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Mounting Sex Pheromone: A Novel Pheromone Responsible for Mate Recognition in the Ixodidae

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MOUNTING SEX PHEROMONE: A NOVEL PHEROMONE RESPONSIBLE FOR MATE RECOGNITION IN THE IXODIDAE.

by

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ABSTRACT

Mounting Sex Pheromone: A Novel Pheromone Responsible for Mate Recognition in the Ixodidae.

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Cholesterol oleate was demonstrated to be the cuticular contact sex pheromone of *Dermacentor variabilis*. This pheromone which has been termed the Mounting Sex Pheromone (MSP) was also demonstrated to be present on the surface of *D. andersoni, Amblyomma maculatum* and *A. americanum.* This contact sex pheromone enables males excited and attracted by 2,6-dichlorophenol to identify the female as a potential mating partner. The MSP is the second in the series of three sex pheromones guiding the hierarchy of behavioral responses which constitute tick courtship behavior. Tests with *D. variabilis* and *D. andersoni* showed that this pheromone could be removed from and replaced on the surface of female ticks as well as transferred to inanimate objects without the loss of male responsiveness. Cuticular ridges, present on the female cuticle were shown to be unimportant as a sex recognition cue. The size of the female was also shown to be not of primary importance in assisting the male in mate recognition.

Cross reactivity was demonstrated particularly within genera and was also demonstrated between genera. The cross reactivity may be related to the presence of sterol esters on all the species of female ticks examined.

DEDICATION

This dissertation is dedicated to my parents James and Mary, my sisters Lenore and Maureen, and my brother Richard. It is also dedicated to my friends in the UK and US who kept me sane most of the time and allowed me the opportunity to be insane the rest of the time.

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Chapter one: INTRODUCTION

Ticks transmit a greater variety of infectious disease agents than any other group of hematophagous arthropods. They are important as pests and as agents of severe toxicoses or fatal paralysis. They affect the public health of many communities and are among the major factors limiting livestock production in tropical and subtropical regions of the world (Arthur, 1962, Harwood and James, 1979). Worldwide losses in agricultural production have been estimated at ten billion dollars per annum. The United States cattle industry alone is estimated to have lost 275·5 million dollars in one year due to ticks and tick associated conditions (Drummond, 1987). In many parts of the world tick transmitted diseases such as the viral Tick Borne Encephalitis, Erythema chronicum migrans and Congo-Crimea Hemorrhagic Fever (CCHF) are endemic and cause significant morbidity and mortality among the population. In the U.S., Rocky Mountain Spotted Fever (RMSF), caused by a pathogenic rickettsia results in numerous fatalities and almost 1,000 cases per year and currently Lyme Disease, caused by a spirochaete, and transmitted primarily by *Ixodes daminii*, has emerged as a major health care concern throughout most of the U.S.

The acari, or mites and ticks, represent one of the largest and most successful arachnid subclasses, comprising a diverse assemblage of free living and parasitic organisms. Ticks (order Metastigmata, Krantz, 1969) evolved as obligate hematophages of reptiles in the late Paleozoic (230 million years before present

hematophages of reptiles in the late Paleozoic (230 million years before present [b.p.]) or early Mesozoic (205 million years b.p.) eras. During the Cenozoic era (75 million years b.p.) these reptilian hosts were gradually replaced by mammals and birds as the dominant hosts for many species of ticks. Tick evolution although paralleling that of their avian and mammalian hosts, is much more conservative, as is typical of parasites in general, and consequently even modern species ('highly evolved' species) are believed to be very similar to their ancestral stock (Hoogstraal and Kim, 1985). The family Ixodidae can be subdivided into two orders, the Metastriata (the hard ticks) and the Prostriata. The Metastriata include all of the Ixodidae apart from the genus Ixodes. The Prostriata include the genus *Ixodes* and the Argasidae. The Metastriata require a blood meal to commence spermatogenesis and oogenesis whereas in the Prostriata gametogenesis occurs independently of feeding.

There are more than 850 species of ticks all of which are parasitic (hematophagous) (Hoogstraal and Kim, 1985). Balashov (1984) classified hard ticks as 'temporary ectoparasites with slow feeding' which leave the host only to molt and oviposit. Feeding generally lasts three to five days in adult females and seven or more days in the immature stages. The body weight of the female may increase more than 100 times. At each of the developmental stages, larval, nymphal, and adult (both male and female), ticks feed only once. The blood meal is sufficient for the molt to occur to the next stage. Females deposit between 1000 to 3000 (*Ixodes* spp. and *Haemaphysalis* spp.) to 10,000 to 20,000 eggs (*Hyalomma* spp. and *Amblyomma* spp.) (Balashov, 1972). Most ixodids are three host species i.e. each

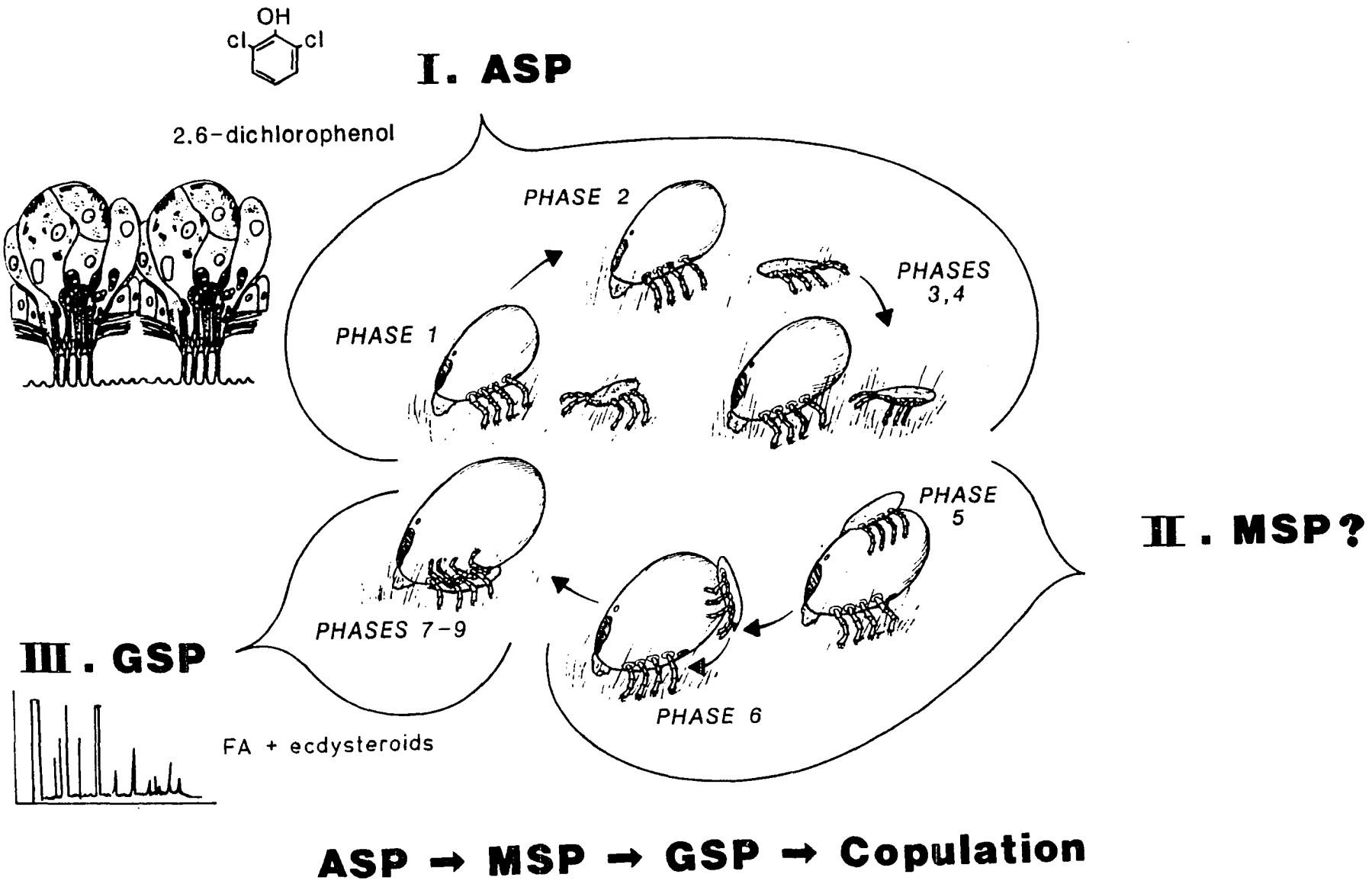
Rhipicephalus, after a blood meal the larvae remain on the host body and molt into nymphs that detach and leave the host after the blood meal (two - host species). In *Boophilus* spp. and some *Hyalomma* spp. all molts occur on the host body (one - host species), and only the adults leave after the blood meal. Ticks are not necessarily host specific, for example, *Ixodes persulcatus* was found to parasitize 200 species of mammals, 120 species of birds and several species of reptiles (Balashov, 1984).

In ticks, as in many insects, mating is regulated by sex pheromones, compounds which are released outside the body of the emitter organism and which direct a mating response in the recipient organism (Rutowski, 1981). In the hard ticks (Metastriata) the dorsally situated foveal glands of the female are the source of a long range or volatile sex attractant pheromone (SAP) which has been shown to be typically a phenol (Wood *et al.*, 1975; Sonenshine, 1984). Berger (1972) showed that 2,6-dichlorophenol (2,6-DCP) was the SAP of the Lone Star Tick, *Amblyomma americanum*. This pheromone has now been identified as being the SAP for 14 species in 5 genera of hard ticks. The restricted chemical evolution of the Acari is unusual especially when compared to the Insecta where a vast repertoire of chemicals are used as volatile and contact sex pheromones. It is clear that this pheromone by itself is insufficient to raise a barrier to heterospecific mating attempts. A further complication in the ixodid volatile sex pheromone paradigm is that a small amount of the volatile pheromone may be produced not only by sexually mature females but also by mature males (Sonenshine, 1987) and, may be found in small amounts in the larval and nymphal stages of other species (McDowell and Waladde, 1986).

Typically mate location in ixodid ticks follows the *Dermacentor* spp. model. Both male and female ticks must be attached to a host for several days and have commenced feeding before becoming sexually mature. Sonenshine (1985) recognized nine discrete mating phases in *D. variabilis* and *D. andersoni* (Fig. 1). In phases one and two the presence of 2,6-DCP, released by the female causes males to become sexually excited and enables them to search for the female. The males then orient towards the female and contact it, (phases three and four), mount and palpate the female dorsal surface (phase five). The males move to the female's ventral surface, interdigitate their legs with those of the female and locate the gonopore (phases seven and eight). Finally the males insert their chelicerae into the female vulva and probe this cavity, and after some time, (10 to 20 minutes), a spermatophore is inserted in the gonopore (phase nine). Sonenshine *et al.*, (1982, 1985) and Allan *et al.*, (1988) showed that the last two phases are guided by the genital sex pheromone (GSP). They demonstrated the presence of a species specific contact genital sex pheromone in the anterior reproductive tract of both *D. andersoni* and *D. variabilis* spp. and established that this determined the point of final species recognition. This 'genital sex pheromone' was also demonstrated to be present in *Hyalomma* spp. (Khalil *et al.*, 1983). The pheromone has been shown to be composed of a series of fatty acids C:14 to C:18 (Allan *et al.*, 1988) and possibly ecdysteroids (Sonenshine *et al.*, 1989).

There are several variations in the tick mating model outlined above. In the first exception, concentration of 2,6-DCP acts as a species identification mechanism allowing species recognition to occur at the beginning of courtship rather than at the

Figure 1. Hypothetical reconstruction of the behavioral stages that occur during courtship in *Dermacentor variabilis* and *Dermacentor andersoni*. (a) Phase 1: feeding female secretes volatile sex attractant, 2,6-dichlorophenol, exciting attached feeding male to detach. (b) Phase 2: detached male commences searching behavior. (c) Phases 3 and 4: sexually excited male detects the pheromone, orients to the emitting sources, and approaches the pheromone-secreting female. (d) Phase 5: male contacts the female, mounts and probes the dorsal surface. The capitulum is flexed and the legs partially folded. (e) Phase 6: the male turns posteriorly, crawls over the female's opisthosoma, and proceeds to the ventral surface. The female lifts its body to facilitate these movements by the male. (f) Phases 7-9: the male positions itself under the females podosoma, places its legs between those of the female, and flexes its capitulum to probe for the genital aperture. The chelicerae are inserted and copulation ensues. (Sonenshine, 1985).



end. In the second exception the physical characteristics of the female tick as well as the active participation of the female in the mate selection process occurs. In the third exception courtship behavior is facilitated by the formation prior to sexual maturity of groups of feeding male and female ticks in very close proximity to each other.

Females of the Camel tick, *Hyalomma dromedarii* produce four times as much 2,6-DCP as *H. anatolicum* excavatum (Silverstein *et al.*, 1984). Male *H. dromedarii* are sensitive to the consequent differences in 2,6-DCP concentration and respond optimally to the concentrations produced by conspecific females (Khalil *et al.*, 1983). A further deviation from the model can be found among three species of Australian reptile tick (Andrews and Bull, 1982). Instead of the simple chemically mediated courtship behavior displayed by *Dermacentor* spp., in *Aponomona hydrosaurii, Amblyomma limbatum* and *A. albolimbatum* courtship is mediated by four different physical and chemical systems. The females secrete an unidentified general attractant and a specific male attractant. Females actively participate in the courtship procedure by recognizing heterospecific males. They respond by clamping themselves against the hosts scales thus denying them access to the gonopore. Finally, the male structure must exactly compliment that of the female so that when the male and female legs are interdigitated the male chelicerae are automatically positioned for insertion in the gonopore.

The aggregation/attachment pheromone of *Amblyomma* spp. serve as examples of the third type of deviation from the *Dermacentor* model. In these species, e.g. *Amblyomma hebraeum*, feeding males secrete an attachment/

aggregation pheromone. This pheromone, which is not sex specific and thus not a sex pheromone induces mixed feeding clusters of males and females. Thus when males and females become sexually mature they find themselves conveniently in extremely close proximity to each other. This pheromone has been shown by Schoni *et al.*, (1984) and Hess and deCastro, (1986) to be a mixture of o-nitrophenol, methyl salicylate, and pelargonic acid. In some species e.g. *A. variegatum* sexually active males physically constrain passing conspecific females preferentially rather than heterospecific females indicating the possibility of a further deviation from the *Dermacentor* model.

The general question arises however, how does a male tick identify a conspecific sexually active female when the same phenolic sex attractant pheromone is present in so many different stadia, species and genera? Part of the answer undoubtedly lies in allopatric speciation, that is, as the species have separate ranges and separate ecological niches the males or females will not encounter their heterospecific counterparts and when males become sexually mature their chances of encountering heterospecific females are low. *D. variabilis* and *D. andersoni* are, however, sympatric and will even feed on the same host yet they do not attempt cross mating. It has been shown that cross mating between *D. variabilis* and *D. andersoni* is extremely rare and could only be forced in laboratory experiments producing sterile hybrids (Oliver *et al.*, 1972; Oliver, 1974). Gladney and Dawkins, (1973), and Rechav *et al.*, (1982) also demonstrated that *Amblyomma hebraeum* and *Amblyomma variegatum* could only be forced to cross-mate in laboratory experiments producing infertile hybrids. Thus it appears that although the sex pheromone is

extremely important in locating the female, presence of this pheromone alone does not confer species identity. Thus it is currently believed that mate location and identification in the Metastriate ticks can be explained by the following factors:- 1) temporal and spatial distribution of species. 2) presence and concentration of the volatile sex pheromone 3) presence and concentration of the species specific genital sex pheromone.

Several anomalies exist in this current paradigm however, to suggest that some further stimulus associated with the female cuticle exists. The presence of sex pheromones and other stimuli associated with the cuticle of the Insecta have been well documented. Khalil *et al.*, (1983), noted that in *H. dromedarii* and *H. anatolicum* excavatum mounting of heterospecific females may be aborted before the male reaches the gonopore, indicating that sexually active males committed to mating may be disrupted before reaching the next stimulus i.e. the gonopore. The male mating response is characterized by several distinct behaviors in which the males' body and mouthparts are pressed against the female body surface (Sonenshine, 1985). Indicating that the male may be involved in some specific behavior to allow recognition of the female. Sonenshine (unpublished) observed that male ticks which were attracted to 2,6-dichlorophenol placed on inanimate objects failed to recognize these objects as potential mates and left quickly after having made physical contact with them. These observations suggested that a second pheromone or other stimulus further mediated tick mating behavior. This contact pheromone, if it exists, is the second pheromone in a hierarchy of three pheromones that the male encounters. It would appear be a gustatory pheromone rather than

an olfactory pheromone and continues the signaling of information beyond that provided by 2,6-dichlorophenol.

The purpose of this study was (1) to determine if a cuticular contact sex pheromone existed on the female tick, and (2) if so to determine the species and generic specificity of the pheromone, (3) to determine the relative importance of the of female tick body characteristics such as size, and surface texture in assisting recognition of the female by male *D. variabilis* and *D. andersoni*. Finally (4) to isolate, characterize and structurally identify the sex pheromone of *D. variabilis*.

chapter two: MATERIALS AND METHODS

2.1 LABORATORY REARING OF TICKS.

Five species of ticks were used in this study. The Rocky Mountain Wood Tick, *Dermacentor andersoni* Stiles; The American Dog Tick, *Dermacentor variabilis* (Say); The Camel Tick, *Hyalomma dromedarii* Koch; The Lone Star Tick, *Amblyomma americanum* (L) and The Gulf Coast Tick, *Amblyomma maculatum* Koch. *D. variabilis* were colonized and reared as described previously (Sonenshine *et al.*, 1977). *H. dromedarii* were reared from a stock obtained from the U.S. NAMRU-3, Cairo, Egypt (U.S. APHIS No. 9433); all stages were fed on albino rabbits (*Oryctolagus cuniculus*). *D. andersoni* were obtained from the U.S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Montana. This species was colonized by feeding on laboratory rabbits. *A. maculatum* were reared from stock obtained from the Livestock Insects Laboratory, U.S.D.A., Kerrville, Texas. Larvae of this species were fed on albino rats (*Rattus norvegicus*), nymphs and adults were fed on albino rabbits. *A. americanum* were colonized and reared from specimens collected in Suffolk, Virginia. Unfed adult ticks of all species were held in an AMINCO-AIRE controlled environment chamber at 27 ± 1°C and 90 ± 1% RH before feeding. When required for extract preparation or bioassays the ticks were allowed to feed on albino rabbits for seven days and then forcibly removed from their hosts.

2.2 PREPARATION OF CRUDE EXTRACT.

Three types of crude extract were prepared from female ticks; dissected tick cuticle extract, cuticular surface wash extract and cuticular scrape extract. Initially, crude extract was prepared from dissected tick cuticle from all species. This was to ensure that as much pheromone as possible was removed from the source (which had not been identified and which could have been internal cuticle surface or the external surface). Extract was also prepared from whole undissected *D. variabilis* females by washing them in hexane. Extract was also prepared by scraping the surface of the cuticle. The last two types of extracts were to localize the presence of any stimulus on the surface of the female. Extract was also prepared from male tick cuticle.

Dissected tick cuticle extract (all species). Partially fed (7-days post attachment) virgin female ticks were used in the preparation of cuticular extract. Extracts were made from 50 to 2000 females. This was done by removing feeding females from host rabbits and freezing them (-20°C) to facilitate removal of the cuticle. Once frozen, the females were cut in two along the anterior/posterior midline and the internal body contents removed. The two cuticular tick halves were placed in Shen's solution for cleaning, Shen's solution was prepared as described by Oliver *et al.*, (1972). Vigorous shaking of the cuticle halves in the Shen's solution dislodged contaminating blood and faeces; this process was repeated three to five times until the visible contamination had been removed. After cleaning, the tick halves were

dried on tissue paper and the cuticle extracted in GC grade hexane (American Burdick and Jackson, Muskegon, MI). The solvent was decanted into another clean vial and the cuticle was extracted another three times with hexane. The volume of extract was then reduced under N_2 so as to provide extract with a concentration of approximately one Female Equivalent (FE) / three μl, and stored at -20°C. until required for use.

Cuticular surface wash extract (D. variabilis). As described above, seven day old post attachment virgin female ticks were removed from immunologically naive hosts. These females were then washed in Shen's solution to remove contaminating feces and blood, dried on tissue paper and then immersed in cold hexane for two to three hours. The hexane was decanted into a precleaned, acid washed beaker and the ticks washed two times with additional hexane, (30 minutes per wash). The hexane washes were combined, concentrated with a rotary evaporator and then concentrated further under N_2 to one ml and stored at -20°C. Concentrations were adjusted to around 0·1 FE/ μl before bioassay.

Extract was also prepared by washing ticks in hexane, by passing hexane (200 ml) over 50 female ticks (designated the 0 minute wash), and by immersing groups of 50 ticks in hexane (200 ml) for 5, 10, and 20 minutes. These cuticular surface extracts were made to determine if the extract prepared from the cuticular surface only would be as effective as extracts prepared from cuticle and underlying tissue.

Cuticular surface scrape extract (D. variabilis). Fifty fed female ticks were cleaned in Shen's solution as described above. The surfaces of these females were then scraped with a razor blade, to remove the outer cuticular layers (epicuticle and endocuticle). Those layers of the cuticle thus removed were extracted in hexane. The hexane solution was concentrated to one ml under N_2 and then filtered through a 0·2 micron Acro LC13 disposable filter (Gelman Sciences Inc, Ann Arbour, MI), to remove pieces of cuticle.

Male cuticular surface extract (D. variabilis and D. andersoni). Male cuticular extract was prepared from fed (seven days post attachment) adults. These were washed in Shen's solution, dried and frozen at -20°C. They were washed in hexane (100 ml) three times and the combined solvent extract reduced to 0·2 ml under N_2.

Glassware. All glassware used in the preparation of extract was acid cleaned in a chromic/sulfuric acid cleaning solution (Chromerge, Manostat, New York, NY) for 24 hours. It was rinsed in distilled water, dried in acetone, rinsed with GC grade hexane and then oven dried at 100°C for 10 hours. After removal from the oven the glassware was allowed to cool to room temperature before use.

