in a glass tube (3.1.5). The mass spectra of the two major components of the poison gland were obtained by GC-MS as described in section 3.1.9. A low bleed 5% SE-30 column (Table 28, column A) was used at 130 °C. The collimating slits of the MS were kept wide open and the multiplier voltage was operated at 3 x 10 kV. Background spectra were recorded before and after the peaks of interest and subtracted from the total spectra. The mass

spectra of synthetic 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, ethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were obtained under the same conditions using solutions in hexane (500 ng/µl).

3.3.8 Quantification of glandular components

The poison glands of ten worker ants were dissected cleanly without the Dufour gland and the sting and sealed separately in glass tubes (3.1.5). The contents of the tubes were chromatographed by solid injection (3.1.6) on the PEG 20M (Table 28, column C) at 130 °C. A computing integrator was employed to calculate the absolute quantities of material using a solution of 2,5-dimethylpyrazine (510 ng/µl) as an external standard (3.1.8).

3.3.9 Bioassay of synthetic substances

Mixturesof 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine in hexane (to give a total of 4 ng per sample) were made separately in 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 proportions. The mixtures were used to lay circular trails as described in section 3.3.2. The trails were presented in the foraging area and the number of arcs run along the trail by each worker ant were recorded for 20 min and the median values were obtained. The means of the median values were calculated after repeating the experiment three times. Blank tests were carried out between each test using hexane only to avoid any cross-contamination of activity. Similarly the activity of one poison gland equivalent of material on a trail was tested for comparison.

The activities of the following compounds to evoke trail following behaviour were tested by bioassay by presenting 4 ng/31.4 cm trail. The compounds tested were 2-ethylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 3-ethyl-2,5-dimethylpyrazine and methyl

4-methylpyrrole-2-carboxylate. The ant colonies used to test the activity were *Tetramorium caespitum*, *T. impurum*, *Myrmica rubra*, and *M. ruginodis*.

The determination of thereshold concentrations at which the worker ants display trail following reactions were performed by presenting them a range of concentrations of synthetic trail substances. The 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were presented separately at 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2} and 10^{3} ng per trail and activities were bioassayed.

To determine whether the 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine act in synergy, a bioassay was carried out. First, using the mixture at 4 ng total/31.4 cm trail level. Then 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were presented separately at 2.8 ng/trail and 1.2 ng/trail levels respectively.

3.4 Chemical Investigation of *Solenopsis geminata*

3.4.1 Sample preparation

The ants were killed by momentary immersion in liquid nitrogen. Either dissected single glands, capillary extracts or individual heads were sealed in small glass tubes (35 mm x 1.8 mm) for subsequent GC analysis. When Dufour glands were cleanly dissected, due care was taken to avoid contamination from the poison gland and sting because large quantities of alkaloids from the latter interfered with the analysis of the Dufour glands. Whole heads were used for the general study of the postpharyngeal glands. When necessary the postpharyngeal glands were excised in water, under the microscope by breaking the cuticle and withdrawing the yellow mass over the brain with tweezers.

3.4.2 Postpharyngeal gland substances

a. *Gas chromatography*

Individual heads or dissected glands were solid injected on the SE-30 (Table 28, column A). The GC oven temperature was programmed from 147 to 260 °C at 3 °C/min. Pieces of cuticle, mandibular glands, various other parts of the head and thorax were also examined for comparison. Quantification of peaks was done using a solution of hexadecane in hexane (770 ng/µl) as an external standard.

b. *GC-MS*

The mass spectra of the three major hydrocarbons of the postpharyngeal gland were obtained by GC-MS using a SE-30 column as described in section 3.1.9.. A single head was solid injected on the column and the GC oven temperature was programmed from 166 to 232 °C at 4 °C/min. The mass spectra of authentic heneicosane, tricosane and (Z)-9-tricosene were also taken under the same conditions.

c. *Double bond position of tricosene*

The tricosene peak obtained from the gas chromatography of a single head of *S. geminata* was collected into a metal U-tube (3.1.7). The trapped material was washed with methanol (50 µl) into a special glass vial (Figure 10). The material thus collected was methoxymercurated and demercurated by the procedure described in section 3.2.5 to yield the methoxy derivatives of tricosene. The product was extracted with ether (5 µl) and the mass spectrum was obtained by GC-MS using the ether extract (1 µl).