2.3 BIOASSAYS.

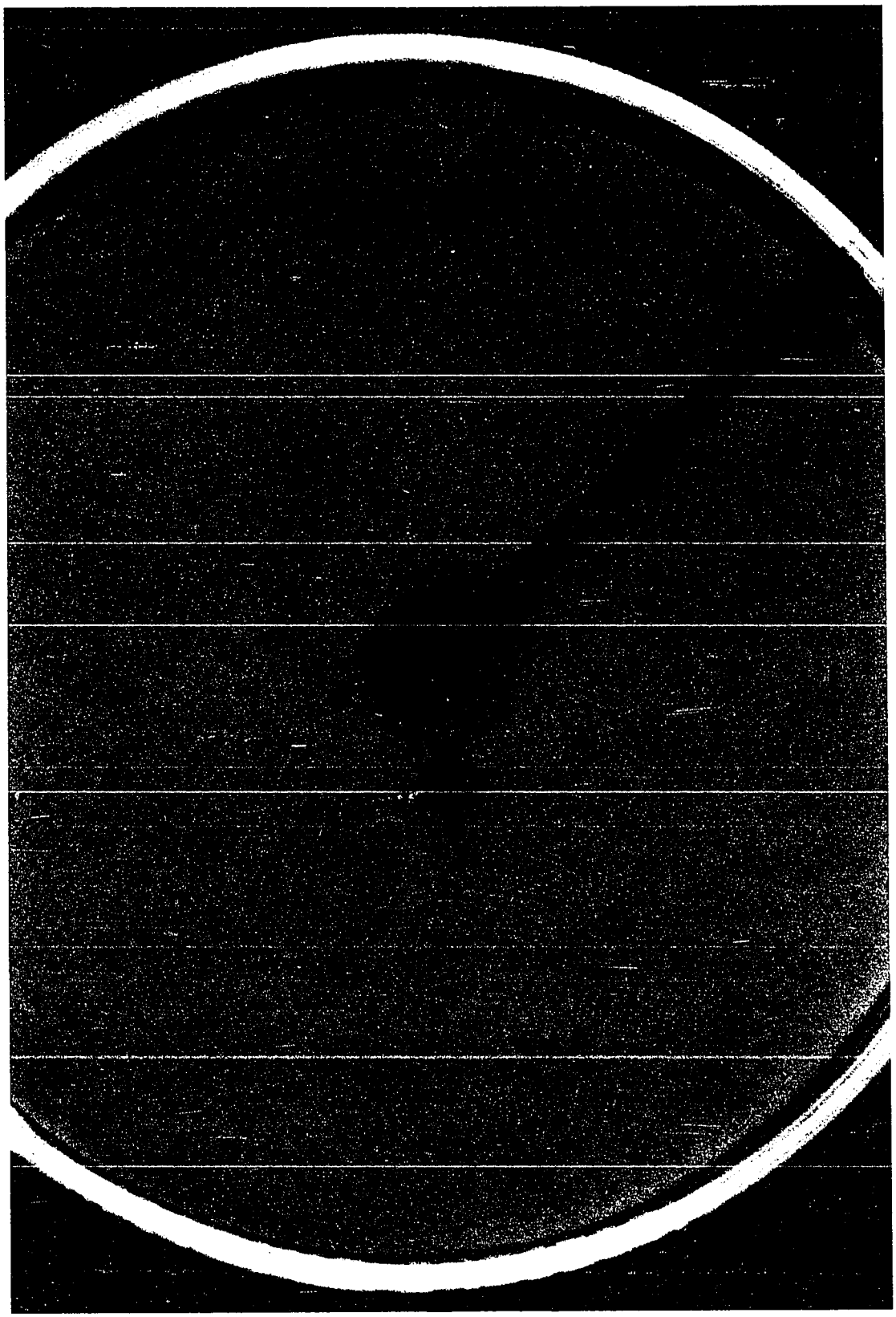
Cleaning and Screening of Males and Females. Fed female ticks (six to seven days post attachment) were immersed in hexane for 24 to 48 hours to remove any surface lipids. All females used in bioassays of fractionated extracts, standards, and the majority of the inter-specific tests were cleaned in this way. Preliminary trials indicated that hexane was effective in removing the biologically active cuticular lipids although chloroform and acetone were also effective. In earlier bioassays females were cleaned by immersion in solvent and scrubbing with a fine, stiff bristle brush. The detached, partially fed females were cleaned in this way for ten to thirty minutes. In all instances these vigorous cleaning regimes resulted in the death of the female. The females were subsequently referred to as 'cleaned females'.

All male ticks were screened to ensure that only those that were responding to females were used in the bioassays. Females were also screened to ensure that they were able to elicit a response from sexually active males (positive control) prior to cleaning. Females cleaned in solvent to remove the pheromone were also screened with sexually active males to ensure that they did not invoke a response (negative control). In all assays approximately six ng of 2,6-DCP (Aldrich Chemical Co. Inc. Milwaukee, WI) in ten μl of hexane was added to the foveal gland area of the dorsal surface of the clean female with a microcapillary pipette. To perform the bioassay, a male was placed alongside the female (less than two cm) or inanimate object and its responses were observed and recorded as described below. Screenings were performed with cleaned females attached with adhesive tape to filter paper

discs in a nine cm diameter petri dish, Plate One. The assays were performed at 27°C ± 1°C, 80% relative humidity, in a walk-in environmental chamber (Western Environmental, Napa, CA.). Incident light was measured with a YSI light meter (LI-Cor Inc., Lincoln, Nebr.) and was found to be 16,500 ± 500 lux.

Scoring of Bioassays. The response of sexually active male ticks when presented to immobilized females and inanimate objects (Dummy Females or Decoys see below), was scored by a system based on the tick mating behavior sequence described by Sonenshine (1985). According to this modified system, male mating behavior can be subdivided into six clearly observable and distinct behaviors. These are; 1/ the male is attracted to the female; 2/ the male touches the female; 3/ the male mounts the dorsal surface of the female i.e. the male climbs onto the dorsal surface of the female; 4/ the male turns on the female (through 180° with its body pressed in close contact with the female); 5/ the male crawls onto the females' ventral surface (with its body pressed in close contact with that of the female); 6/ the male positions itself at the females' gonopore, flexes its capitulum, probes the gonopore and inserts its chelicerae. The male's legs are interdigitated with the females to assist with the positioning of the male, at this time. The first two stages i.e. behaviors one and two were not included in the final analysis as they are a measure of 2,6-DCP attractiveness. As the male performed each of the four behaviors three through six, it received one point for a maximum total of four points. The number of points gained by each male was recorded. Each bioassay was replicated 20 times; at first each replication used a fresh male and fresh female. In later bioassays of fractionated extract, standards and the five species inter-specific

Plate I. Petri dish arena in which bioassays were performed with attached females and attendant male.



tests one female was tested with five males and a total of 20 males were used with four females. Thus, in all instances, the final 'total score' was out of 80 points, i.e., four points/male tick × 20 repetitions (= 80 points total) for the completed bioassay. The raw scores were then converted to a percentage of 80 points, this was the 'percent score'. (Both 'percent score' and 'total score' i.e. the absolute number of points scored are given in the results tables). The greater the value of the percentage score the more closely the test males behavior represented the ideal situation (i.e. all males positioned for mating = 100%). The total number of the 20 males tested which successfully completed all four behaviors was also noted, (total positioned) this value along with the corresponding percentage (percentage positioned) are given in the tables of results.

Time Analysis. Time analysis was a comparison of the time spent on the real female tick or dummy female tick (DFT) by males when mounting sex pheromone (MSP) (the putative contact sex pheromone) was present and when it was not.

2.4 EXPERIMENTAL: TO DETERMINE THE POSSIBLE EXISTENCE OF A PHEROMONE.

Bioassays were performed as described above for screening ticks. The experimental protocol was divided into two sections, test and control. Generally in the test portion females or inanimate objects (DFTs) were coated with extract and male response to this (or other treatment) was recorded. In the control portion clean females or inanimate objects with no extract added were tested with sexually active males.

2.4.1 *Conspecific Assay.*

Removal of Stimulus: To determine if a pheromone was present the cuticular surface compounds were removed from whole *D. variabilis* female ticks by cleaning as described in Section 2.3. These 'cleaned females' were treated with six ng of 2,6-DCP and were then screened with sexually active males to determine if the females still induced a mating response. The experiment was repeated with *D. andersoni*. Controls were untreated females i.e. females which had not been cleaned, tested with the same males challenged previously with the surface-cleaned females. Data were analyzed using a paired one tailed t-test. (SAS Statistical Package, SAS Institute Inc, Cary, NC).

Replacement of Stimulus. Crude extract was prepared from both *D. variabilis* and *D. andersoni* female cuticle. Cleaned *D. variabilis* females were tested with sexually active *D. variabilis* males to determine if the stimulus had been removed (control). These females were then coated with *D. variabilis* female crude extract (1·5 to 2·0 FE/cleaned female). Once the solvent had evaporated and ca. six ng. of 2,6-DCP had been added the same males that had been used in the control were used to determine if the stimulus had been restored. Data were analyzed using a paired one tailed t-test, (SAS Statistical Package). The experiment was repeated with *D. andersoni* females and males.

Transfer of Stimulus. Extract prepared from female cuticle was placed on inanimate objects to determine if the male response could be transferred by moving the stimulus. The DFTs were inert plastic, spherical beads, (18 mm diameter). These beads were prepared by roughening their surface with sandpaper and were

attached to a cardboard support with a fine copper wire (Plate Two A). A sample of crude extract (16 μl ca. equal to four to five FE) was added incrementally to the surface and the hexane solvent allowed to volatilize. The treated specimen was held in an incubator (30°C). Each species extract was tested with conspecific males. [Also the heterospecific males were tested (see below)]. Controls were plastic beads with hexane only added. The same males were used in both control and test portions of the experiment. Scoring of this bioassay was as described above. Data were analyzed with a paired one tailed t-test.

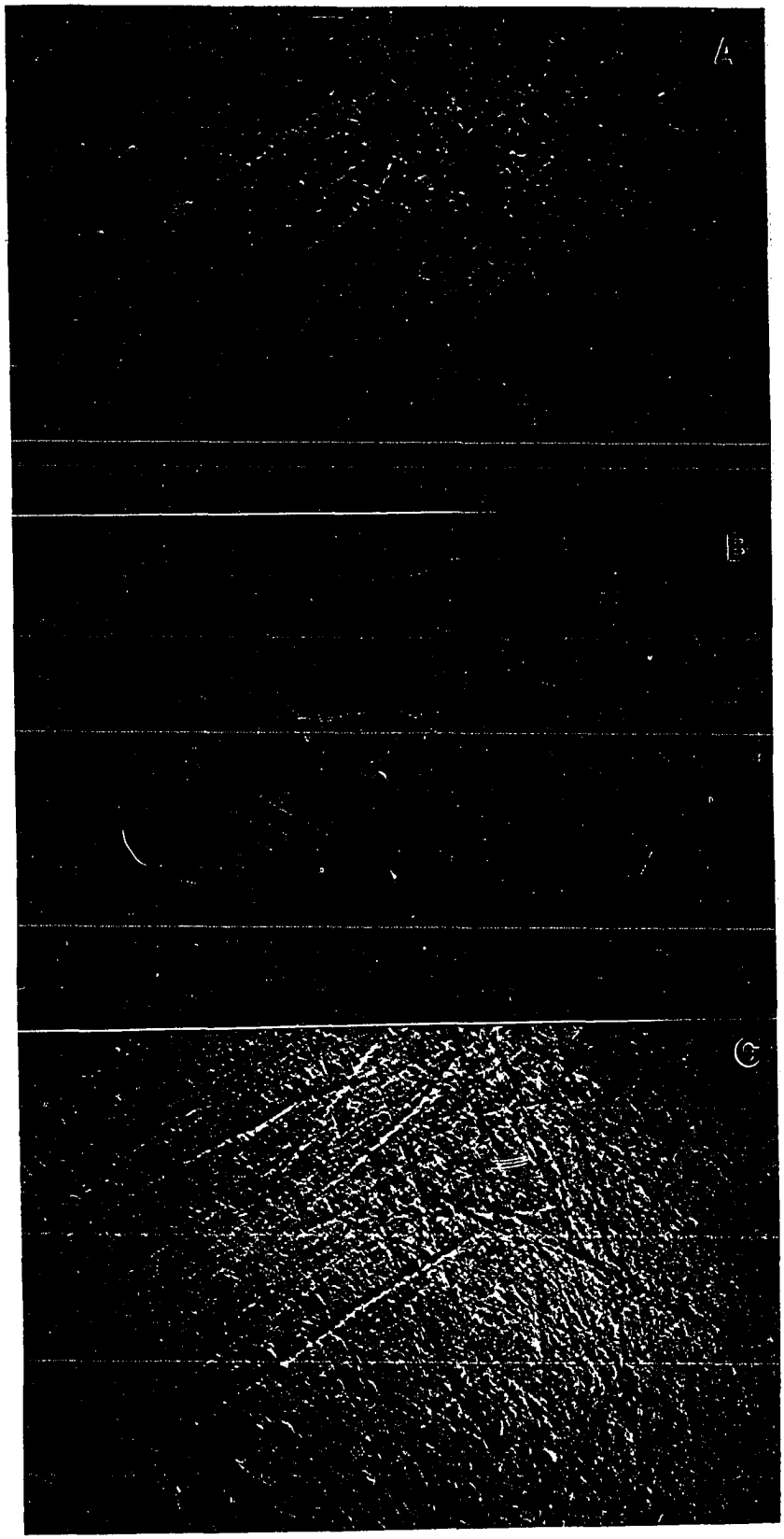
In addition, the amount of time spent by males in contact with extract-coated DFTs and control DFTs was recorded. Data collected were analyzed by paired t-tests.

Several other bioassays were performed. Cuticular wash extracts (0, 5, 10 and 20 minutes) were prepared as described above. These extracts were then added to clean females (1FE) and bioassayed as normal in the 'clean female' bioassay with conspecific males.

Cuticular surface scrape extract was prepared as described above and added to cleaned females. One FE of extract was added to each cleaned female and bioassays conducted as normal.

Cuticular extract prepared from male cuticle was placed on conspecific 'clean females' and bioassayed with conspecific males. Concentration of extract applied was ten male equivalents (ME) per clean female.

Plate II. SEM of (A) plastic bead (DFT) surface roughened with fine sandpaper, (B) Normal surface of female tick, and (C) surface of female abraded with fine sandpaper.



2.4.2 Heterospecific assay: species and generic specificity of the pheromone. This was done by preparing extract from both *D. andersoni* and *D. variabilis* females as described above. *D. andersoni* female extract was placed on cleaned *D. variabilis* females and tested with both *D. andersoni* and *D. variabilis* males. *D. variabilis* female extract was placed on cleaned *D. andersoni* females and tested with both *D. andersoni* and *D. variabilis* males. Controls were cleaned females with hexane and 2,6-DCP only added. In this experiment the conspecific males were also tested as controls. Extract prepared from female cuticle was placed on inanimate objects (described above). These experiments acted as controls for any physical stimulus (either positive or negative) provided by the body of the heterospecific female. The total amount of time that the male spent in contact with the dummy female tick was also determined. Data collected were analyzed by t-test.

The experiment was then expanded and repeated using *D. andersoni*, *D. variabilis*, *A. maculatum*, and *A. americanum* females, female extract and fed males. The experiment was performed on females which had been cleaned and coated with conspecific extract. Extract was added at several concentrations (1·0 FE, 0·1 FE and 0·01 FE) to control for the effect, if any, differences in pheromone concentration between species and genera and other cuticular cues provided by the female body.

D. andersoni female extract was placed on cleaned *D. andersoni* females and then tested with each of the four species of male. Extract was applied at each of the three concentrations indicated above. This procedure was then repeated for each of the other species of ticks. Time spent by the males in contact with the females

was not recorded. Controls were the cleaned females tested with fed males before extract was added.

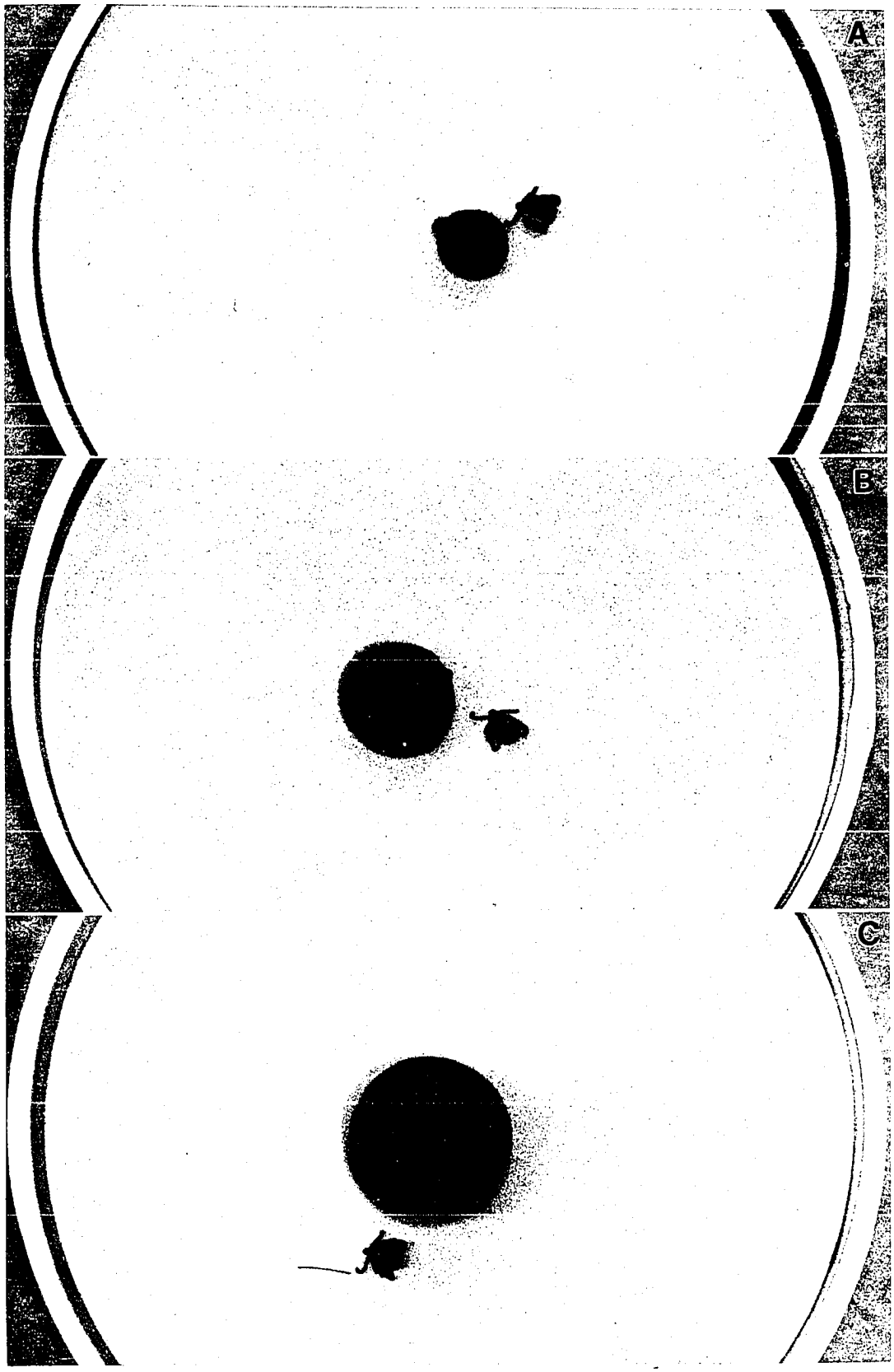
2.5 BIOASSAYS TO DETERMINE THE IMPORTANCE OF FEMALE BODY TEXTURE, SIZE, AND PHEROMONE CONCENTRATIONS.

Body Texture. To determine the importance of female surface texture in providing a mechanical stimulus to male ticks, cuticular ridges were physically removed from female *D. andersoni* and *D. variabilis* by abrasion with fine sandpaper. These abraded females were then tested with sexually active conspecific and heterospecific males. To replace any chemical cue which might have been removed conspecific female extract was replaced on the abraded females and allowed to dry before testing with males. Controls were the females used in the test portion before removal of the cuticular ridges tested with sexually active conspecific males. Plate Two B and C shows the surface of a normal and abraded female. The cuticular ridges can be seen to be completely removed.

Size of female and concentration of extract. Size of the female and concentration of extract may be an important determining factor in conspecific mate recognition. These variables were tested as described below.

Extract of both *D. andersoni* and *D. variabilis* females was prepared as described above and placed onto the surface of three different sized balls, A, B and C. The radius of ball A was 9·0 mm (surface area 1018 mm^2); ball B, 5·9 mm (surface area 437 mm^2) and ball C, 3·85 mm (surface area 186 mm^2). The surface

Plate III. Petri dish arena in which size of DFT and concentration of extract experiments were performed. (A) 3·85 mm radius DFT, (B) 5·9 mm radius DFT and (C) 9·0 mm radius DFT.



area of ball B was similar to that of a feeding (seven day) *D. andersoni* female. The surface area of ball C was similar to that of a feeding (seven day) *D. variabilis* female. Four different concentrations of extract were applied, namely, one, two, four, and eight FE. 2,6-DCP was placed onto the balls prior to the release of male ticks to initiate mate finding behavior. Both *D. andersoni* and *D. variabilis* males were tested against all variables, controls were provided by balls without extract and by internal comparisons in the subsequent statistical analysis. The total amount of time in contact with the ball was recorded (longest time out of three trials). The male mating response was scored as previously described. The balls were placed in the center of a petri dish. They were attached by a piece of copper wire to a cardboard disk which was covered with a piece of filter paper. This resulted in a glass rimmed, paper lined arena with a ball in the center (Plate Three). Twenty males of each species were tested at each of the three sizes, four concentrations and for each of the two species of extract.

The experiment was designed as a Pure Model One Four-Way Analysis of Variance with uneven sampling size (Sokal and Rohlf, 1981). Size, concentration, species of male tested and species of extract tested were the main effects. This design allowed independent assessment of the main effects as well as of their interaction. Data were transformed using the square root transformation. Data were analyzed on an IBM model 4381 computer using the SAS statistical package, General Linear Models routine. Means were compared using Duncan's Multiple Range test. Data were also plotted in a three dimensional histogram using the SAS Chart routine.

2.6 CHEMICAL CHARACTERIZATION OF TICK EXTRACT.

Sections 2.6 through 2.11 describe the chemical separations undertaken to isolate and identify the pheromone. The procedures are summarized in the flow diagram below (Fig 2).

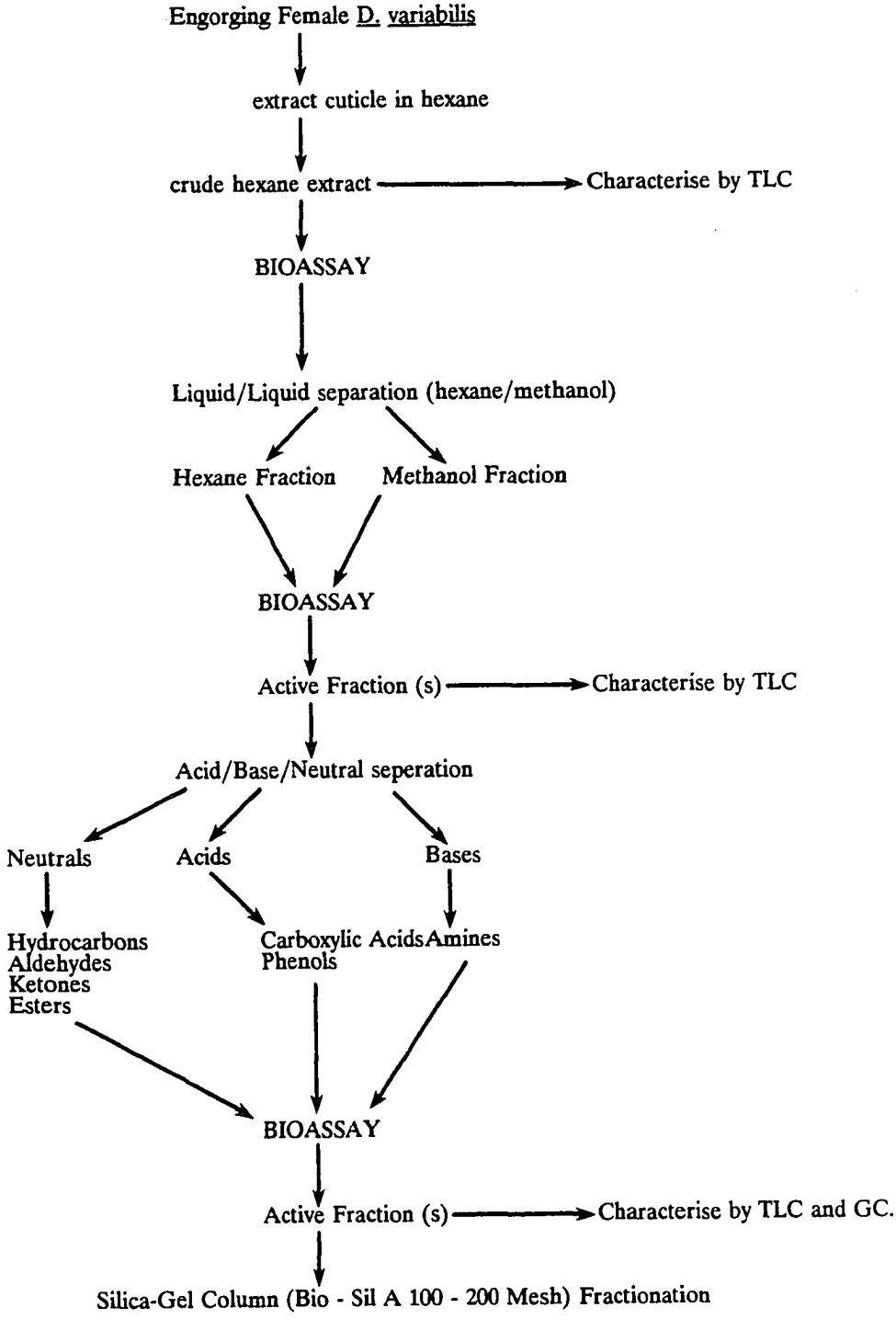
2.6.1 Thin Layer Chromatography. Thin Layer Chromatography (TLC) was performed throughout the chemical part of this study. It was used to characterize the crude extract and also to monitor the efficiency of the fractionation of the crude extract through the various separation processes described in the sections below.

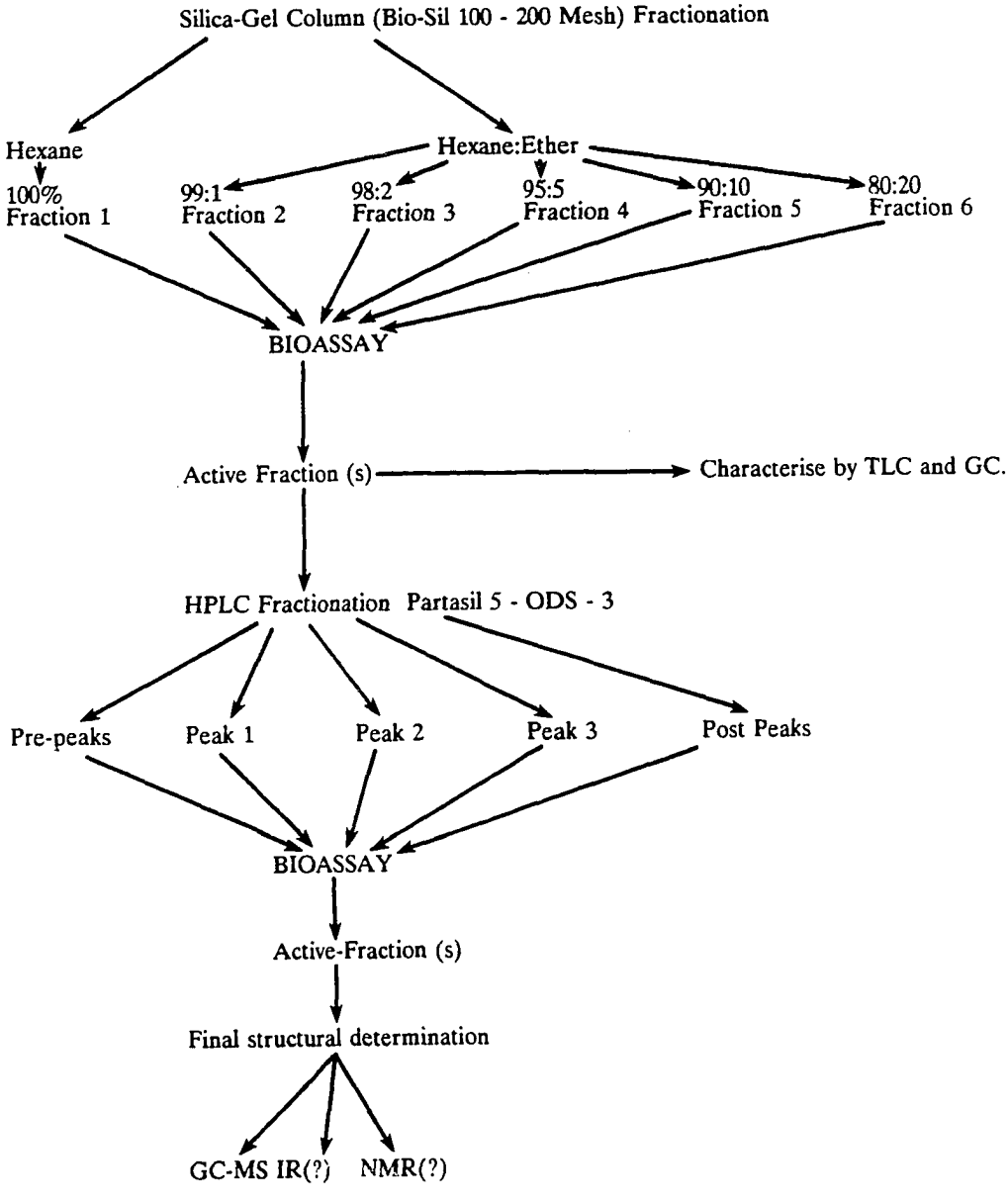
TLC was also used for preparative purposes, individual spots representing chemicals or classes of chemicals were removed from the TLC plate for extraction and further analysis by Gas-Chromatography (GC) and bioassay.

Prior to analysis, TLC plates were pre-cleaned in hexane twice and then twice more in the solvent system in which they were to be developed. Sample was applied with a two μl microcapillary pipette (Dade Volupette Pipet, American Dade, American Hospital Supply Corporation, Miami, FL.). Spotting of High Performance TLC (HPTLC) plates was carried out according to the manufacturers instructions for both large and small amounts of sample.

Developing tanks ($27 \cdot 5 \times 7 \cdot 3 \times 24 \cdot 5$ cm) were lined with absorbent paper and 200 cms^3 of solvent added. Once TLC plates were placed in the tanks solvent was allowed to ascend to a line 13 cm from the origin. When the non-polar solvent system was being used the first solvent was allowed to rise to 14 cm and the second

Figure 2. Flow diagram detailing chemical separation and identification steps of Mounting Sex Pheromone.





solvent to 10 cm. Once fully developed, the plate was removed, dried, sprayed with ethanol:H_2SO_4 (50:50, v/v) and visualized by charring (120°C for 20 minutes) (this procedure reveals all organic compounds but is destructive). Alternatively, visualization was by exposure to I_2 vapor, or by illumination with 254 nm ultra-violet (UV) light (these are non-destructive procedures).

Analytical Thin Layer Chromatography: Characterization of the crude extract. Initial characterization of crude extract was performed using Baker-Flex silica IB-2 (20 × 20 cm) plates (JT Baker Chemical Co., Phillipsburg, NJ.), and on C-18 Reverse Phase plates, (Baker Si-C-18F (19C) TLC plate 20 cm × 20 cm, 20 lanes). Later analyses were performed using High Performance TLC plates (10 × 20 cm, 200 μm thick silica layer with preadsorbent area) (Whatman Chemical Separation Inc, Clifton, NJ). Solvent systems used to characterize the crude extract were:

Solvent 1/ A one dimensional, two solvent system; the first solvent was isopropyl ether: acetic acid (96:4 v/v). The second solvent was petroleum ether:diethyl ether:acetic acid (90:10:1 v/v), (Skipski and Barclay, 1969).

Solvent 2/ A one dimensional, one solvent system; the solvent was petroleum ether:diethyl ether:acetic acid (80:20:1 v/v), (Mangold, 1969).

Solvent 3/ A one dimensional, two solvent system; the first solvent was hexane 100%. The second solvent was hexane:diethyl ether (80:20 v/v)(Non-polar solvent system).

Solvent systems used to characterize the partially purified fractions were, (1) hexane:diethyl ether, 90:10, v/v; (2) heptane:benzene, 90:10, v/v, and (3) the non-polar solvent system described above.

Crude extract from *D. variabilis, D. andersoni, A. americanum, A. maculatum, and H. dromedarii* were used. Seventy μl (approximately 14 FE) of each were spotted on the plate. Standards used were; cholesterol 50 μg, Non Polar Lipid Mix (NPLM)(containing cholesterol oleate, methyl oleate, triolein [a triglyceride], oleic acid and cholesterol, 20 % of each) 70 μg. Hydrocarbon (n-tetradecane and n-tetradecene) 70 μg of each. Lipid Standards containing linoleic, linolenic, oleic, palmitic, and stearic fatty acid methyl esters. Seventy μg of fatty acids e.g. oleic or arachidonic acid were used where indicated.

Preparative Thin Layer Chromatography. For preparative TLC the same procedures described above for HPTLC were used to prepare and develop the plate. Spots were visualized using I_2 and UV light. Once the spot of interest had been located, it was scraped off the TLC plate and placed in a Soxhlet micro-extractor (Ace Glass, Vineland, NJ), and extracted from the silica by refluxing in approximately 25 ml of hexane. The extract was then concentrated under N_2 and the sample further analyzed. This preparative technique was also used as a sample clean-up procedure for material collected by packed column GC, and microderivatisation analysis (see below).

Removal and Extraction of Putative MSP Spot From HPTLC Plates. Crude *D. variabilis* extract, and the neutral fraction of the *D. variabilis* extract were spotted on HPTLC plates (54 FE of each extract, six lanes); after development the putative

MSP spots were extracted as described above. A control plate which contained no sample material was also developed and extracted as for the other plates. The extracts were bioassayed, and successful extraction was verified by analysis on HPTLC.

2.7 IDENTIFICATION OF BIOLOGICALLY ACTIVE CHEMICAL CLASS.

2.7.1 Liquid:Liquid separation.

This was the first fractionation of the crude *D. variabilis* female extract. The technique separates organic compounds on the basis of their solubilities in solvents of differing polarities. For the purposes of this separation two solvents were chosen, on the basis of their immiscibility and their widely differing polarities. The polarity index (P') of methanol is 5·1 and hexane is 0·1 on a 10·2 scale (polarity of water = 10·2).

Extract was prepared as described above. Equal volumes of hexane (200 ml) (GC Grade American Burdick and Jackson, Muskegon, MI) and methanol (200 ml) (GC Grade, American Burdick and Jackson) were placed in a 400 ml separating funnel. The crude *D. variabilis* cuticular extract was added and the separating funnel firmly stoppered. The funnel was inverted and vigorously shaken for several minutes. The two layers were allowed to separate with the hexane layer on top and the methanol layer below. The methanol layer was run off into a second separating funnel and a further 200 ml of hexane were added to this methanol and the process

was repeated. The hexane layers were collected, combined and reduced under N_2 as was the methanol layer. The two fractions designated 'methanol fraction' and 'hexane fraction' were then tested for their biological activity, by the clean female bioassay with negative controls. The efficiency of the separation was monitored by TLC (IB-2 plates) with both heptane:benzene 90:10 and hexane:diethyl ether 90:10 as the solvent systems.

2.7.2 acid/base/neutral separation.

The acid/base/neutral separation is a well known extraction technique which separates molecules on the basis of their relative acidity. In this extraction process carboxylic acids, phenols and other weak acids were deprotonated by a base (in this case sodium bicarbonate (saturated $NaHCO_3$) and sodium carbonate (saturated Na_2CO_3), the anions were thus water soluble. Bases were rendered water soluble by protonation in acidic media (2N HCl). The experimental procedure used in this extraction was that described by Keese *et al.*, (1982).

The bioassay positive fraction from the liquid:liquid partition (in two ml of hexane) was added to a 500 ml separating funnel containing 200 ml of saturated $NaHCO_3$ and 200 ml of freshly distilled diethyl ether. The funnel was stoppered and shaken vigorously. Two layers were formed, an aqueous layer containing the acid components of the extract and a non-aqueous layer (diethyl ether) from which the acids had been extracted. After separation of the layers the aqueous layer was run off into another 500 ml funnel containing a further 200 ml of freshly distilled

diethyl ether. This funnel was then stoppered and vigorously shaken. The aqueous layer was collected and acidified with 2N H_2SO_4. The aqueous solution was added to another separation funnel and extracted with distilled diethyl ether. This was the 'acid' fraction and the ether extract was concentrated to one ml under N_2. The 'acids extracted' ether layer from above was then added to a clean separating funnel and 200 ml of 2N HCl. The above process was then repeated for this base fraction. The aqueous layer containing the bases was basidified by adding 2N NaOH and this layer was in turn extracted with distilled diethyl ether. The material remaining in the original ether layer from which both acids and bases had been extracted was the 'neutrals'. This fraction as well as the other fractions was evaporated to dryness under N_2 and reconstituted in GC-MS grade hexane after drying with anhydrous $MgSO_4$ or Na_2SO_4.

All glassware used in the above separation was acid cleaned prior to use and all solutions used in the process were freshly prepared. Three fractions were generated; 1/ the neutral fraction, which would contain molecules such as n-alkanes, branched alkanes, aldehydes, ketones and neutral esters. 2/ The acid fraction, which was actually the combined weak acids, such as carboxylic acids, and stronger acids such as phenols, and 3/ The basic fraction which would contain bases such as amines.

Bioassay for biological activity. The three fractions generated by this separation were bioassayed to determine their biological activity using the standard 'clean female' bioassay. Approximately one FE of each fraction was placed on the clean female.

Chromatography of the acid/base/neutral fractions. The active fraction was analyzed by HPTLC to monitor the efficiency of the separation and by Gas Chromatography to determine the complexity of the mixture. The 'non-polar' (method three) TLC solvent system was used.

2.7.3 Column chromatography.

Preliminary Separation: Mallinkrodt Silica Column. A silica column was prepared by adding four ml of silica (Mallinckrodt Silicic Acid 100 Mesh Powder, Mallinckrodt Inc, St. Louis, MO) in a hexane slurry to a ten ml acid cleaned pipette. The column was pre-cleaned by sequentially eluting with ten column volumes of each of five solvents; hexane, hexane:diethyl ether (80:20 v/v), ether, chloroform and methanol. This solvent choice was based on a stepwise elution sequence described by Kates (1975). The elution procedure was repeated, the eluates collected and the volumes reduced under N_2 to one ml. These eluates were then retained for bioassay as column blank controls. A very low bioassay response would indicate that no material with biological activity was being eluted from the column. Crude extract (300 FE) was prepared as described above and added to the top of the column in one ml of hexane. The column was then eluted using the stepwise solvent elution scheme described above. The fractions were collected, reduced under N_2 to one ml, and bioassayed at six concentrations (30, 15, 3·7, 1·5, 0·7 and 0·4 FE). (TLC was used to monitor elution success, results are not presented). Results of this preliminary fractionation were used in conjunction with the polarity

information gained from the liquid extraction and acid/base/neutral extraction to decide which solvent system to elute active components from the Bio-Sil A column.

Separation on Bio-Sil A Column. A silica (Bio-rad Silicic Acid, Bio-Sil A 100 - 200 Mesh, Bio-Rad Laboratories, Richmond, CA) column was prepared by adding five mg of Bio-Sil A in a hexane slurry to an acid cleaned 20 ml pipette. The volume of silica in the column and the length of the Bio-Sil A column were determined by the amount of sample to be loaded onto the column as described by Still *et al.*, (1978). (The ratio of weight of Silica-gel to extract was 40:1. The ratio of length to diameter of the column was 9:1, and the solvent flow rate was approximately two ml per minute for optimal separation). The column was flushed through with 20 column volumes of hexane and hexane:diethyl ether in the following ratios, 99:1, 98:2, 95:5, 90:10, and 80:20 v/v to pre-clean the column. The Bio-Sil A column was standardized using, n-octacosane, octadecene, docosanoic acid, and tetracosanol as standards. One mg of each standard was loaded onto an appropriately sized column and eluted with a variety of solvents as above. The standardization procedure using the stepwise elution scheme, revealed that, n-alkanes and n-alkenes eluted first (100% hexane fraction), fatty acids eluted next (95:5) [hexane:diethylether fraction], and alcohols in the 90:10 v/v fraction. Using the results from this standardization it was possible to calculate which standard was eluted with which solvent and the volume of solvent required to elute the particular chemical class.

After the column had been cleaned with solvent, the sample [123.2 mgs (2000 FE) of neutral extract, obtained as described in Sections 2.8 and 2.9 above] was

loaded in two ml of hexane to the top of the column. The column was eluted with 3 × 5 ml aliquots of each of the solvents above. Also, after the last aliquot of 80:20 v/v hexane:diethyl ether was eluted, the Bio-Sil A was removed from the column and extracted in a soxhlet extractor. This column extract allowed the amount of material retained by the column to be determined. Aliquots were reduced under N_2 and the separation was subsequently monitored by HPTLC. The fractions were then examined for biological activity (clean female bioassay, negative controls), at two concentrations (one FE and ten FE). (Fractions were also monitored by Gas Chromatography See).

2.8 BIOASSAY OF STANDARD COMPOUNDS.

Several groups of non-polar compounds represented in the *D. variabilis* crude extract, were bioassayed at a range of concentrations, for biological activity in the 'clean female' bioassay. Alkanes from C:14 to C:40 were also bioassayed at the same concentrations described above. Fatty acids from C14:0 to C22:1, were bioassayed at concentrations of 10·0, 2·5 and 0·5 μg / clean female. Sterol ester standards from C2:0 to C20:0 were bioassayed at five concentrations, 10·0, 2·5, 1·0, 0·5 and 0·1 μg per clean female. Cholesterol at 10·0 μg/cleaned female and tick extract (MSP fraction) at 10·0, 1·0 and 0·1 μg as well as hexane were also bioassayed as controls.

2.9 DERIVATIZATION EXPERIMENTS.

Once a single spot on a TLC plate had been isolated and identified with biological activity as a result of the Bio-Sil A silica column separation, attempts were made to characterize the active molecule by reacting the MSP fraction with a variety of reagents targeting potential specific molecular structures. Biological activity was tested before and after the reactions.

Oxidation of olefinic and acetylinic linkages. Potassium permanganate acts as an oxidizing agent and it targets both acetylinic and olefinic bonds. Ten gm of NaCl and 0·05 gm of $KmNO_4$ were mixed in 50 ml of H_2O (distilled). The solution was placed in a 125 ml separating funnel along with 50 ml of benzene and 0·1 gm of tetra butyl ammonium bromide (phase transfer agent). Several drops of the resultant purple benzene solution were added to 60 μl of the MSP fraction. The color change was noted and the solution was extracted in excess distilled diethyl ether, concentrated under (N_2) and then used for bioassay and analysis on a HPTLC plate. Hexane (60 μl) was treated in the same way as the tick extract for the control.

Transmethyl esterification. In this reaction free carboxyl groups are targeted and methyl esterified. Additionally ester linkages are broken generating a FAME and an alcohol. In both the transmethyl esterification of fatty acids and tick extract the following procedures were undertaken. Ten μl (20 FE) of tick extract, MSP fraction, acid fraction or other fractions were placed in an acid cleaned two cm^3 vial and the solvent evaporated under N_2. Thirty μl of BCl_3 in excess methanol was

added. The vial was tightly sealed and heated to 60°C for 45 minutes in a water bath. After cooling the mixture was washed twice with saturated NaCl. The hexane fraction was concentrated to 20 μl and then analyzed by HPTLC and bioassayed.

2.10 ISOLATION AND PURIFICATION OF THE ACTIVE COMPOUND.

2.10.1 High pressure liquid chromatography.

High Pressure Liquid Chromatography (HPLC) analysis was performed on the MSP fraction (Fraction three) eluted from the Bio-Sil A column. This fraction had previously been shown to be bioassay positive. The analysis was carried out using a Waters model 721 HPLC system controller coupled with a Waters model 730 data module (Waters, Milford, MA). Detection was performed by a 214 nm Ultra Violet light (Zinc lamp) in a Waters model 441 fixed wavelength detector. The detector sensitivity was set at 0·02 absorbance units full scale (AUFS). Solvent, which had been filtered and sparged prior to use was delivered by two Waters model 510 pumps connected to a U6K injector. The column used was a 25 cm Whatman Partasil 5-ODS-3 reverse phase analytical column (Whatman, Hillsboro, OR). The solvent used was a 60:40 v/v mixture of HPLC grade acetonitrile (Mallinckrodt, ChromAR) and isopropanol (Burdick and Jackson, American Scientific Products, Muskegon, MI). The solvent was delivered isocratically at a flow rate of two ml/minute.

Analytical HPLC. Analysis was performed by injecting one μl aliquots of MSP fraction (two FE) eluate into the injector. Sterol ester standards in hexane were injected under the same conditions in varying amounts from 0·5 μg to 10·0 μg. Co-injections of sterol esters and the MSP fraction were performed to help establish peak identities. Using peak area data amounts of material in the MSP fraction aliquots and individual peaks were also determined. Peak areas of the unknown compounds were compared with peak areas of the corresponding sterol ester standards which co-eluted with the MSP fraction peaks.