d. *Configuration of 9-tricosene*

Argentation-TLC was employed to determine the configuration of 9-tricosene from the postpharyngeal glands. the tricosene peak obtained from the gas chromatography of two heads of *S. geminata* was collected as described in section 3.1.7. The trapped material was washed with hexane (50 µl) into a Reacti-vial (0.33 ml) and applied to the origin of a 10% $AgNO_3$-silica gel layer (20 cm x 5 cm x 0.3 mm). It was developed with diethylether (1% v/v) in light pertroleum (B. Pt. 40-60 °C). The spots were visualized by spraying with 10% H_2SO_4 and heating in an oven at 120 °C for 10 min. The authentic (Z)-9-tricosene (Aldrich) and a mixture of synthetic (Z)-and (E)-9-nonadecene were also chromatographed under the same conditions.

e. Ethological activity of (Z)-9-tricosene

(Z)-9-tricosene (1 µg) was applied to a piece of white paper (1 cm x 1 cm) and placed in the foraging area of *S. geminata*. The behaviour of the worker ants towards the piece of paper was observed for 20 min.

3.4.3 Poison gland substances

a. *Gas chromatography*

The poison glands were cleanly dissected as described in section 3.1.5. The individual glands were gas chromatographed on either the 10% PEG 20M column (Table 28, column C) at 185 °C or the 5% SE-30 column (Table 28, column A) at 190 °C, using the solid injection technique(3.1.6). The

authentic samples of *cis*-and *trans*-2-methyl-6-undecylpiperidine (synthesized by A. P. Billington) were also chromatographed under the same conditions. For the examination of the trace constituents, a poison gland was ground and extracted with hexane (5 µl) and the solution was chromatographed (0.5 µl) on the OV-1 capillary column with the oven temperature programmed from 40 to 300 °C at 10 °C/min. The components were quantified using a solution of 2,5-dimethylpyrazine in hexane (511 ng/µl) as an external standard (3.1.8).

b. *GC-MS*

The mass spectra of the two major components of the poison gland were obtained by GC-MS using the SE-30 column (Table 28, column A) as described in section 3.1.9. One poison gland was solid injected on the column at 170 °C and the mass spectra of the two peaks and the valley in between were recorded. The spectra of authentic samples were also recorded under the same conditions.

3.4.4 Dufour gland substances

a. *Gas chromatography*

A mixture of boric acid (20%, w/w) and 5% SE-30 on Chromosorb W (100-120 mesh) was prepared for a pre-column (12 cm x 4 cm) for the subtraction of piperidines, used with either the 5% SE-30 column (Table 28, column A) or 10% PEG 20M column (Table 28, column C). The Dufour glands were solid injected (3.1.6) on the SE-30 column at 190 °C (attenuation x50) or on the PEG 20M column at 185 °C (attenuation x50). The Dufour gland extracts in hexane (5 µl) were also examined by on-column injection (0.5 µl, 40 °C) on the OV-1 capillary column (Table 28, column G) and the oven temperature was increased from 40 to 300 °C at 10 °C/min.

b. *GC-MS*

The mass spectra of the major components of the Dufour gland were obtained by GC-MS using the SE-30 column (Table 28, column A) as described in section 3.1.9. Twelve Dufour glands were solid injected on the column and GC

oven temperature was programmed from 166 to 232 °C at 4 °C/min. The mass spectra of authentic heptadecane, heneicosane and (Z)-9-tricosene were also taken under the same conditions.

c. *Bioassay of trail following behaviour*

The glandular extracts in acetone were bioassayed in the same manner as described for *Tetramorium caespitum* in section 3.3.2. A cross-activity study was carried out using Dufour gland extracts of *Solenopsis geminata*, *Myrmica scabrinodis* and *Monomorium pharaonis*. A solution of synthetic faranal (gift of Prof K. Mori) was also tested by bioassay (4 ng/31.4 cm trail) on the above three species of ants.