Preparative HPLC. Peaks of interest were collected for bioassay and further chemical analysis. Preparative HPLC was performed using the same equipment as described above and under the same conditions. Collection parameters were established by injecting 5 μl of a hexane soluble dye (Oil Red O, Sigma Chemical Company, St. Louis, MO). When the dye passed through the detector a peak deflection was observed on the chart recorder, the time taken for the dye to begin to appear in the collecting vial was noted. Using this information it was possible to collect peaks one, two and three as well as pre-peak and post peak eluate. Confirmation of the purity of the collected fractions was obtained by reinjecting collected material after it was reduced under N_2 to a known concentration of two FE/μl.

2.10.2 Analytical gas chromatography.

Gas-chromatography (GC) analysis of extracts was performed on a Hewlett Packard Model 439 gas chromatograph (Hewlett Packard Instrument Company, Downers Grove, IL). Peak integration was performed with a Shimadzu model C-R6A Chromatopac integrator (Shimadzu Scientific Instruments, Columbia, MD). Analyses were performed on the capillary and packed columns, detailed below.

1/ SP-2330 0·2 μm Fused silica capillary, 30 m, 0·32 mm ID. (Supelco INC., Bellefonte, PN). A medium polarity column used for the preliminary analysis of partially refined material and for fatty acid methyl ester analysis.

2/ DB-5 0·25 μm Fused silica capillary 30 m, 0·32 mm ID. (J & W Scientific INC., Folsam, CA.). A low polarity column used primarily for the analysis of non-polar compounds such as hydrocarbons.

3/ DB-1 0·25 μm Fused silica capillary 15 m, 0·32 mm ID. (J & W Scientific, Folsam, CA). A non-polar column used for the analysis of non-polar compounds.

4/ SP-2250 Packed column 6 ft × 0·25 inches OD × 2 mm ID Deactiglas column. (Alltech, Deerfield, IL). This column was packed with 3 % SP-2250 stationary phase on 100/200 Mesh Supelcoport support (Supelco).

In all instances (except where otherwise indicated) sample injections were made with a Hamilton 10 μl syringe (Hamilton Company, Reno, NV). Sample sizes were of one FE in one μl of hexane, except where otherwise indicated.

Analytical Gas Chromatography: The analyses undertaken are indicated below along with column and GC conditions.

1/ The DB-1 and the SP-2250 (packed) columns were used for analyses of sterol esters, MSP, and peak numbers two and three from the HPLC separation were undertaken. Analyses were carried out on cholesterol oleate and other sterol esters standards at a range of temperatures between 100°C and 320°C to determine the retention times of these compounds.

2/ The DB-5 and SP-2330 columns were used to determine the presence of alkanes, alkenes and alcohols in the unfractionated crude extract. GC conditions were as indicated in the Results. Several alkane, alkene and alcohol standards were injected and a Kovats (Kovats, 1965) series generated for each type of compound.

3/ The SP-2330 column was used for analysis of the biologically active fraction generated by the acid/base/neutral separation including the methyl esterified and untreated fractions. The non-biologically active fractions were also analyzed for comparison.

4/ The SP-2330 and DB-5 columns were used for analyses of fractions generated by the Bio-Sil A column. Analyses of both methyl esterified and non methyl esterified fractions were performed at a lower temperature regime, (180°C to 280°C) on an SP-2330 column. At the higher temperature regime (320°C, and 280°C to

320°C), the untreated column blank, hexane (100 %), and hexane:diethyl ether (99:1) were analyzed on the SP-2330 and DB-5 columns respectively.

2.11 IDENTIFICATION OF THE ACTIVE COMPOUND BY GC-MS.

Samples for coupled Gas Chromatography-Mass Spectrometry (GC-MS) were collected from the HPLC. These samples were derived from separations of MSP fraction peak number three as described previously. Peak three was shown to be the dominant constituent of the mixture (see HPLC results). GC-MS analysis was performed by William Lusby at the United States Department of Agriculture, Beltsville, Maryland to confirm the identifications made by co-injections on the HPLC and GC. GC-MS analyses on the collected peak three and authentic cholesterol oleate and their derivatives were performed on a Finnigan-MAT model 4510 GC-MS. The instrument was equipped with a direct exposure probe and fitted with a 30 m x 0·32 mm (id) fused silica column with a 0·25 μm film of DB-1 (Bonded dimethylsilicone; J&W Scientific, Folsom, CA).

Analysis of the HPLC purified sample peak number three. The HPLC purified sample peak and cholesterol oleate standard were examined by Electron Ionization (EI) GC-MS and Chemical Ionization (CI) GC-MS. EI spectra were collected at an indicated source temperature of 150°C and an ionizing voltage of 70 eV. Chemical ionization mass spectra were obtained using methane, ammonia, perdeutro ammonia and ^{15}N-ammonia as reagent gases at an indicated source temperature of 60°C. Probe samples were desorbed from the probe tip loop by application of a heating

current of 20 mA per second. GC-MS samples were injected at a helium carrier head pressure of 11 PSI and a column temperature of 160°C.

Saponification and Formation of Fatty Acid Methyl Ester. The saponification products of the sample peak and the cholesterol oleate standard were examined by EI only, using the conditions described for intact MSP above. Approximately 10 μg of the material collected from the HPLC (Peak three) was saponified to cleave the ester linkage by refluxing with 4% methanolic potassium hydroxide at 65°C for four hours.

The tick extract and cholesterol oleate reaction mixtures were extracted with hexane and concentrated for analysis of sterol content by EI-GC-MS. A comparison of the sterol component obtained by the saponification of cholesterol oleate then made.

The fatty acid moiety from the saponification reaction was methyl esterified and identified as described below. The methanolic layers obtained in the above reactions were acidified with HCl (1 N), and extracted with hexane. The hexane mixture was concentrated to 0·5 ml and reacted with diazomethane (MeOH catalyst). The resultant fatty acid methyl esters were analyzed by EI-GC-MS, and retention time comparisons were made with standard compounds on GC.

Chapter three: RESULTS

3.1 EXPERIMENTAL: TO DETERMINE THE POSSIBLE EXISTENCE OF A PHEROMONE.

The results of this section are presented in three tables detailed below. The first column of the tables, 'Extract added', specifies which extract (*D. variabilis* or *D. andersoni* female), if any, was added to either the cleaned female or inanimate objects. The second column, 'Species of Female Treated' shows the species of female, if any, to which the extract was added, (this column was not included in Table three as extract was added to inanimate objects only). This column also gives other relevant information on any other modification, i.e., if the female tick in column one was cleaned (clean) or not cleaned (n/clean) or if it was abraded (abraded) or not abraded (n/abraded). The third column, 'Species of Male Used', shows which males were used in the bioassays.

Conspecific assay; Removal, replacement and transfer of stimulus. Results are presented in Table 1. The male mating response was significantly reduced ($P < 0 \cdot 01$) when solvent cleaned *D. variabilis* and *D. andersoni* were tested with conspecific males, (experiments one and two). When conspecific cuticular extract was applied to the cleaned (previously screened) females, the male response was shown to be significantly greater than when it was absent, ($P < 0 \cdot 01$) (experiments three and four). *D. andersoni* males responding to conspecific extract-coated dummy female ticks showed a significantly higher percentage score than those males

Table 1 Removal, restoration and transfer of male mounting responses to female ticks in *D variabilis (DV)* and *D. andersoni (DA)* as determined in bioassay with conspecific partners[a].

Exp	Extract	Female	Male	E	N	Position		Score		Time Analysis
						Tot	%	Tot	%[b]	
1	None	DV clean	DV	T	20	0	0	17	21.3**	
	None	DV n/clean	DV	C	20	20	100	80	100.0	
2	None	DA clean	DA	T	20	0	0	24	30.0**	
	None	DA n/clean	DA	C	20	20	20	80	100.0	
3	DV	DV clean	DV	T	20	20	100	80	100.0**	
	hexane	DV clean	DV	C	20	0	0	13	13.8	
4	DA	DA clean	DA	T	20	16	80	69	86.3**	
	hexane	DA clean	DA	C	20	0	0	11	13.8	
5	DV	—	DV	T	20	7	35	53	66.3**	P = 0.0001[c]
	none	—	DV	C	20	0	0	16	20.0	
6	DA	—	DA	T	20	4	20	48	60.0*	P = 0.0031[c]
	none	—	DA	C	20	0	0	22	27.8	

a Exp = experiment number; Extract = extract or other treatment applied to the female; Female = species of female treated; Male = species of male used in the bioassay; E = test (T) or control (C); N = number of repetitions; Position, Tot = total number of males reaching the gonopore, stage six; Position, % = % of all males tested that reached the gonopore; Score, Tot = the sum of all points gained by males completing part or all of the test; Score, % = (total points gained by males completing part or all of the test/total points possible) × 100.

b * $P < 0.05$; ** $P < 0.01$. (Paired T-test comparing test and control for each experiment).

c Results of paired T-test comparing time spent on DFT coated with extract vs those wothout extract.

— = DFT rather than real female was used.

presented with controls ($P < 0 \cdot 05$). *D. andersoni* female extract induced *D. andersoni* males to attempt the complete mating sequence (stage six) in four cases, (experiment five). *D. variabilis* males responding to conspecific extract-coated dummy female ticks showed a highly significantly increased percentage score compared to those males responding to controls ($P < 0 \cdot 01$)(experiment six). Male *D. variabilis* ticks reached stage six, seven times when responding to *D. variabilis* female extract.

Analysis of the total time spent by each male in contact with the dummy tick revealed that *D. andersoni* males spent a highly significantly ($P = 0 \cdot 0031$) increased amount of time on *D. andersoni* female extract coated ticks, when compared with controls. The amount of time spent by male *D. variabilis* ticks in contact with female *D. variabilis* extract-coated dummy ticks was highly significant ($P = 0 \cdot 0001$) when compared to time spent in contact with the controls.

Male response to cuticular wash extracts (0, 5, 10 and 20 minutes) placed on clean females, were 78 % (DV) 55 % (DA); 74 % (DV) 54 % (DA); 90 % (DV) 64 % (DA); 86 % (DV) and 59 % (DA); respectively. Control responses for cleaned females were all less than 5 %. Male *D. variabilis* response to cuticular scrape extract was 88 %. Male *D. andersoni* response to cuticular scrape extract was 79 %. Males did not respond to extracts made from the body surfaces of other conspecific males at the same concentrations that were found to be effective for female extracts. Male *D. variabilis* response to male *D. variabilis* extract (four male equivalents, ca. $0 \cdot 5$ FE, by weight) was $37 \cdot 5\%$. Male *D. andersoni* response to male *D. andersoni* extract (four male equivalents, ca. $0 \cdot 5$ FE by weight) was $26 \cdot 3\%$.

Heterospecific assay. *Species and generic specificity of the pheromone.*

Results of these bioassays are presented in Table 2. Male *D.variabilis* showed a significant response to *D. andersoni* female extract placed on cleaned *D. variabilis* females ($P < 0 \cdot 01$ experiment one). *D. andersoni* males also showed a significant response to *D. andersoni* female extract when placed on *D. variabilis* females ($P < 0 \cdot 01$, experiment two). *D. variabilis* males showed a significant response to *D. variabilis* extract placed on *D. andersoni* females ($P < 0 \cdot 01$, experiment three). *D. andersoni* males also showed a significant response to *D. variabilis* female extract placed on *D. andersoni* females ($P < 0 \cdot 05$, experiment four). *D. variabilis* males showed a significantly higher response to conspecific female extract (100 %) than to heterospecific female extract (83 · 8 %) ($P < 0 \cdot 05$). *D. andersoni* males showed a significantly higher response to conspecific female extract (86 · 3 %) than to heterospecific female extract (61 · 3 %) ($P < 0 \cdot 05$). *D. variabilis* males were more responsive to conspecific female extract (100 %) than *D. andersoni* males (86 · 3 %) ($P < 0 \cdot 05$). *D. andersoni* males and *D. variabilis* males were equally responsive to heterospecific extract (no significant difference between percentage scores). *D. andersoni* male response to DFTs coated with *D. variabilis* female extract was not significant (experiment five). *D. variabilis* males showed a significantly increased response to *D. andersoni* female extract when compared with the controls ($P < 0 \cdot 05$). Analysis of the total time spent by each male in contact with the DFT revealed that *D. andersoni* males did not spend a significantly greater amount of time in contact with *D. variabilis* extract than the controls ($P = 0 \cdot 2742$). The amount of time spent by *D. variabilis* males in contact with *D. andersoni* extract coated DFTs

Table 2 Removal, restoration and transfer of male mounting responses to female ticks in *D variabilis (DV)* and *D. andersoni (DA)* as determined in bioassay with heterospecific partners[a].

Exp	Extract	Female	Male	E	N	Position		Score		Time Analysis
						Tot	%	Tot	%[b]	
1	DA	DV	DV	T	20	16	80	67	83.8**	
	hexane	DV	DV	C	20	0	0	11	13.8	
2	DA	DV	DA	T	20	14	70	70	87.5**	
	hexane	DV	DA	C	20	0	0	17	21.3	
3	DV	DA	DV	T	20	17	85	74	92.5**	
	hexane	DA	DV	C	20	0	0	9	11.3	
4	DV	DA	DA	T	20	8	40	49	61.3*	
	hexane	DA	DA	C	20	0	0	24	30.0	
5	DV	——	DA	T	20	1	5	30	37.5ns	P = 0.2742[c]
	none	——	DA	C	20	0	0	22	27.8	
6	DA	——	DV	T	20	3	15	41	51.3*	P = 0.0047[c]
	None	——	DV	C	20	0	0	16	20.0	

a Exp = experiment number; Extract = extract or other treatment applied to the female; Female = species of female treated; Male = species of male used in the bioassay; E = test (T) or control (C); N = number of repetitions; Position, Tot = total number of males reaching the gonopore, stage six; Position, % = % of all males tested that reached the gonopore; Score, Tot = the sum of all points gained by males completing part or all of the test; Score, % = (total points gained by males completing part or all of the test/total points possible) × 100.

b * $P < 0.05$; ** $P < 0.01$. ns = not significant (paired T-test comparing test and control for each experiment).

c Results of paired T-test comparing time spent on the DFT coated with extract vs those without extract.

was significantly greater than the time spent in contact with the controls (P = 0·0047).

Table 3 summarizes the results of the tests using males of four different species to conspecific and heterospecific crude extracts. In general, cross reactivity between species and both genera of hard ticks was evident. Control values, i.e., male responses to cleaned ticks were all zero, (not shown in the table). For example *D. variabilis* males did not distinguish between *D. variabilis* and *D. andersoni* extracts, but they showed much weaker or insignificant responses to extracts of the other genera. *D. andersoni* males, however, responded strongly to extracts of all species. In general *A. americanum* and *A. maculatum* males responded more strongly to extracts of the same genus than to those of other genera at one FE concentration. Although *A. maculatum* response to *D. variabilis* female extract (43 %, one FE) was not significantly different (P < 0·01) from *A. americanum* female response to *A. maculatum* (54 %, one FE), *A. americanum* and *A. maculatum* responses to *D. andersoni* extract at all three concentrations were significantly less than their responses to other extracts (except *A. maculatum* male response to *D. variabilis* and *D. andersoni* female extract at 0·01 FE.

3.2 BIOASSAYS TO DETERMINE THE IMPORTANCE OF FEMALE BODY TEXTURE, SIZE, AND PHEROMONE CONCENTRATIONS.

Body Texture. The results of the experiment to determine the importance of the female surface texture in providing a mechanical stimulus to male ticks are provided in Table 4. Plate 1 shows the cuticular ridges before and after abrasion.

Table 3 Bioassay responses of male ticks of four species of Ixodidae to cleaned females treated with crude extract from females[1].

Tick Species (♀)	MSP Conc. (FE)	DV ♂	DA ♂	AA ♂	AM ♂
D. variabilis	1.0	$90^{a,\alpha}$	$47^{b,\beta}$	$31^{c,\epsilon}$	$43^{b,c,\beta}$
	0.1	$80^{a,\alpha}$	$43^{b,\alpha}$	$29^{b,c,\beta,\epsilon}$	$23^{c,\epsilon}$
	0.01	$3^{b,\alpha}$	$23^{a,b,\beta}$	$0^{b,\beta}$	$3^{b,\beta}$
D. andersoni	1.0	$84^{a,\beta}$	$54^{b,\beta}$	$5^{c,\delta}$	$6^{c,\epsilon}$
	0.1	—	$29^{a,\alpha}$	$18^{a,b,\epsilon}$	$4^{b,\delta}$
	0.01	—	$19^{a,\beta}$	$0^{b,\alpha}$	$0^{b,\beta}$
A. americanum	1.0	$29^{c,\beta}$	$58^{b,\alpha,\beta}$	$80^{a,\alpha}$	—
	0.1	$25^{c,\beta}$	$40^{b,c,\alpha}$	$84^{a,\alpha}$	$46^{b,\beta}$
	0.01	$6^{a,b,\alpha}$	$3^{b,\epsilon}$	$19^{a,\alpha}$	—
A. maculatum	1.0	$41^{b,\beta}$	$72^{a,\alpha}$	$54^{b,\beta}$	$71^{a,\alpha}$
	0.1	$23^{c,\beta}$	$43^{b,\alpha}$	$39^{b,\beta}$	$79^{a,\alpha}$
	0.01	$8^{c,\alpha}$	$58^{a,\alpha}$	$15^{c,\alpha}$	$34^{b,\alpha}$

1 Females were cleaned by immersion in hexane for 48 hours; the tick extract fraction co-eluting with steryl ester standards was collected and diluted to the dilutions shown in the table. Samples of each extract were applied to cleaned conspecific females. Following solvent evaporation (5 - 10 min), behavioral assays were performed with sexually active males (each tested with five males, three trials/male. The values in the table represent the mean percent response.

a,b,c Male response (%) of each of the four species were compared with each other for each species of female extract at a given concentration in the same row. Responses with the same superscript were not significantly different from one another by the Duncan's multiple range test. ($P < 0.01$).

$\alpha,\beta,\epsilon,\delta$ Male responses (%) of a given species were compared with each other for each of the four species of female extracts at a given concentration in the same column. Responses with the same superscript were not significantly different from one another by the Duncan's multiple range test. ($P < 0.01$).

Table 4 Removal and restoration of male mounting responses to abraded female ticks in *D variabilis (DV)* and *D. andersoni (DA)* as determined in bioassay with conspecific partners[a].

						Position		Score	
Exp	Extract	Female	Male	E	N	Tot	%	Tot	%[b]
1	none	DV abraded	DV	T	40	13	33	65	40.6**
		DV	DV	C	40	40	100	80	100.0
	DV	DV abraded	DV	C	20	16	80	73	92.5ns
2	none	DA abraded	DA	T	20	2	10	11	13.8**
		DA	DA	C	20	20	100	80	100.0
	DA	DA abraded	DA	C	20	18	90	70	87.5ns

[a] Exp = experiment number; Extract = extract or other treatment applied to the female; Female = species of female treated; Male = species of male used in the bioassay; E = test (T) or control (C); N = number of repetitions; Position, Tot = total number of males reaching the gonopore, stage six; Position, % = % of all males tested that reached the gonopore; Score, Tot = the sum of all points gained by males completing part or all of the test; Score, % = (total points gained by males completing part or all of the test/total points possible) × 100.

[b] * $P < 0.05$; ** $P < 0.01$. ns = not significant.

When the surfaces of both *D. andersoni* and *D. variabilis* were abraded with fine sandpaper, the male mating response was very significantly reduced ($P < 0{\cdot}01$) when compared to females which were not abraded. When extract was applied to these abraded females the male mating response was restored and was not significantly different from the untreated females. This indicates that male *D. variabilis* and *D. andersoni* were not utilizing the cuticular ridges as specific mating cues.

Size of female and concentration of extract. Results of this analysis are presented in Table 5 which is divided into two parts (*D. variabilis* male responses and *D. andersoni* male responses). The results are also presented graphically in Fig. 3.

DFT size: When size of DFT was considered separately and both species of male response were grouped together, the size of the DFT appeared to be unimportant in determining male response, ($F = 1{\cdot}23$, $P = 0{\cdot}2940$). However, when each species of male response to DFT size was considered separately it was seen that DFT size was important, ($F = 39{\cdot}98$, $P = 0{\cdot}0001$). Comparisons of means by Duncan's Multiple Range Test indicated that *D. variabilis* males had a significantly greater response ($P < 0{\cdot}05$) to DFT C than to DFT A. This effect was more pronounced in those tests with *D. variabilis* males responding to DFTs coated with *D. andersoni* extract. *D. andersoni* males, conversely, had a significantly higher response ($P < 0{\cdot}05$) to DFT A than to DFT C. This effect was only marginally more pronounced with *D. andersoni* male response to *D. variabilis* female extract.

Table 5 Mean time (seconds ± S.E.) spent by male *D. variabilis* and *D. andersoni* in contact with DFTs coated with female extract at one of four concentrations for three sizes of DFT.

Species of Extract	DFT Size	Concentration of Extract (FE) 1	2	4	8
		D. variabilis males			
D. variabilis	A	$291.7^{b,\alpha}$ ±69.7	$938.6^{a,\alpha,\beta}$ ±281.5	$488.3^{b,\alpha}$ ±181.7	$50.9^{c,\beta}$ ±19.2
	B	$65.8^{b,\beta}$ ±32.2	$390.5^{a,\beta}$ ±150.9	$436.0^{a,\alpha}$ ±151.7	$473.7^{a,\alpha}$ ±143.9
	C	$226.4^{c,\alpha}$ ±89.1	$1265.2^{a,\alpha}$ ±285.1	$495.9^{b,c,\alpha}$ ±128.8	$681.5^{a,b,\alpha}$ ±155.0
D. andersoni	A	$145.8^{a,\alpha}$ ±49.6	$102.8^{a,\beta}$ ±48.4	$120.0^{a,\beta}$ ±48.3	$48.3^{a,\beta}$ ±10.3
	B	$100.3^{b,\alpha}$ ±38.7	$107.7^{a,b,\beta}$ ±22.8	$242.4^{a,\alpha,\beta}$ ±68.5	$165.6^{a,b,\beta}$ ±56.4
	C	$220.4^{a,\alpha}$ ±66.6	$385.5^{a,\alpha}$ ±92.7	$445.0^{a,\alpha}$ ±163.4	$625.2^{a,\alpha}$ ±183.8
		D. andersoni males			
D. variabilis	A	$87.0^{a,\alpha}$ ±23.0	$129.0^{a,\alpha}$ ±59.6	$103.5^{a,\alpha}$ ±40.7	$384.4^{a,\alpha}$ ±224.4
	B	$76.0^{b,\alpha}$ ±24.9	$81.3^{a,b,\alpha}$ ±20.2	$161.2^{a,\alpha}$ ±41.4	$75.6^{a,b,\alpha,\beta}$ ±19.2
	C	$7.1^{b,\beta}$ ±0.8	$10.1^{a,b,\beta}$ ±2.0	$16.1^{a,\beta}$ ±3.4	$17.9^{a,\beta}$ ±5.0
D. andersoni	A	$274.7^{a,\alpha}$ ±130.8	$57.2^{a,b,\alpha}$ ±14.0	$47.2^{b,\alpha}$ ±12.0	$104.1^{a,\alpha}$ ±4.5
	B	$109.8^{a,\alpha,\beta}$ ±73.4	$38.0^{a,\alpha}$ ±12.1	$26.3^{a,\alpha}$ ±5.1	$36.0^{a,\beta}$ ±5.9
	C	$34.3^{a,\beta}$ ±6.8	$43.5^{a,\alpha}$ ±17.9	$13.2^{b,\beta}$ ±6.9	$20.5^{a,b,\beta}$ ±12.3
		D. variabilis	*D. andersoni*		
Controls	A	47.4^{α} ±6.6	46.2^{α} ±10.5		
	B	37.0^{α} ±5.0	$26.6^{\alpha,\beta}$ ±5.7		
	C	33.2^{α} ±6.0	22.4^{β} ±6.3		

a,b,c Male bioassay response (%) to each of the concentration levels were compared with each other for each of the ball sizes, species extract and for each species of male. Responses with the same superscript were not significantly different from each other (Duncans Multiple Range Test $P < 0.05$).