d. *Trapping of GC effluent*

Two Dufour glands were injected onto either SE-30 or PEG 20M columns at 190 or 185 °C respectively. Fractions of the GC effluent were collected separately in metal U-tube as described in section 3.1.7. The trapped material was washed with hexane (50µl) and the solutions were made upto 5 ml with hexane. The activity of each solution was bioassayed separately using an aliquot (50 µl) by the method described in section 3.3.2.

e. *Micro-reactions*

The total contents of a Dufour gland were brominated as described in the section 3.2.7. The hydrogenation of the Dufour gland contents was performed by the method described in the section 3.2.4. The products of both reactions were examined on both SE-30 and PEG 20M columns. The total effluent of the reaction products were collected and tested for activity by bioassay as described in the section 3.3.2. The tricosene peak was collected (3.1.7) from 5 Dufour glands and ozonolyzed as described in section 3.2.3.

f. *Thin layer chromatography*

An extract of two Dufour glands was made in hexane (500 µl). An aliquot of the extract (50 µl) was applied to the origin of a silica gel layer (20 cm x 5 cm x 0.3 mm) and the plate was developed with hexane. The solvent front was allowed to run for 15 cm. The silica was cut into ten

bands (1.5 cm each) and extracted with hexane. The activity of each band was tested as described in the section 3.3.4. A Dufour gland extract treated with Br_2 in hexane (100 µl, 1% v/v) was tested in the same manner. A sample of synthetic α-farnesene was also chromatographed under the same conditions for comparison.

3.5 Dufour Gland Substances of the Genus *Myrmica*

3.5.1 Sample preparation

The ants were killed by momentary immersion in liquid nitrogen. The Dufour glands were removed as described in section 3.1.5. Either dissected single glands, capillary extracts or whole gasters were sealed in small glass tubes (35 mm x 1.8 mm) for subsequent GC analysis by solid injection (3.1.6).

3.5.2 Gas chromatography

The following GC conditions were used to study the Dufour gland constituents of the four *Myrmica* species under investigation. Unless otherwise stated nitrogen was used as the carrier gas at a flow rate of 50 ml/min.

a. *Myrmica sulcinodis*

(i) 5% w/w OV-101 silicone (Table 28, column B) with GC oven temperature programmed from 140 to 192 °C at 4 °C/min.

(ii) 10% w/w PEGA (Table 28, column D) with GC oven temperature at 127 °C isothermal or programmed from 125 to 162 °C at 2 °C/min.

(iii) Porapak Q (Table 28, column E) at 170 °C isothermal.

b. *Myrmica rugulosa*

(i) 5% w/w OV-101 silicone (Table 28, column B) with GC oven temperature programmed from 130 to 192 °C at 4 °C/min.

(ii) 10% w/w PEGA (Table 28, column D) with GC oven temperature programmed from 120 to 160 °C at 2 °C/min.

(iii) Porapak Q (Table 28, column E) at 170 °C isothermal.

c. *Myrmica schencki*

The columns and conditions were the same as for *M. rugulosa*.

d. *Myrmica albuferensis*

(i) 10% w/w PEGA (Table 28, column D) with GC oven temperature programmed from 120 to 160 °C at 3 °C/min.

3.5.3 Gas chromatography-mass spectrometry (GC-MS)

The identification of all major components in the Dufour gland contents was confirmed by GC-MS (3.1.9). Five Dufour glands of the species under study were cleanly dissected and sealed in a small glass tube (3.1.5). The glass tubes were solid injected (3.1.6), separately, using the same columns and conditions (3.5.2) employed for quantification of the components.

3.5.4 Analytical evidence for structure assignment of GC peaks

a. Bromination

The glandular components were brominated as described in section 3.2.7 and the products were examined under the same conditions used for identification and quantification of the compounds (3.5.2).

b. Ozonolysis

The Dufour glands were cleanly dissected from the ants (3.1.5), gas chromatographed using the solid injection technique (3.1.6) and the alkenes of interest were trapped separately (3.1.7). The alkenes were ozonolyzed and the products were examined on the 10% PEG 20M column (Table 28, column C). The carbonyl compounds formed were identified by the comparison of the retention times and mass spectra with those of respective authentic samples.

c. Hydrogenation

The individual, cleanly dissected Dufour glands were injected on onto the 10% PEGA column (Table 28, column D), containing a pre-column packing (6 cm) of 1% palladium catalyst (3.2.4) at 127 °C isothermal. Hydrogen was employed as the carrier gas with the flow rate adjusted to produce the same retention times for the saturated alkene peaks as when using nitrogen as carrier.

3.5.5 Determination of glandular dimensions

The dimensions of the glands were determined using a scale engraved on glass reading to 0.1 mm. The glands were placed in a drop of water on a microscopic cover slip (18 mm x 18 mm) placed over the scale. The glands were examined under the microscope (3.1.5) and the dimensions were recorded.

3.6 Structures of Farnesenes

3.6.1 GC-MS of farnesenes

The total volatile material of 5 gasters of *M. scabrinodis* were examined on the 10% PEG 20M column (Table 28, column C) at 160 °C and the mass spectra of the three major components present, were obtained by GC-MS (3.1.9).

3.6.2 Ozonolysis of farnesenes

Farnesene (4 µg), homofarnesene (10 µg) and bishomofarnesene (10 µg) were collected separately in metal U-tubes (3.1.7) by solid injecting (3.1.6) 20 gasters of *M. scabrinodis* on 10% PEGA column (Table 28, column D) at 140 °C. The collected farnesenes were washed separately with carbon tetrachloride (x3, 30 µl, preparative GC-purified) into glass micro-test

tubes and ozonolyzed (3.2.3, Figure 49). The carbonyl products formed were examined on the 10% PEG 20M column (Table 28, column C) at 140 °C (attenuation x 200) and the Chromosorb 102 column (Table 28, column F) at 160 °C. The mass spectra of the carbonyl products formed were obtained by GC-MS (3.1.9) using the same columns.

A sample of synthetic (Z,E)-and (Z,Z)-α-farnesene (synthesized by Miss Lorna Thompson) and (E)-or (Z)-nerolidol (Koch-Light) were also ozonolyzed and the products were examined similarly for comparison.

Alternatively farnesene (200 ng), homofarnesene (500 ng) and bishomofarnesene (500 ng), from one cleanly dissected Dufour gland of *M. scabrinodis* were separately trapped from the 10% PEGA column (Table 28, column D) at 140 °C into glass capillaries (3.1.7b). The trapped materials were ozonolyzed separately using the solvent less ozonolysis technique (3.2.3) and the products were examined as described for ozonolysis in solvent.

3.6.3 Hydrogenation of farnesenes

The total volatile material from 20 whole gasters was hydrogenated on the 10% PEGA column, at 140°C, (Table 28, column C) containing a pre-column packing (6 cm) of 1%, palladium catalyst (3.2.4). Hydrogen was used as the carrier gas at a flow rate of 40 ml/min. The total hydrogenated effluent was collected in metal U-tubes (3.1.6a)

The collected mixture of farnesenes was taken up in hexane (25 µl) for subsequent GC-MS examination on 10% PEG 20M column (Table 28, column C) at 160°C.

3.7 Mandibular Gland Substances of Genus *Myrmica*

3.7.1 Myrmica albuferensis

The worker ants were killed instantaneously, by holding them with a pair of soft feather light tweezers (290 mm, Gallenkamp) and momentary immersion in liquid nitrogen, to ensure that the ants did not become too disturbed. The heads were separated from the bodies and sealed in a thin walled glass tube (20 mm x 1.8 mm o.d.). The samples were introduced into the gas chromatograph by the solid injection technique (3.1.5). The tubes were kept at 200 °C at the injection port for 3 min before crushing. The mandibular gland contents were analysed on 10% PEG 20M (Table 28, column C) at 130°C isothermal. The quantification of individual chemical components was achieved as described previously (3.1.8).