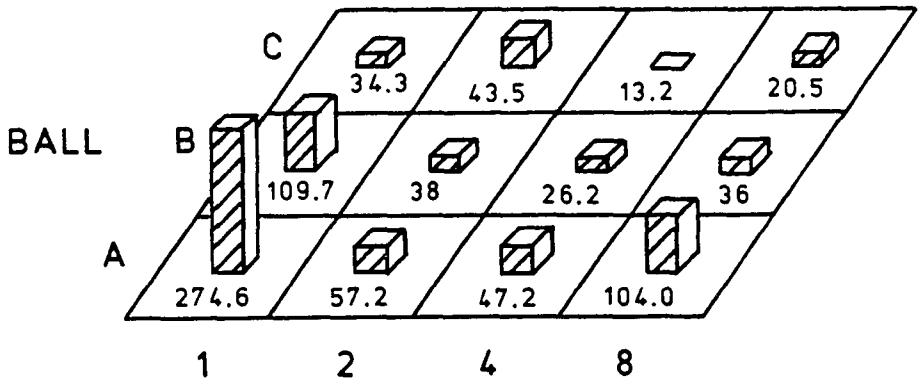
α,ß Male bioassay response (%) to each of the ball sizes were compared with each other for each species extract at each concentration and for each species of male. Responses with the same superscript were not significantly different from each other (Duncans Multiple Range Test $P < 0.05$).

Figure 3. Block chart of mean times (seconds) spent by male *Dermacentor variabilis* or *D. andersoni* males in contact with DFTs (three sizes) coated with *D. variabilis* or *D. andersoni* female extract (four concentrations).

A

DA♂WITH DA♀EXTRACT

BLOCK CHART OF TIME MEANS

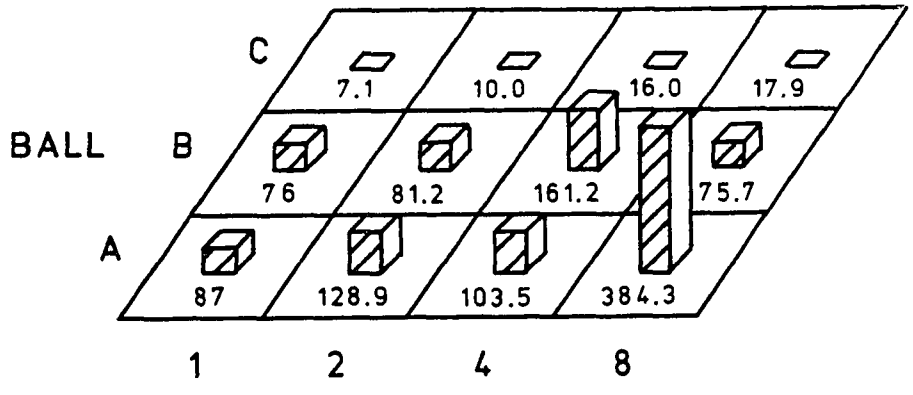


CONCENTRATION (FE)

B

DA♂WITH DV♀EXTRACT

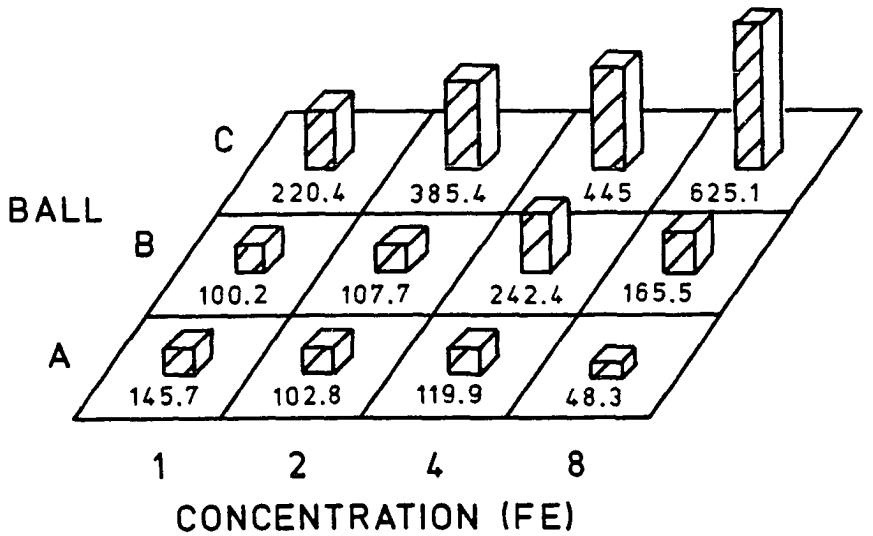
BLOCK CHART OF TIME MEANS



CONCENTRATION (FE)

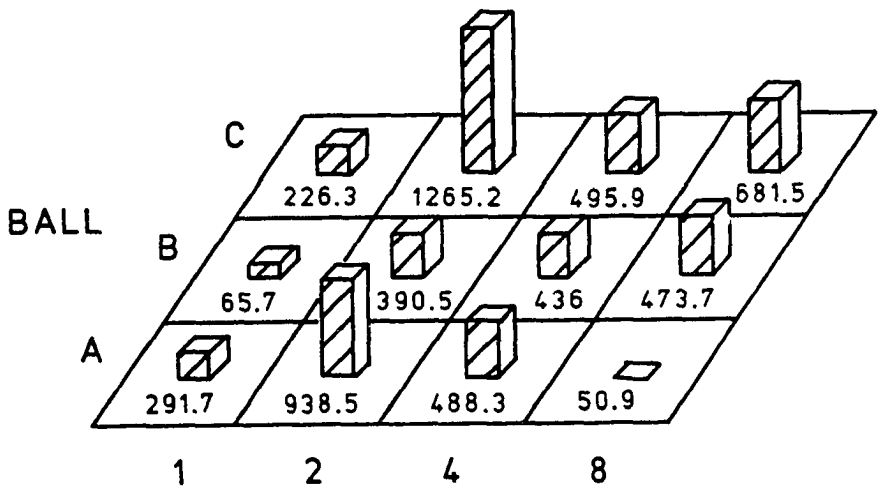
C DV♂WITH DA♀EXTRACT

BLOCK CHART OF TIME MEANS



D DV♂WITH DV♀EXTRACT

BLOCK CHART OF TIME MEANS



Comparison of control results indicated that *D. andersoni* males preferred DFT A than DFT C. *D. variabilis* males did not discriminate between control DFTs.

Concentration of Extract: Analysis indicated that there was a significant added variance component due to the concentration of extract, ($F = 6 \cdot 09$, $P = 0 \cdot 0004$), i.e. concentration of female extract was important in determining the amount of time spent by *D. variabilis* or *D. andersoni* males in contact with the DFTs. Comparisons of means by Duncan's Multiple Range Test indicated that all four concentrations of extract cause males to spend significantly greater time in contact with the DFTs coated with extract than those decoys without extract. Two FE induced a greater response ($P < 0 \cdot 05$) than one FE and in some instances the male response was observed to decrease with increasing concentration. The concentration effects were mediated by the effects of species of male tested ($F = 9 \cdot 82$, $P = 0 \cdot 0001$), species extract used ($F = 7 \cdot 87$, $P = 0 \cdot 0001$) and size of the DFT tested ($F = 4 \cdot 32$, $P = 0 \cdot 0003$).

Species of Male Tested: There was a significant added variance component due to the species of male tick tested ($F = 95 \cdot 86$, $P = 0 \cdot 0001$), i.e. one species of male tick was more responsive to extract than the other. The data showed that *D. variabilis* males spent a significantly greater amount of time in contact with DFTs than *D. andersoni* males did. This amount of contact time was influenced by DFT size ($F = 39 \cdot 98$, $P = 0 \cdot 0001$), extract type ($F = 13 \cdot 64$, $P = 0 \cdot 0002$), and by concentration of extract ($F = 9 \cdot 82$, $P = 0 \cdot 0001$).

Extract used: There was a significant added variance component due to the species of extract coated on the DFTs ($F = 30 \cdot 52$, $P = 0 \cdot 0001$). This time was

also influenced by size of DFT, concentration of extract and the species of male which was being tested, as indicated above.

3.3 CHEMICAL CHARACTERIZATION OF THE TICK EXTRACTS.

Results of the characterization of the crude extract are summarized in Table 6 below for ease of comparison. Drawings of the TLC chromatograms are shown in Fig 4 A (Solvent system 1, IB-2 plate); Fig 4 B (Solvent system 2, IB-2 plate); Fig 4 C (Non Polar solvent system, IB-2 plate) and 4 D (non polar solvent system, HPTLC plate). Although C18 plates were used in the characterization the results are not presented here as no additional classes of compounds were resolved. Spots marked on the plates with an 'M' turned magenta, those marked with a 'P' turned purple after being sprayed with sulphuric acid/ethanol (50:50 v/v) and charred (100°C for ten minutes). Spots which turned purple contained sterol components. Cross-hatched spots were more intensely charring than those which were represented with a continuous line, which in turn were more intense than those represented by a discontinuous line.

Numerous distinct spots were resolved on the TLC plates by the different solvent systems. Comparisons with the authentic standards and R*f* values for all the identifiable classes of lipids resolved by these systems are given in the Appendix A, Tables 1-4.

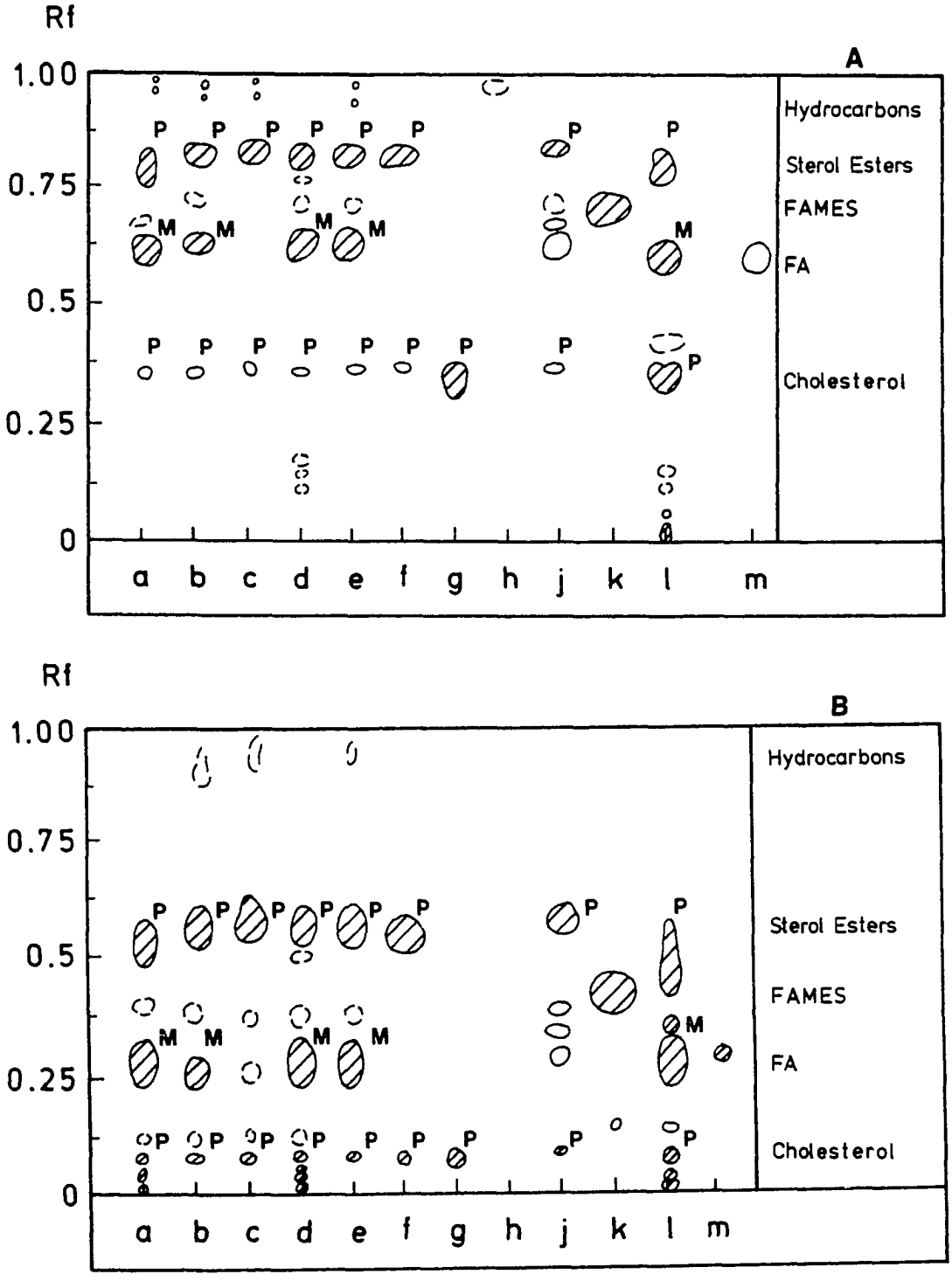
Hydrocarbons were observed in all species of tick extract tested although not all solvent systems resolved hydrocarbons for each species. The hydrocarbons

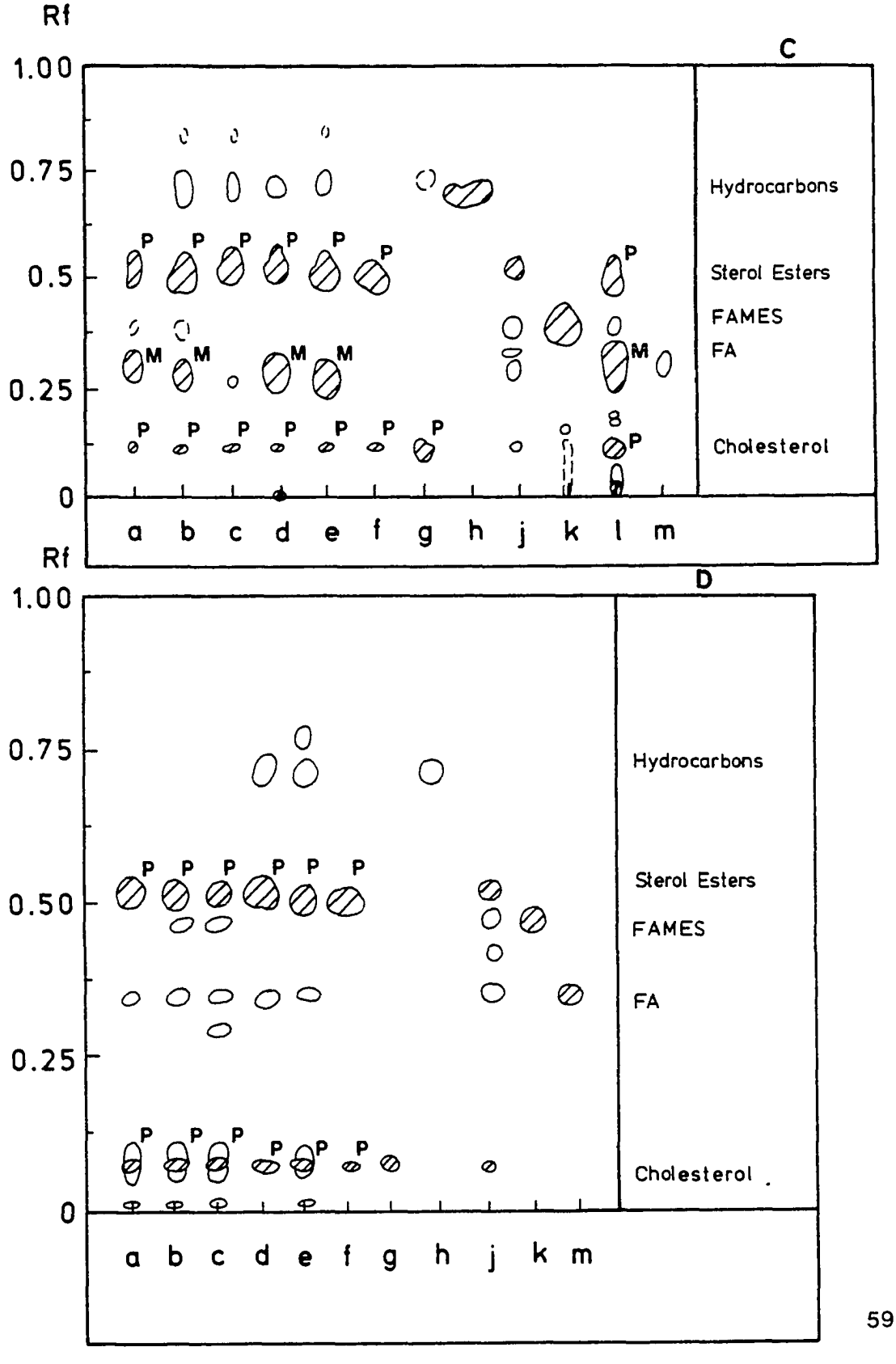
Table 6. Summary of charaterization of non-polar lipid classes of five species of Ixodid tick by TLC analysis.

			Compound Classes and Relative Intensity of spots.					
			hydro-carbons	hydro-carbons	Sterol Esters	FAMEs	FA	Chole-sterol
Ext	Plate	Solvent System						
	IB-2	Skipski	++	+++	+++	+	+++	+++
DV	IB-2	Mangold	—	—	+++	+	+++	+++
	IB-2	Non-polar	—	—	+++	+	+++	+++
	HPTLC	Non-polar	—	—	+++	—	++	+++
	IB-2	Skipski	++	++	+++	+	+++	++
DA	IB-2	Mangold	+	—	+++	+	+++	++
	IB-2	Non-polar	+	++	+++	—	+++	+++
	HPTLC	Non-polar	+	+	+++	—	++	+++
	IB-2	Skipski	—	—	+++	+	+++	++
AM	IB-2	Mangold	—	—	+++	+	+++	+++
	IB-2	Non-polar	—	++	+++	—	+++	+++
	HPTLC	Non-pola-	—	++	+++	—	++	++
	IB-2	Skipski	++	++	+++	—	—	++
AA	IB-2	Mangold	+	—	+++	+	+	+++
	IB-2	Non-polar	+	++	+++	—	++	+++
	HPTLC	Non-polar	—	—	+++	++	++	+++
	IB-2	Skipski	++	++	+++	+	+++	++
HD	IB-2	Mangold	+	—	+++	+	+++	+++
	IB-2	Non-polar	+	++	+++	+	+++	+++
	HPTLC	Non-polar	—	—	+++	++	++	+++

DV = Dermacentor variabilis; DA = Dermacentor andersoni; AM = Amblyomma maculatum; AA = Amblyomma americanum; HD = Hyalomma dromedarii. +++ = intensely charring spot; ++ = moderately charring spot; + = lightly charring spot; -- = spot not present. Skipski = solvent syste one; Mangold = solvent system two; Non-polar = solvent system three.

Figure 4 (A-D). Drawings of chromatograms of TLC analyses of non-polar cuticular lipids and standards on silica IB-2 plates and silica High Performance TLC plates. (A = IB-2 plate; Skipski solvent system. B = IB-2 plate; Mangold solvent system. C = IB-2 plate; Non-polar solvent system. D = HPTLC plate; Non-polar solvent system). (a) *D. variabilis* extract; (b) *Hyalomma dromedarii* extract; (c) *Amblyomma americanum* extract; (d) *A. maculatum* extract; (e) *D. andersoni* extract; (f) sterol ester standard; (g) cholesterol standard; (h) hydrocarbon standard; (j) non polar lipid mix; (k) lipid (fatty acid methyl ester) standard; (l) *D. variabilis* cuticle soxhlet extract; (m) fatty acid standard. Spots indicated by M and P appeared magenta and purple respectively when visualized. Cross hatched spots were the most intensely charred.





typically migrate farthest of all the compounds present and tended to resolve into two spots which may represent alkanes and alkenes.

Sterol esters were observed in all tick fractions and in all solvent systems. Spots were large, and intensely charring. They turned purple after being sprayed with the ethanol/sulphuric acid mixture, indicating the presence of the sterol moiety.

Spots which co-migrated with FAME standards were observed in all species but were not resolved on all plates in all solvent systems. Spots were faint indicating small amounts of material.

Fatty acids were observed in all the five extracts. Large quantities of material apperaed to be present in four of the extracts as indicated by the large intensely charring spots. The *A. americanum* extract appeared to contain little or no fatty acid by comparison. Cholesterol was observed in all five extracts. Quantities appeared to be quite large as indicated by the very dense, intensely charred spot.

3.4 IDENTIFICATION OF THE BIOLOGICALLY ACTIVE CHEMICAL CLASS

Bioassay of the fractions resulting from the liquid:liquid separation indicated that almost all of the biological activity remained in the non-polar (hexane) layer. This fraction contained 88·75% of the biological activity versus 8·75% for the methanol fraction. Controls were 12·5% and 11·25% respectively and were not considered to be significantly different from the blank controls. These results along with the TLC results below suggested that the biological activity was likely to be

associated with either the hydrocarbons, sterol esters, FAMEs or fatty acids but not cholesterol.

TLC analysis (IB-2 plate, heptane:benzene 90:10 v/v) revealed that the hexane fraction contained primarily non-polar material, (*Rf* = 0·94), sterol esters (*Rf* = 0·55), fatty acids (*Rf* = 0·46) and cholesterol (*Rf* = 0·1). The methanol fraction (i.e., the polar layer) did not appear to contain these compounds. Some more polar material was present although it did not migrate far from the origin (*Rf* = 0·04), Fig. 5.