3.7.2 Myrmica rubra queens

A large number of heads of *M. rubra* queens was supplied by Mme. M.C. Cammaerts (University of Brussels, Belgium). The amounts of 3-octanol and 3-octanone present in the mandibular glands were determined by the solid injection of the individual heads (3.1.5) on the 10% PEG 20M column (Table 28, column C) at the 93 °C isothermal.

3.7.3 Reduction of 3-octanone

The 3-octanone from mandibular glands was trapped into a glass capillary (3.1.7) by the injection of an individual head of *M. rubra* onto 10% PEG 20M column at 130 °C. The reduction of the 3-octanone was performed by solid sodium borohydride as described in section 3.2.6, without using a solvent. The 3-octanol product formed was examined on the same column under the same conditions. Alternatively the trapped 3-octanol in the glass capillary was reduced using a solution of sodium borohydride in tetrahydrofuran (3.2.6) and the products were examined on the OV-1 capillary

column (Table 28, column G).

3.7.4 Isolation of 3-octanol

3-Octanol was collected separately from the mandibular glands of the ants, *M. rubra*, *M. ruginodis* and *M. scabrinodis*. The ants were killed by momentary immersion in liquid nitrogen. The individual heads were separated and 30 heads were sealed in a small section of soda glass capillary tubing (35 mm x 1.8 mm). The vial was kept for 5 min in the injection port at 200 °C and injected (3.1.6) onto 10% PEG 20M column (Table 28, column C) at 120 °C. The 3-octanol peak was collected in a metal U-tube (3.1.7). The material collected was washed with dichloromethane (x2, 50µl) into a Reacti-vial (0.33 ml) containing anhydrous magnesium sulphate (1 mg). The supernatent liquid was withdrawn and used for derivatization.

Optically pure (S)-3-octanol was collected from oil of Japanese peppermint (*Menthae japonicae*) (Maruishi Seiyaku, Tokyo) in the same way by injecting (5 µl x 5) samples on the PEG 20M column (Table 28, column C) at 120 °C, and the small octanol peak (Figure 44) was collected (3.1.7).

3.7.5 Preparation of N-trifluoroacetyl-(S)-alanyl esters

N-Trifluoroacetyl-(S)-alanyl chloride was prepared by a variation of the method of Souter[379]. (S)-Alanine (100 mg, Aldrich) in a dry, glass stopped flask was cooled in an ice bath and completely dissolved in trifluoroacetic anhydride (1 ml, Aldrich) by occasional shaking. The excess trifluoroacetic anhydride was evaporated by a stream of dry nitrogen and freshly distilled thionyl chloride (1 ml) was added to the chilled flask. The excess thionyl chloride was evaporated under dry nitrogen and the residue was dissolved in dichloromethane (500 µl).

3-Octanol in dichloromethane (50 µl) was added to N-trifluoroacetyl-(S)-alanyl chloride solution (50 µl) in a Reacti-vial (0.33 ml). The vial was stoppered and kept for three days at room temperature and

the products were examined on the OV-1 capillary column (Table 28, column G) at 150 °C isothermal.

3.7.6 Preparation of N-trifluoroacetyl-(S)-prolyl esters

The N-trifluoroacetyl-(S)-prolyl ester of 3-octanol was prepard by a variation of the method of Halpern and Westley[380]. 3-Octanol in chloroform (50 µl) was placed in a Reacti-vial (0.33 ml) and N-trifluoroacetyl-(S)-prolyl chloride (15 µmol) in chloroform (100 µl) (Regis, Chicago, IL, U.S.A.) and pyridine (10 µl) were added. The sealed vial was heated (90 °C, 10 min), when cool, hydrochloric acid (100 µl, 1 M) was added and shaken. The lower organic layer was separated, dried over sodium sulphate and examined by GC on OV-1 capillary column (Table 28, column G) at 150 °C.

3.7.7 Preparation of (+)-*trans*-chrysanthemate esters

The 3-octanol was chrysanthemoylated by a previously described method[293]. Typically, (+)-*trans*-chrysanthemic acid (gift of Prof C. J. W. Brooks) (2 mg) was treated (60 °C, 1 h) with freshly distilled thionyl chloride (200 µl, BDH). Excess thionyl chloride was removed in a stream of dry nitrogen. The alcohol in toluene (50 µl, sodium dried) was treated with chrysanthemoyl chloride (three molar proportions) in toluene (50 µl). The ester was purified by TLC (silica gel G, using diethyl ether-hexane, 96:4, R_f same as methyl palmitate) and examined on the OV-1 capillary column (Table 28, column G) at 150 °C.