When the material was spotted on an IB_2 plate and developed in a hexane:diethyl ether (90:10 v/v) system which is slightly more polar than heptane:benzene (90:10 v/v) the separation was seen clearly to have occurred although some polar material was included in the non-polar (hexane) fraction, Fig. 6. The hexane fraction contained hydrocarbons (*Rf* = 0·69), sterol esters (*Rf* = 0·61), fatty acid methyl esters (*Rf* = 0·52), fatty acids (*Rf* = 0·38) and cholesterol (*Rf* = 0·12). The methanol fraction contained a major spot at *Rf* = 0·15 (cholesterol standard *Rf* = 0·15) and a minor spot at *Rf* = 0·62 (sterol esters) and *Rf* = 0·36 (fatty acids). The hexane fraction contained 2·47 $\mu g/\mu l$ of dissolved material. The methanol fraction contained 2·91 $\mu g/\mu l$ of dissolved material.

Bioassay of the acid, base and neutral fractions (from the hexane fraction of the liquid:liquid separation) revealled that almost all almost all of the biological activity was found in the neutral fraction (77·5 % and 90 % at 1 and 5 FE respectively). Male responses to the acid and base fractions were not significant, (11·9 % and 3·8 % respectively). Controls gave an average response of 5 %.

Figure 5. Drawing of chromatogram of TLC analysis (IB-2 plate, heptane:benzene 90:10 v/v) of five hour methanol and hexane liquid : liquid separation fractions of crude *D. variabilis* extract with standards. (a) hydrocarbon mix; (b) fatty acid (arachidonic acid); (c) methanol fraction (five hour extract); (d) hexane fraction (five hour extract); (e) sterol esters; (f) cholesterol.

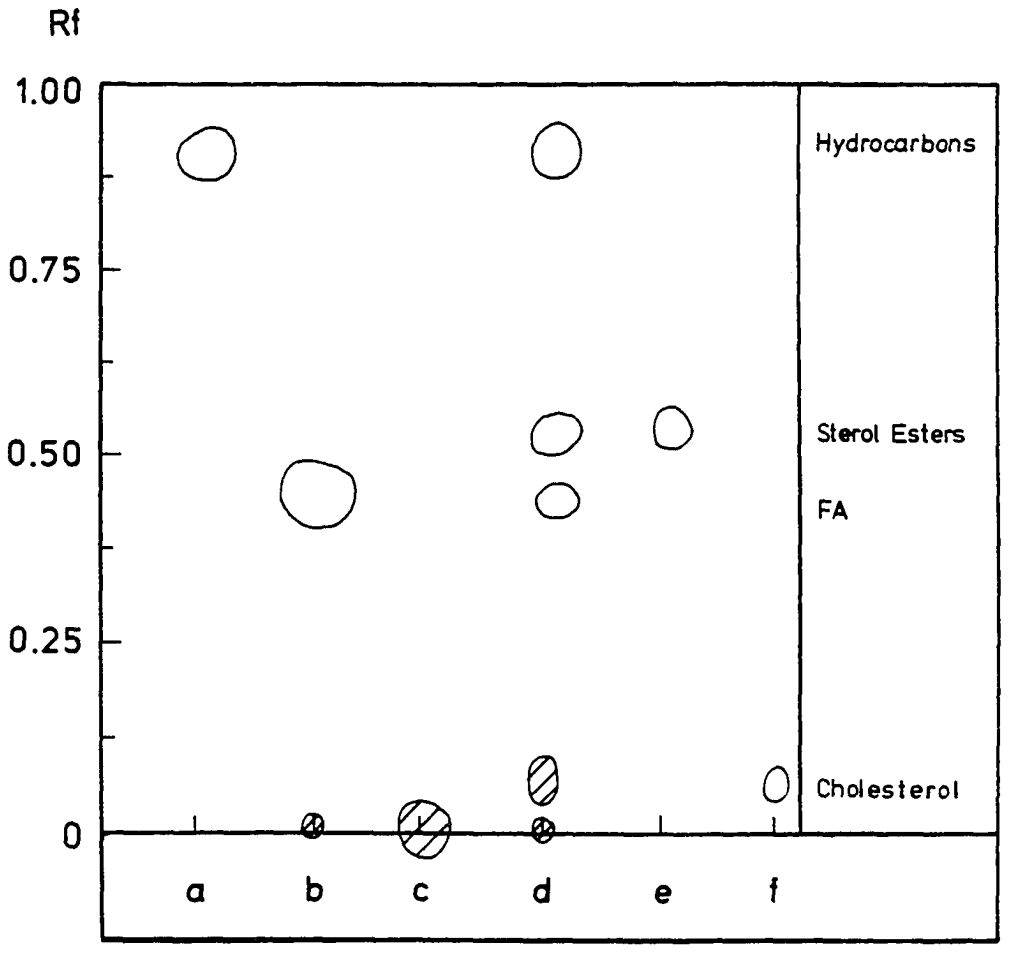
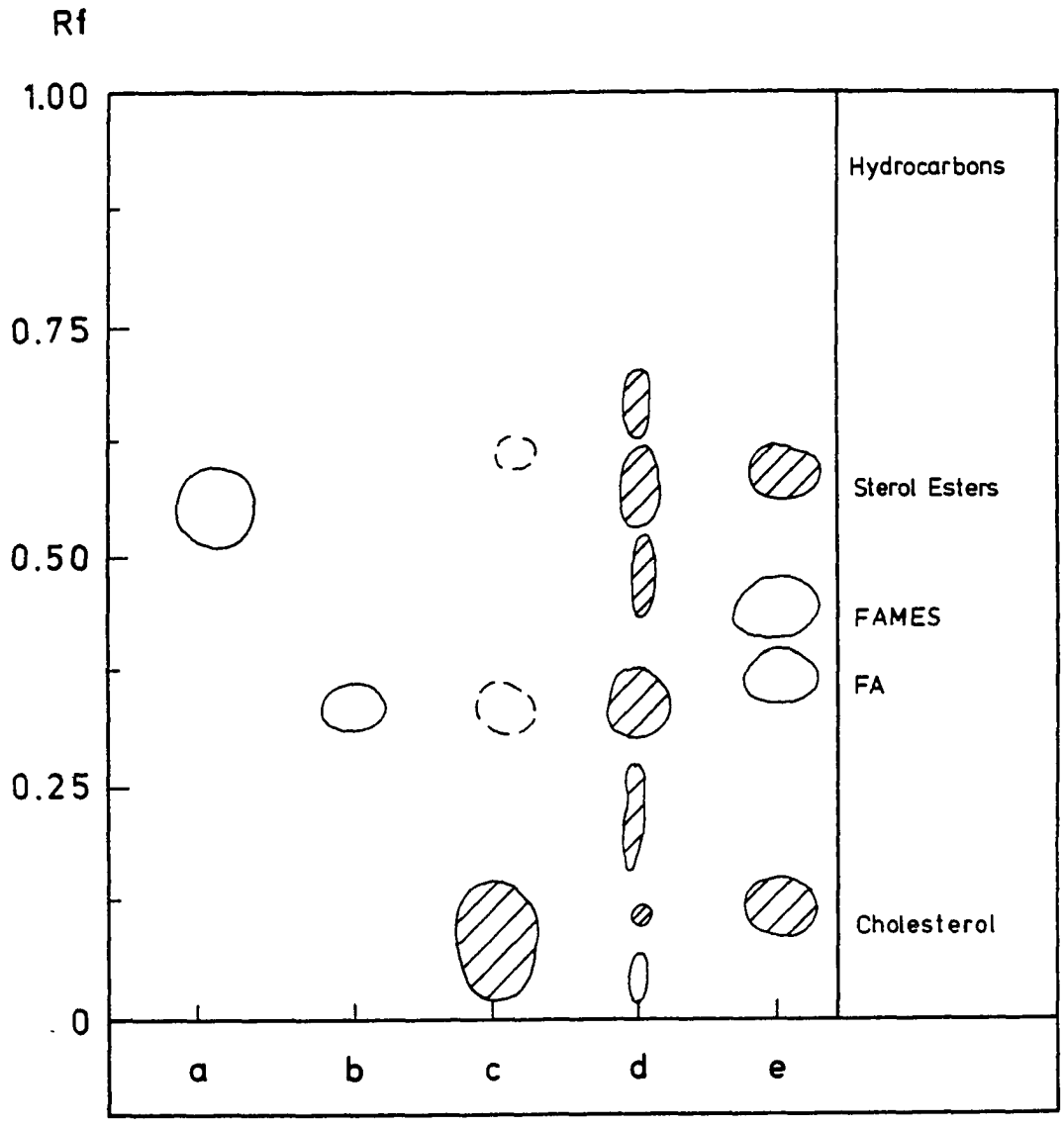


Figure 6. Drawing of chromatogram of TLC analysis (IB-2 plate, hexane:diethyl ether 90:10 v/v) of five hour methanol and hexane liquid:liquid separations of crude *D. variabilis* extract with standards. (a) hydrocarbon mix; (b) fatty acid (arachidonic acid); (c) methanol fraction; (d) hexane fraction; (e) non polar lipid mix (sterol ester, fatty acid, methyl ester, fatty acid, cholesterol).



Chromatography of the acid, base and neutral fractions by TLC (IB-2 plate, non-polar solvent system) revealed that the acid fraction contained material that gave four spots Rf value = 0·56, 0·42, 0·29, and 0·05, (Fig. 7). The base fraction contained no material that was apparent under this TLC regime. The neutral fraction contained material that resolved into five spots. The material contained in spot Rf = 0·93 is believed to be hydrocarbons. The spot at Rf = 0·61 is believed to be sterol esters. Those at Rf = 0·32 and Rf = 0·09 are fatty acids and cholesterol respectively. The location of authentic standards are also shown on the plate. All these spots are represented to a lesser extent in the acid fraction. The sterol ester spot is however much more intense in the neutral fraction.

The results of the bioassay of fractions generated by column chromatography (Mallinkrodt silica column, Table 7) revealed that most of the male bioassay response was to the hexane fraction; except for the 15 FE concentration of the hexane:diethyl ether (80:20) eluate, no significant activity was found in any of the other fractions. These results made it possible to optomize the choice of column support and solvent systems for the most efficient separation of the active fraction.

Using Bio-Sil A as the column support and a range of non-polar solvent systems from hexane (100%) to hexane:diethyl ether 80:20, v/v, the 99:1 hexane:diethyl ether fraction was found to contain virtually all of the biological activity in the neutral *D. variabilis* fraction. This fraction when analyzed by TLC was found to contain the sterol ester components of the extract only. Figure 8 shows a TLC of all the compounds present in all the fractions generated. When 1FE of

Figure 7. Drawing of chromatogram of TLC analysis (IB - 2 plate, non-polar solvent system) of the acid, base and neutral fractions of the hexane fraction of *D. variabilis* extract with standards. (a) acid fraction; (b) base fraction; (c) neutral fraction; (d) sterol ester; (e) n-alkane and n-alkene; (f) fatty acids (oleic acid and arachidonic acid); (g) cholesterol; (1) hexane solvent front, (2) hexane : diethyl ether (80 : 20) solvent front, (3) origin.

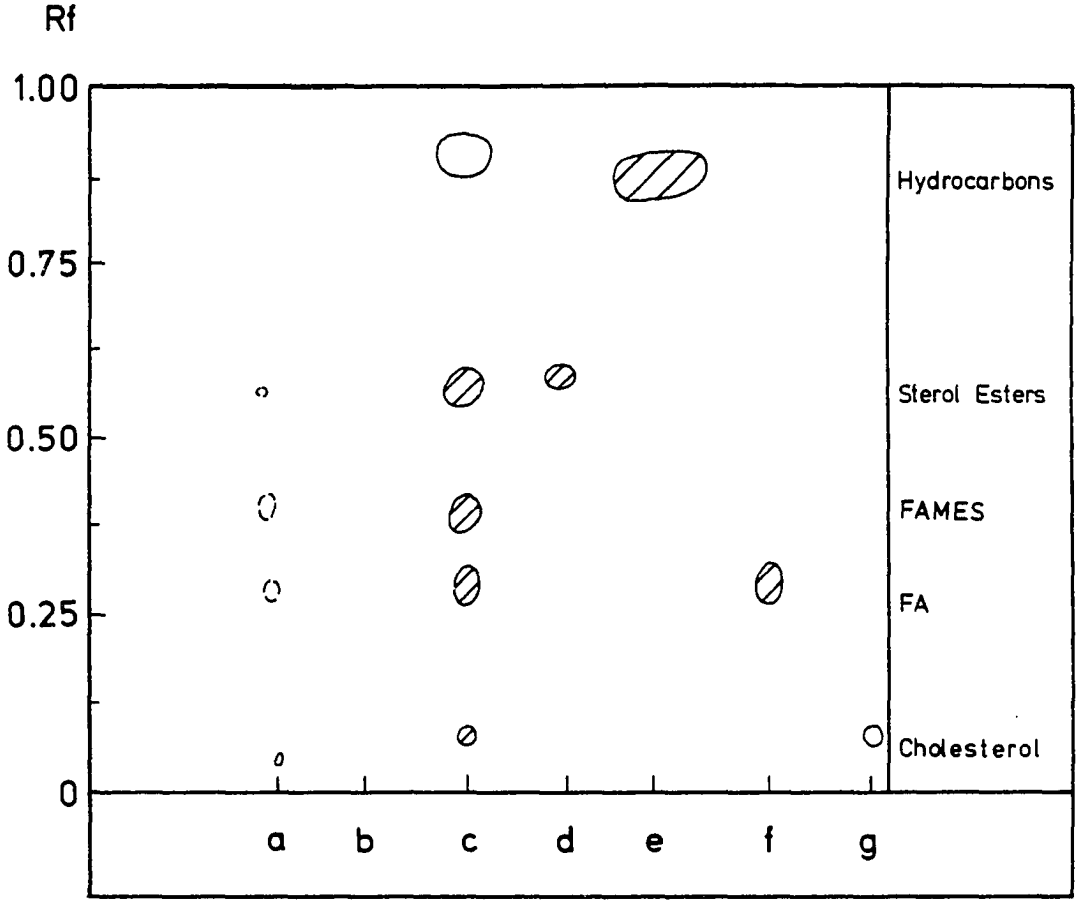
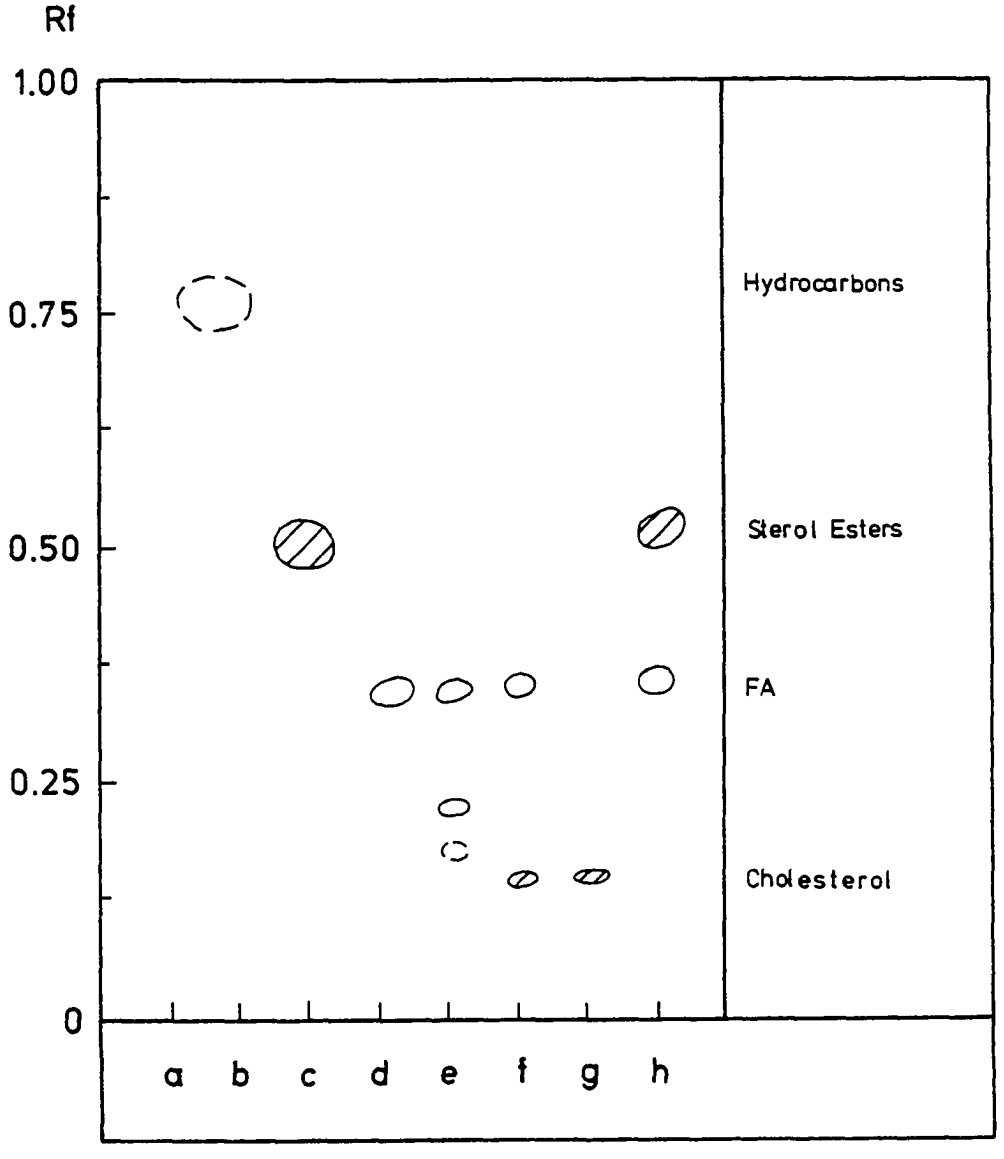


Table 7 Average response[1] (%) of *D. variabilis* males when bioassayed with crude *D. variabilis* female extract. Fractionated on a Mallinkrodt silica column.

	Concentration of Eluate (in Female Equivalents)					
Eluate	30	15	3.7	1.5	0.7	0.4
hexane	78[a]	88[a]	60[c]	58[c]	88[a]	66[a]
hexane:diethyl ether (80:20)	—	36[c]	14[b]	0[b]	1[b]	11[b]
ether	—	1[b]	0[b]	1[b]	0[b]	0[b]
chloroform	—	0[b]	0[b]	9[b]	0[b]	0[b]
methanol	—	0[b]	0[b]	0[b]	0[b]	0[b]

[1] Average of sixty observations for each value.
[a] Not significantly different ($P < 0.01$) from the male bioassay response obtained from the crude unfractionated extract.
[b] Not significantly different ($P < 0.01$) from the corresponding solvent blank controls.
[c] Not significantly different ($P < 0.01$) from other values denoted by [c].
[d] Significantly different ($P < 0.01$) from 0% response.
— Not done.

Figure 8. Drawing of chromatogram of TLC analysis (HPTLC plate, non polar solvent system) of the fractions generated by separation of the neutral fraction of *D. variabilis* extract on a Bio-Sil A column. (a) **F1**, Column blank, 20 volumes eluted and collected before addition of biological material, concentrated and spotted on the TLC plate; (b) **F2**, hexane 100 %; (c) **F3**, hexane:diethyl ether, 99:1; (d) **F4**, hexane:diethyl ether, 98:2; (e) **F5** hexane:diethyl ether, 95:5; (f) **F6**, hexane:diethyl ether 90:10; (g) **F7**, hexane:diethyl ether, 80:20; (h) **F8**, soxhlet extract of Bio-Sil A column after elution of extract.



each of the Bio-Sil A fractions was spotted on a HPTLC plate, developed and visualized as previously described, it was immediately apparent that F1 (a, column blank) contained no discernable material. F2 (b, hexane 100 %) contained a very small amount of hydrocarbon, R*f* = 0·80. F3 (c, hexane:diethyl ether 99:1) contained a densely charring spot, R*f* = 0·53, while F4 (d), F5 (e) and F6 (f), contained faint spots, R*f* = 0·36. F5 and F6 also contained several other spots at the positions where fatty acids, R*f* = 0·23, and alcohols, R*f* = 0·15, respectively would be expected to be found; F7 (g) also contained a spot that co-chromatographed with alcohols, R*f* = 0·15. F8 (h), the soxhlet column extract also contained the denser spot seen in F3 and this may account for its relatively high bioassay results.

Results of the bioassay of the fractions from the Bio-Sil A column are shown in Table 8. Fraction F3, the 99:1 hexane:diethyl ether fraction, contained all of the biological activity at 1 FE. Although males responded on occassion to other fractions, none of those responses were significant. However, at ten FE biological activity was found in fractions three, six and seven. Biological activity, however, was significantly higher in fraction three than in the other active fractions.

Overall, it was clear that, F3, the only fraction that showed significant biological activity, had an intensely charring spot which suggested that it may comprise a single compound or class of compounds responsible for the biological activity. It was clear from previous analysis that this 'active' spot contained sterol esters. This result strongly suggested that Mounting Sex Pheromone (MSP) was either a single sterol ester or a mixture of sterol esters. This fraction, which had

Table 8 Average response (%) of *D. variabilis* males when bioassayed with fractions of crude *D. variabilis* female extract eluted from the Bio-Sil A column.

Eluate	Concentration of Eluate in Female Equivalents	
	1	10
F1 column blank	4[a]	4[c]
F2 hexane	6[a]	5[c]
F3 99:1 (hexane:diethyl ether)	83[b]	81[f]
F4 98:2	11[a]	21[c]
F5 95:5	7[a]	21[c]
F6 90:10	5[a]	34[d]
F7 80:20	0[a]	44[d]
F8 column extract	11[a]	31[c]

a These values are not significantly different from each other ($P < 0.01$).
b This value is significantly greater ($P < 0.01$) from those indicated by *a*.
c These values are not significantly different from each other ($P < 0.01$).
d These values are significantly greater ($P < 0.01$) than those values denoted by *c*.
f This value is significantly greater than all other values ($P < 0.01$).

been designated neutral 99:1, hexane:diethyl ether fraction was redesignated 'MSP containing fraction' or 'MSP fraction'. Based on these results it was decided to bioassay sterol esters as well as hydrocarbons and fatty acids for comparison.

When the whole tick extract and MSP fraction spot were analyzed by HPTLC it was seen that the MSP fraction migrated to the same position on the plate as the tick sterol ester spot, indicating that the MSP material could be removed and rechromatographed. No other materials were observed in the MSP fraction lane and no material was observed in the control lanes, indicating that the crude extract spot was stable under these conditions. Bioassay results indicated that the crude extract spot induced a male response of 88%. The neutral 99:1 extract gave a bioassay response of 84% and the control value was 0%.

3.5 BIOASSAY OF CHEMICAL STANDARDS.

Male ticks responded most intensely to sterol esters, but much less so to fatty acids and hydrocarbons (Tables 9-11).