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APPENDIX I

The amount of 3-octanone and 3-octanol in the mandibular glands of *Myrmica rubra* queens

Nest	Replicate number	3-octanone (ng)	3-octanol (ng)
G	1	-	15.8
	2	trace	66.3
	3	-	21.3
	4	-	13.4
	5	3.6	207.5
	6	-	43.4
H	1	17.0	276.9
	2	13.4	246.2
	3	4.3	94.7
	4	11.8	306.1
	5	6.7	177.5
	6	-	2.4
	7	3.2	166.7
	8	4.3	190.6
	9	3.6	99.4
	10	36.7	453.7
	11	6.7	184.6
	12	13.0	145.2
	13	26.8	295.1
	14	-	3.9
	15	45.0	411.1
	16	2.0	34.7
	17	26.0	293.5
	18	7.9	206.1
	19	1.6	159.8
	20	6.3	175.2
	21	9.4	134.1
I	1	115.2	813.7
	2	1.6	34.7
	3	-	-
	4	0.4	18.1
	5	0.8	53.7
	6	-	5.5
	7	18.9	216.5
	8	1.6	45.0
	9	-	3.2
	10	-	5.5
	11	-	21.3
	12	28.0	96.3
	13	1.2	66.3
	14	1.6	79.7
	15	18.1	469.5
	16	1.2	23.7
	17	39.8	201.2
	18	106.5	447.4
	19	8.7	80.5
	20	7.9	95.5
	21	trace	26.8

APPENDIX I (continued)

Nest	Replicate number	3-octanone (ng)	3-octanol (ng)
J	1	0.4	27.2
	2	-	15.0
	3	6.3	107.3
	4	65.1	423.1
	5	11.4	299.8
	6	9.9	90.7
	7	2.8	96.3
	8	-	22.9
	9	8.7	85.6
	10	1.6	51.3
	11	12.6	189.4
	12	4.7	36.3
	13	49.7	258.8
	14	-	7.1
	15	3.9	195.7
	16	0.2	18.9
	17	7.9	132.6
	18	31.6	301.4
	19	88.4	494.7
	20	24.5	268.7
	21	90.3	430.0
	22	46.2	456.1
	23	14.6	202.8
	24	7.9	98.6
	25	1.6	89.8
	26	5.5	26.0
	27	17.8	343.2
	28	9.9	142.8
	29	71.4	383.5
	30	3.2	62.3
	31	3.2	184.6
	32	73.0	239.1
	33	4.7	49.7
K	1	14.5	199
	2	9	124
	3	2.1	62.5
	4	23	359
	5	16.9	317
	6	69.7	419.8
	7	5.9	214
	8	43.9	318.4
	9	25.7	257
	10	trace	56.3
	11	11.8	232
	12	1	85.8
	13	30.7	492
	14	3.2	172.4
	15	2.2	92.9
	16	4.3	202.4
	17	28.9	367
	18	4.8	161.7
	19	11.2	269.2
	20	-	-
	21	trace	5

APPENDIX I (continued)

Nest	Replicate number	3-octanone (ng)	3-octanol (ng)
	22	trace	25.9
	23	18.2	251.9
	24	115	623.6
	25	3.7	139.3
	26	1.5	33.6
	27	120	596
	28	9.7	105
	29	1.5	52.5
	30	88.4	476.8
	31	71.8	287.5
	32	0.5	39.2
	33	0.5	14.0
	34	1.0	24.6
	35	0.7	58.7
	36	74.2	401.3
	37	29.8	375.3
	38	31.3	366.9
	39	4.5	234.4
	40	12.8	223.7
	41	5.9	74.4
	42	120	500
	43	-	-
	44	1.5	62.7
	45	190	385.9
	46	32.1	351.8
	47	1.9	24.6
	48	45.5	390