Males responded to all sterol esters, especially cholesterol oleate which was not significantly different than MSP (Table 9). The data presented for sterol esters (Table 9) is very different from the others. For all the sterol esters except for those indicated by a '*d*' the male response level was significantly greater than 0. At the 10 μg/cleaned female concentration level cholesterol oleate (C18:1) elicited a male response of 92 %. This value was not significantly different from the bioassay response elicited by the MSP fraction, ($P > 0 \cdot 05$). All other sterol esters were

Table 9 Bioassay responses (%) of male ticks to cleaned females treated with sterol esters, cholesterol and MSP[a]

Sterol esters		Concentration per cleaned female				
		10μg	2.5μg	1μg	0.5μg	0.1μg
Cholesterol Oleate	(C18:1)	92[b]	78	81[b]	36	18[c,d]
Cholesterol Linoleate	(C18:2)	67[c]	63	63[c]	57	27[b]
Cholesterol linolenate	(C18:3)	71[c]	64	46[c]	53	33[b]
Cholesterol Stearate	(C18:0)	58[c]	53	50[c]	35	16[c,d]
Cholesterol Arachidate	(C20:0)	66[c]	65	56[c]	50	34[b]
Cholesterol Palmitate	(C16:0)	52[c]	56	28[c]	16	7[c,d]
Cholesterol Laurate	(C12:0)	70[c]	64	65[c]	51	20[c,d]
Cholesterol Acetate	(C2:0)	48[c]	40	41[c]	13	7[c,d]
Cholesterol		0	—	—	—	—
Hexane		14[c]	0	2[c]	0	0
MSP		100	—	95	—	40

a Females were cleaned by immersion in hexane for 48 hours; standards or MSP and 2,6-DCP (10 ng) dissolved in hexane were pipetted onto the cleaned females. Following solvent evaporation (5 - 10 min), behavioral assays were done with fed males. Each female was assayed with 20 males, with 3 trials/male, as described by Hamilton and Sonenshine (1988); values in the body of the table represent the mean % positive response; — = not done.

b Not significantly different from MSP ($P > 0.05$) by Duncan's Multiple Range test.

c Significantly different from MSP ($P < 0.01$) by Duncan's Multiple Range test.

d Not significantly different from 0 ($P < 0.01$) by test of Proportions.

Table 10 Bioassay responses (%) of male *D. variabilis* ticks to cleaned females treated with fatty acids[a].

Fatty acid		Concentration per cleaned female. 10 μg	2.5 μg	0.5 μg
Euric	(C22:1)	0	0	2
Behenic	(C22:0)	0	1	0
Arachidic	(C20:0)	0	0	1
Stearic	(C18:0)	—	0	0
Oleic	(C18:1)	21	10	8
Linoleic	(C18:2)	21	21	6
Linolenic	(C18:3)	3	—	—
Palmitic	(C16:0)	0	0	1
Myristic	(C14:0)	0	8	3

a Females were cleaned by immersion in hexane for 48 hours; standards or MSP and 2,6-DCP (10 ng) dissolved in hexane were pipetted onto the cleaned females. Following solvent evaporation (5 - 10 min), behavioral assays were done with fed males. Each female was assayed with 20 males, with 3 trials/male, as described by Hamilton and Sonenshine (1988); values in the body of the table represent the mean % positive response; — = not done.

Table 11 Bioassay responses (%) of male *D. variabilis* ticks to cleaned females treated with n-alkanes[a].

n-Alkane		Concentration per cleaned female. 10 μg	2.5 μg	0.5 μg
Tetracontane	(C40)	2	2	0
Dotriacontane	(C32)	5	13	4
Hexacosane	(C26)	2	5	0
Docosane	(C22)	1	1	0
Eicosane	(C20)	3	2	1
Octadecane	(C18)	4	3	1
Hexadecane	(C16)	2	1	3
Tetradecane	(C14)	1	3	2

a Females were cleaned by immersion in hexane for 48 hours; standards or MSP and 2,6-DCP (10 ng) dissolved in hexane were pipetted onto the cleaned females. Following solvent evaporation (5 - 10 min), behavioral assays were done with fed males. Each female was assayed with 20 males, with 3 trials/male, as described by Hamilton and Sonenshine (1988); values in the body of the table represent the mean % positive response.

significantly different ($P < 0 \cdot 01$) from the response elicited by MSP fraction, as denoted by 'c'. This pattern was repeated at the $1 \cdot 0$ μg/cleaned female concentration. The results at the $0 \cdot 5$ and $0 \cdot 1$ μg/cleaned female concentration were less clear cut indicating that these were threshold concentrations. Cholesterol was not shown to be active in eliciting a male mating response.

The results presented in both Tables 10 and 11 indicate that fatty acids in the range C:14 to C:22 and n-alkanes in the range C:14 to C:40 do not elicit the characteristic male mating response when placed on clean females with appropriate concentrations (ca 10 ng/female) of 2,6-DCP. In both the fatty acid test and the n-alkane tests there was no significant difference in responses between the lower concentration ($0 \cdot 5$ μg/cleaned female) and the highest concentration (10 μg/cleaned female). In all cases the male response was not significantly different from 0.

3.6 DERIVATIZATION EXPERIMENTS.

Oxidation of olefinic or acetylinic linkages. When the 'purple benzene' solution was added to the tick extract there was a color change, the control did not change color. The TLC results (Fig. 9) indicated that the MSP fraction spot had not moved position. This suggested that although olefinic groups had been oxidized the molecular structure had not been significantly altered by the reaction. Bioassay results indicated that the active component had not been significantly affected as the bioassay response was $77 \cdot 5$ % which was significantly greater than the control value ($P < 0 \cdot 01$). Oxidation of the 1-tetradecene control, $Rf = 0 \cdot 91$, appeared to result

Figure 9. Drawing of chromatogram of TLC analysis (HPTLC plate, non polar solvent system) of the products of oxidation reactions; (a) MSP fraction oxidized with $KMN0_4$; (b) 1-tetradecene; (c) 1-tetradecene oxidized with $KMNO_4$; (d) repetition of oxidation of MSP fraction.

in the creation of alcohols, Rf = 0·10 as well as other compounds, probably aldehydes confirming that the reagents were acting normally.

Transmethyl esterification. Analysis of transmethyl esterified crude extract indicated the presence of fatty acids, (See gas-chromatography section below).

HPTLC analysis of the products of the transmethyl esterification of the MSP fraction indicated that some of the material in this fraction had reacted (see Fig. 10). A sterol ester linkage is clearly indicated by the presence of the expected reaction products cholesterol and FAMEs. Some material did not react and this may explain the bioassay score of 80 % for the extract after the reaction. The 100 % pure cholesterol ester standard did not completely react either, the products of the reaction appear to be the same as for the MSP fraction.

3.7 ISOLATION AND PURIFICATION OF ACTIVE COMPONENT.

HPLC revealed that the MSP fraction contained three major components (Fig. 11). When one μl (two FE) of MSP fraction was injected three peaks (peak one, two, and three) were apparent at retention times of 8·18, 9·84 and 12·34 minutes. Based upon the previous findings, comparisons were made with authentic sterol ester standards. These peaks were found to correspond to the retention times of cholesterol linolenate (C18:3), linoleate (C18:2) and oleate (C18:1) standards respectively. Coinjection of one μl of MSP fraction and each of the standards in turn resulted in increased peak sizes for each of the three naturally occurring compounds i.e. they co-eluted (Fig. 12A, B and C).

Figure 10. Drawing of chromatogram of TLC analysis (HPTLC plate, non polar solvent system) of products of transmethylesterification results; (a) MSP fraction; (b) transmethyl esterified MSP fraction; (c) cholesterol palmitate; (d) transmethyl esterified cholesterol palmitate; (e) fatty acid methyl ester; (f) cholesta 2,4 diene standard.

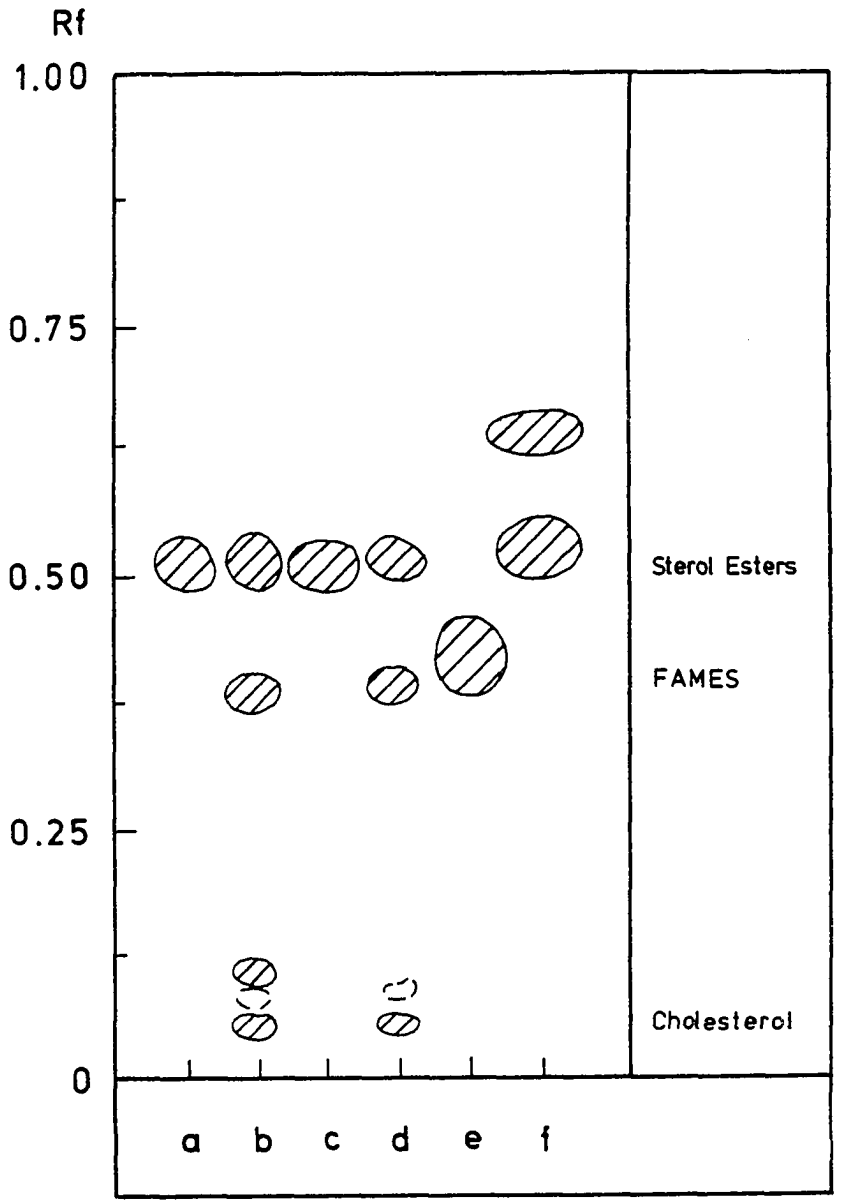


Figure 11. Drawing of chromatogram of HPLC analysis of one μl MSP (two FE) fraction. (Reverse phase C-18 Whatman 5-ODS-3 Partasil column. Flow rate = two ml $\cdot$ min^{-1} acetonitrile:isopropanol 60:40.

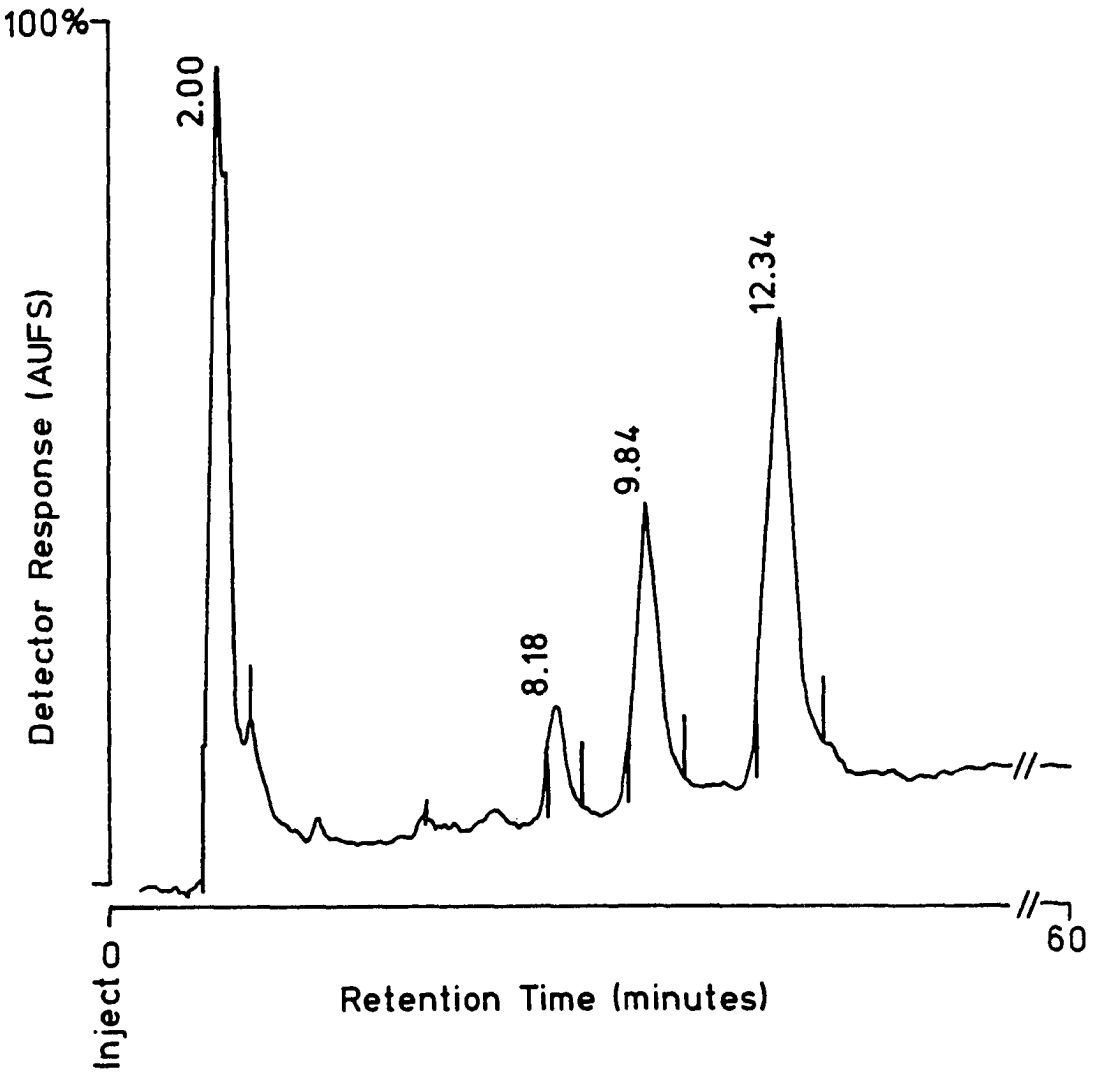
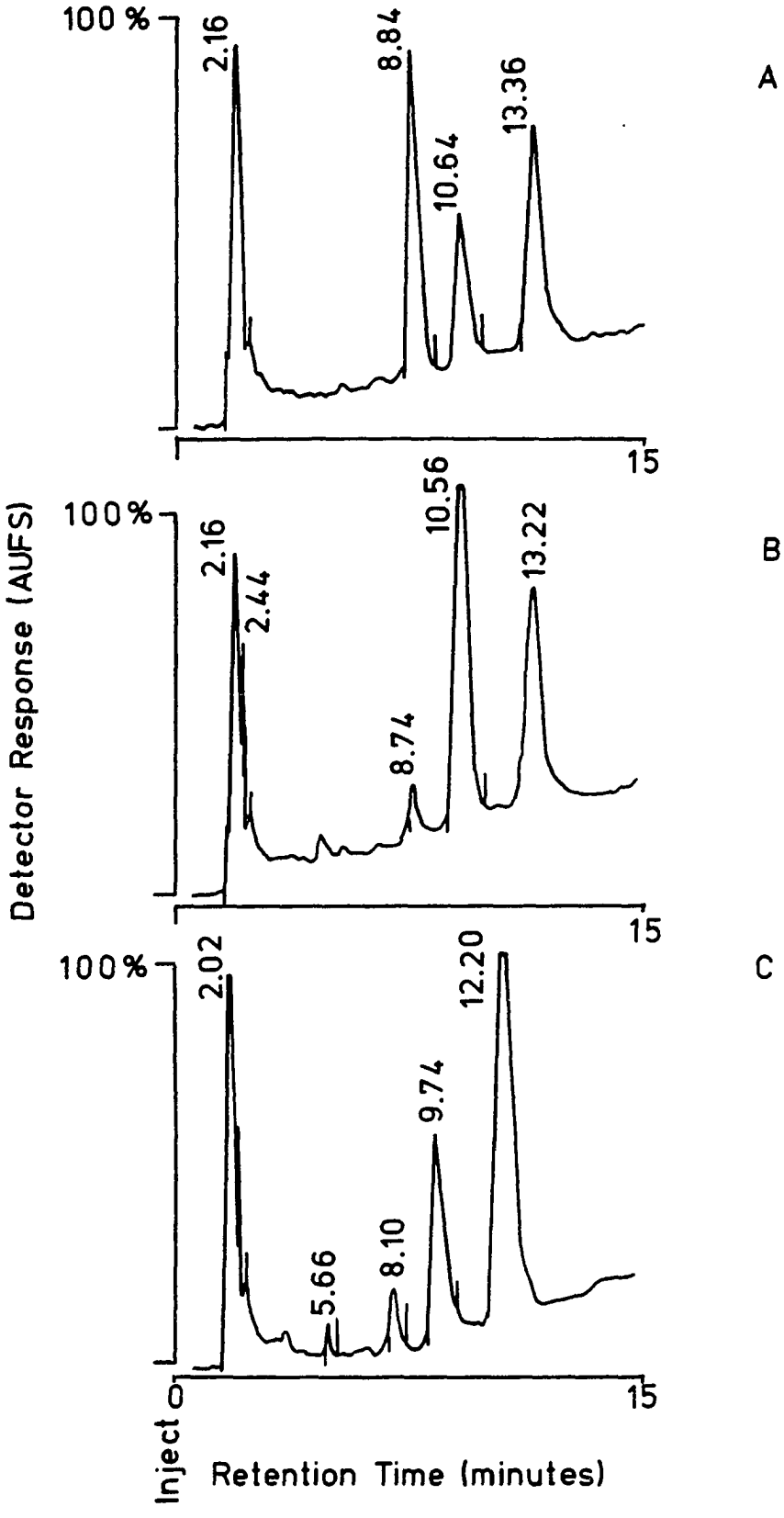


Figure 12 (A,B, and C). HPLC chromatograms of coinjections of cholesterol linolenate (A), cholesterol linoleate (B) and cholesterol oleate (C) with one μl (two FE) of MSP fraction.



A one FE aliquot of the MSP fraction was found to contain 0·16 μg of the material in peak one, 4·23 μg of the material in peak two and 12·10 μg in peak three.

Peaks two and three (Fig. 11) were collected for further study. These peaks were reinjected on the 5-ODS-3 column to confirm their successful collection (Fig. 13 C,D). Peak one was not collected successfully due to molecular degradation as indicated by reinjection of the collected fraction (Fig. 13 B). In addition pre and post peaks were also collected for bioassay and were reinjected to confirm collection and 'purity' (Fig 13 A,E). No significant quantities of material were observed in this these fractions by HPLC.

The collected material was bioassayed and male bioassay response to peaks two and three were both active giving bioassay responses of 85 and 95 % respectively. Male bioassay response to pre and post peaks was found to be not significantly different from the controls (controls were 5 % in both cases). Peak one was not successfully collected and the material which was, did not give bioassay responses significantly greater than the controls.

Gas Chromatography revealled that the MSP fraction resolved into three peaks when analysed on the DB-1 column, two of these peaks were resolved by the integrator. The predominant peak was a peak at Rt 23·18 minutes (Fig. 14 A). The second peak had a Rt of 11·223 minutes. The Rt of the 23·18 minute peak corresponded to Rts of sterol ester standards with conjugated C:18 fatty acid moieties i.e. cholesterol linolenate, cholesterol linoleate and cholesterol oleate all

Figure 13 (A,B,C,D and E). HPLC Chromatograms of collected and reinjected pre-peaks, peaks 1, 2 and 3, and post peaks.

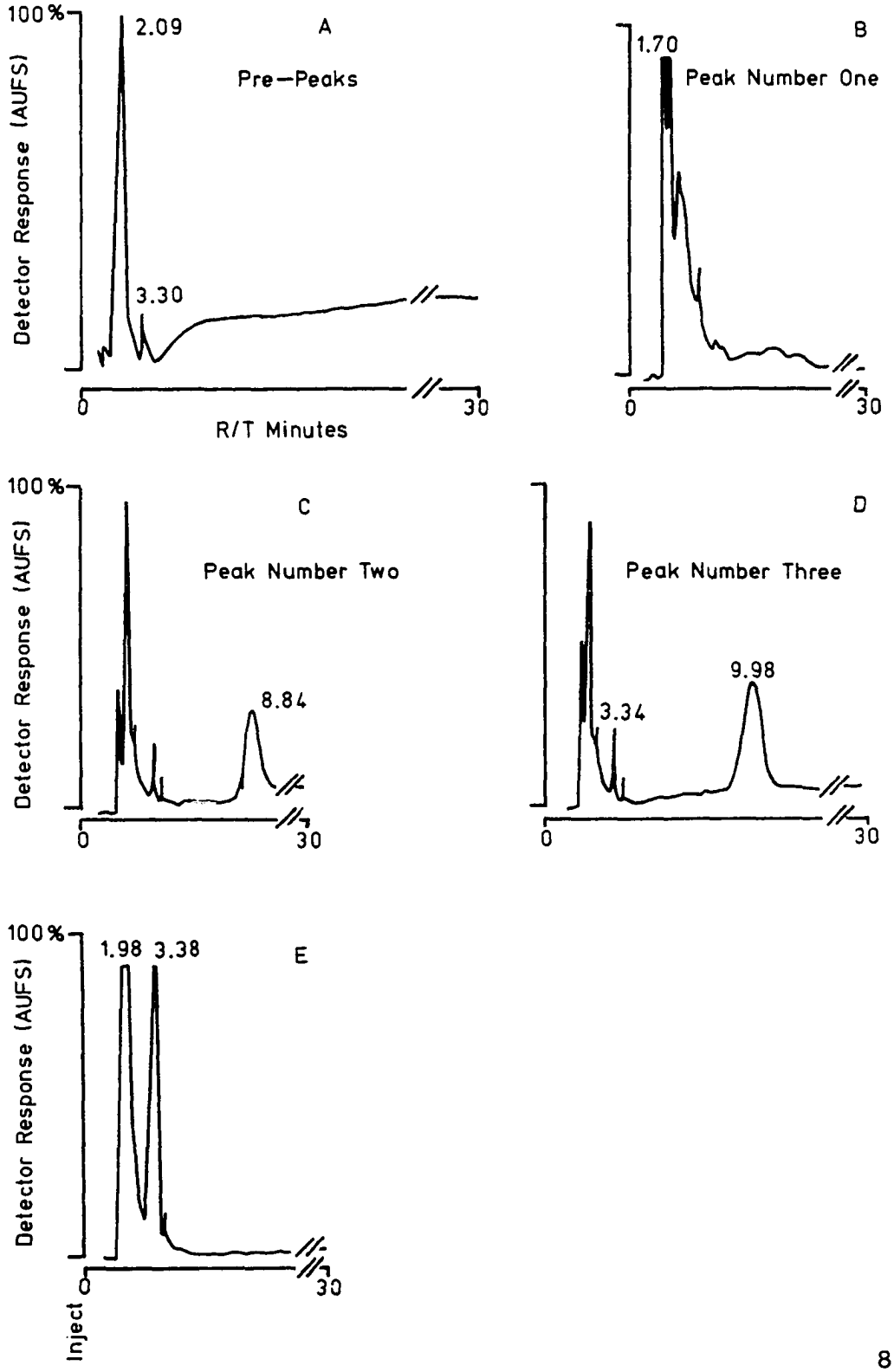
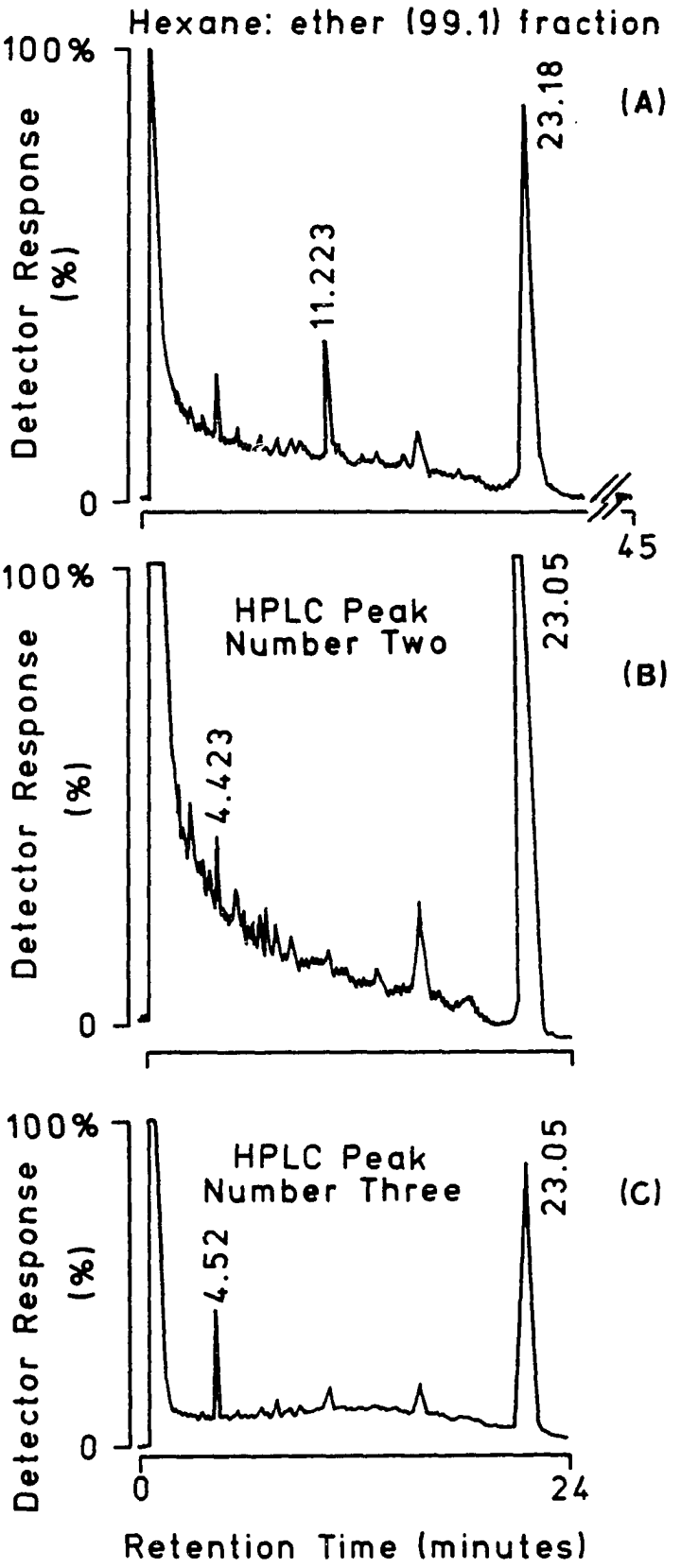


Figure 14. Gas-chromatographic analysis of (a) MSP fraction (hexane:diethyl ether 99:1), (b) HPLC peak # 2 and (c) HPLC peak # 3.



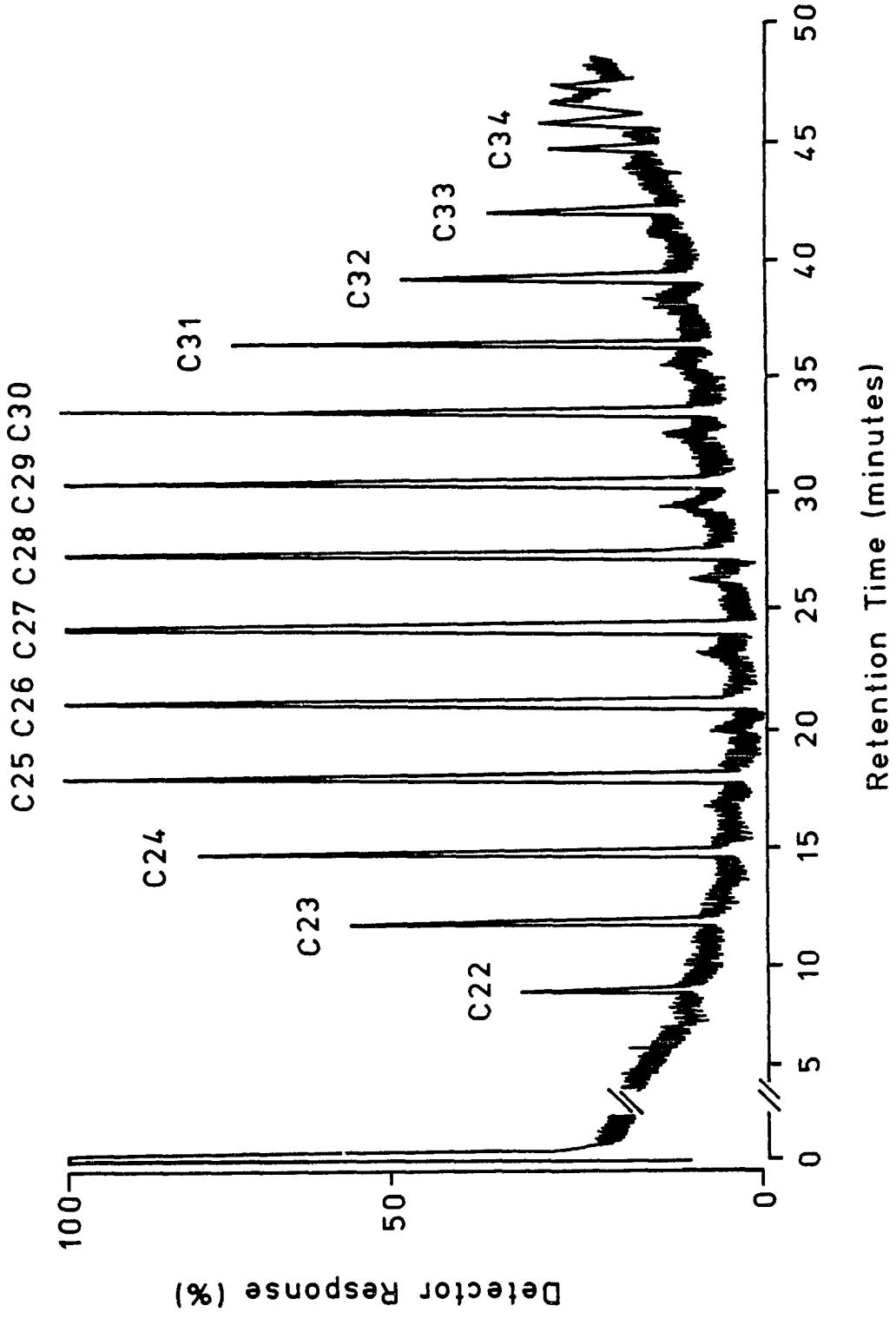
of which have the same Rt. Sterol esters with fatty acid moieties of different chain lengths have different retention times. The 11·223 minute peak corresponded with cholesterol myristate.

The collected HPLC peaks (peaks number two and three) were also injected on the GC (DB-1 column) (Fig. 14 B and C). When peak number three was injected a peak which was incompletely resolved was seen to be present at 23·04 minutes. A much smaller peak was also present at 4·52 minutes. This 23·04 minute peak co-eluted with the sterol esters with the conjugated C:18 fatty acid moiety i.e. with cholesterol linolenate, linoleate, oleate and octanoate respectively. When peak number two was injected, one dominant peak at 23·05 minutes was observed. This peak also had the same Rt as sterol esters with the conjugated C:18 fatty acid moieties.

Gas-chromatographic analysis of the crude tick extract indicated the presence of n-alkanes. Injection of standards and the subsequent comparison of their retention times and those of compounds in the crude tick sample suggested the presence of a series of n-alkanes from C:18 to C:35. Fig. 15 Shows the series from C:22 to C:34 observed in a five minute, unfractionated cuticular wash. Tentative identities were made by comparisons with standards. No evidence was found for the existence of either alkenes or alcohols in tick cuticle extract.

Results of GC analysis of the acid, base and neutral fractions revealed numerous peaks of unknown identity. GC results are given in Fig. 1 in Appendix B. Although significant differences in the contents could be seen it was not possible to account for the differences in biological activity on this basis.

Figure 15. n-Alkane series C:20 to C:35 from an unfractionated *D. variabilis* five minute hexane cuticle wash.



Similarly GC analysis of non methyl esterified low temperature (180 to 260°C.) analysis of the eight fractions generated from the Bio-Sil A column revealed no significant difference in F3 that could be associated with the biological activity when compared with the other fractions [Fig. 2 (A-H) Appendix B]. When the fractions were transmethyl esterified some small amounts of FAMEs were revealed on GC analysis [Fig. 3 (A-H) Appendix B]. A rapeseed oil standard was analyzed for comparison. Again, compounds present in the active fraction were present in the inactive fractions and so could not be responsible for the observed behavior.

The high temperature (SP 2250 column, 320°C. isothermal) analysis of the first three fractions (Fig. 16) revealed the presence of significant quantities of material in the active fraction [Retention time (Rt) = 35 minutes] and smaller amounts at regular intervals before this (Fig. 16 B). This material was not present in the other fractions analyzed (Fig 16A and 16C). Analysis on the DB-5 column (280 - 320°C) revealed the same profiles in each fraction (Fig. 17).

3.8 IDENTIFICATION OF PHEROMONE BY GC-MS.

The GC-MS analysis compared the fragmentation patterns of HPLC peak #3 (MSP molecule) with those of cholesterol oleate and confirmed this peak as cholesterol oleate.

The analysis by GC-MS of the purified peak #3 of the MSP fraction collected by HPLC using direct exposure probe ammonia chemical ionization (CI) mass spectrometry yielded a base peak at m/z 668 (100%) and corresponded to the

Figure 16. Gas-chromatographic analyses of the first three Bio-Sil A column *D. variabilis* extract fractions at high temperature (320°C isothermal SP-2250 column). (a) **F2**, hexane 100 %; (b) **F3**, hexane:diethyl ether, 99:1; (c) **F4**, hexane:diethyl ether, 98:2.

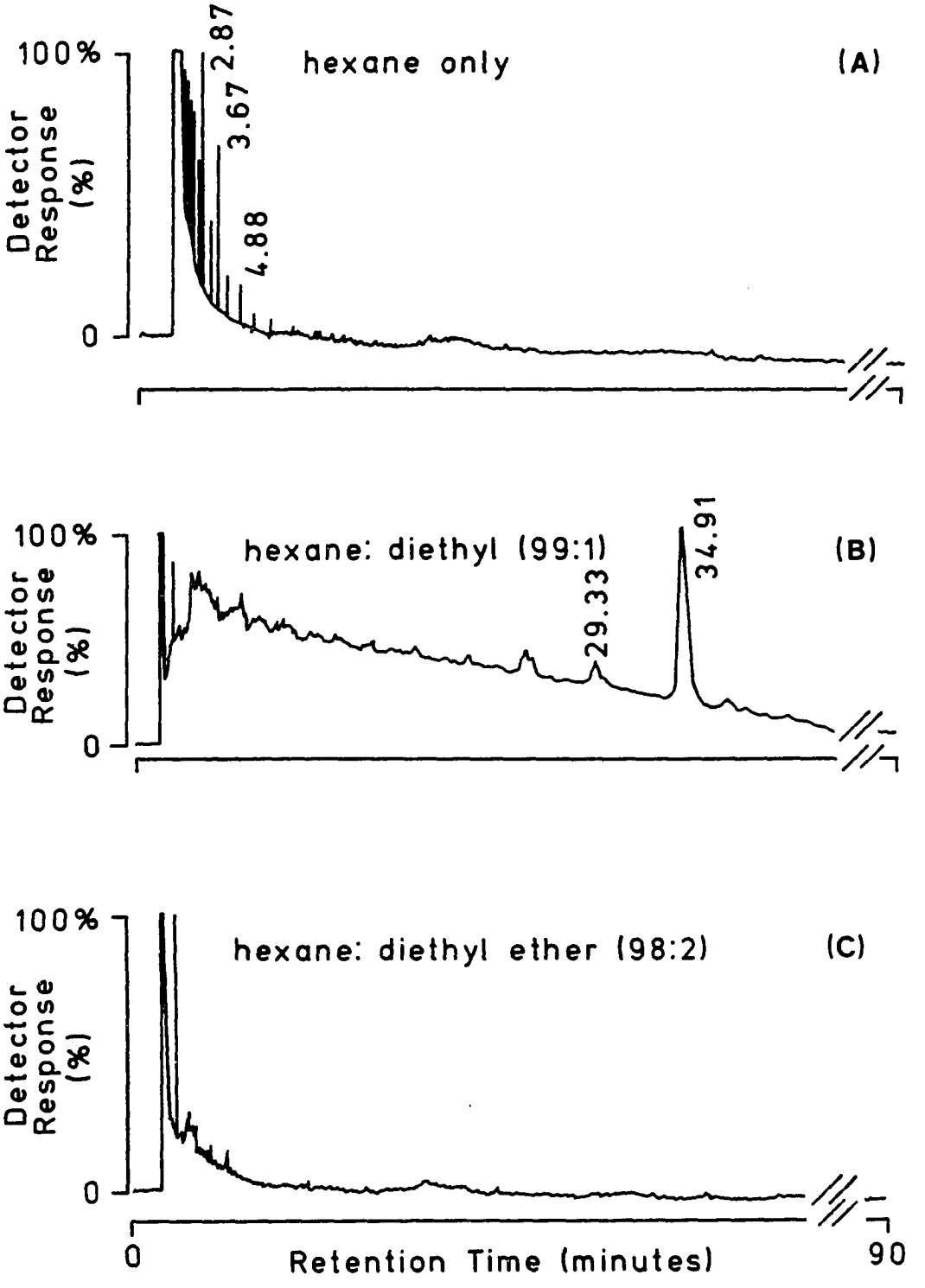
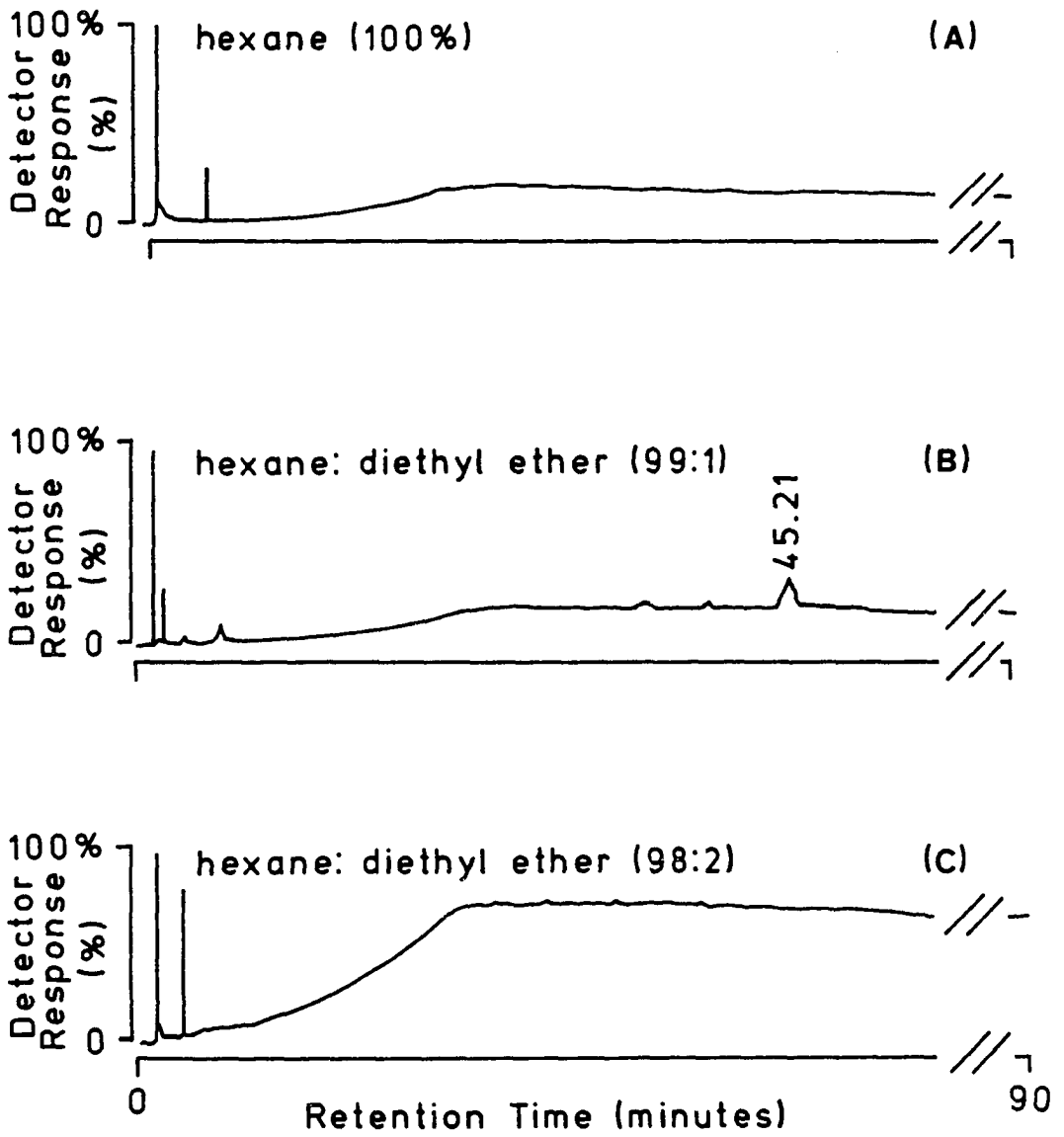


Figure 17. Gas-chromatographic analyses of the first three Bio-Sil A column *D. variabilis* extract fractions at high temperature (260° to 320°C isothermal DB-5 column). (a) **F2**, hexane 100 %; (b) **F3**, hexane:diethyl ether, 99:1; (c) **F4**, hexane:diethyl ether, 98:2.



presumed ammonia adduct ion; this was equivalent to the molecular weight of MSP (650 mass units (mu)) plus the molecular weight of protonated NH_3 = 668 mu (NH_4^+ = 18 mu).

A similar analysis using $^{15}NH_3$ confirmed that $^{15}NH_4^+$ was adducted to the MSP molecule and gave the dominant peak at m/z 669 (100%) confirming also that the molecular weight of the MSP molecule was 650 mu.

Using deuterated ammonia (ND_3, ND_4^+ = 22 mu) as the reagent gas the ammonium adduct ion gave the dominant peak at m/z 672 (100%) again confirming the MSP as having a molecular weight of 650. This physical data is summarized in Table 12 below.

When methane (CH_4) was used as the reagent gas a slightly more vigorous ionization occurred and the molecule was cleaved. A base peak of m/z 369 (100%) indicated that this component of the molecule had a molecular weight of 368 mu. The peak at m/z 649 (9%) and 651 (6%) corresponded to the M - H^+ and M + H^+ ions respectively indicating and confirming the molecular weight of the MSP to be 650 mu. The loss of the 282 mu component of the MSP molecule indicated the loss of oleic acid. This fragment does not appear in the mass spectra as it would be rapidly and completely ionized into much smaller fragments.

Analysis of the MSP molecule by electron ionization mass spectrometry provided a spectrum with a base peak at m/z 368 (100%) with the next most abundant ion at m/z 57 (22%). All of the above CI spectra as well as the EI spectrum were identical to the corresponding spectra for cholesterol oleate. See

TABLE 12 Physical Data from analysis of the *D. variabilis* biologically active fraction purified by HPLC.

MSP
NH_3-CI-MS m/z: 668[100%, $(M+NH_4)^+$].
$^{15}NH_3$-CI-MS m/z: 669[100, $(M+^{15}NH_4)^+$].
CH_4-CI-MS m/z: 651[6, $(M+H)^+$], 649[9, $(M-H)^+$], 369[100, $(M+H-282)^+$].
ND_3-CI-MS m/z: 672[100, $(M+ND_4)^+$].
EI-MS m/z: 368(100%), 353(8), 260(7), 255(6), 247(7), 147(11), 95(10), 83(10), 81(11), 71(10), 69(13), 57(22), 55(15).
SAPONIFICATION PRODUCTS
Cholesterol EI-MS m/z: 386(100%), 371(25), 368(24), 353(26), 301(55), 275(60), 255(25), 231(39), 159(45), 133(48), 119(48), 207(75), 105(76), 81(69), 69(41), 55(73).
Cholestanol EI-MS m/z: 388(55%), 373(6), 355(8), 234(59), 233(93), 215(100), 165(36), 147(22), 108(68), 95(83), 81(84), 79(24), 69(33), 57(47), 55(76).
Methyl Oleate EI-MS m/z: 296(4%), 264(28), 222(13), 180(9), 123(14), 110(21), 97(48), 83(49), 74(53), 69(58), 55(100).

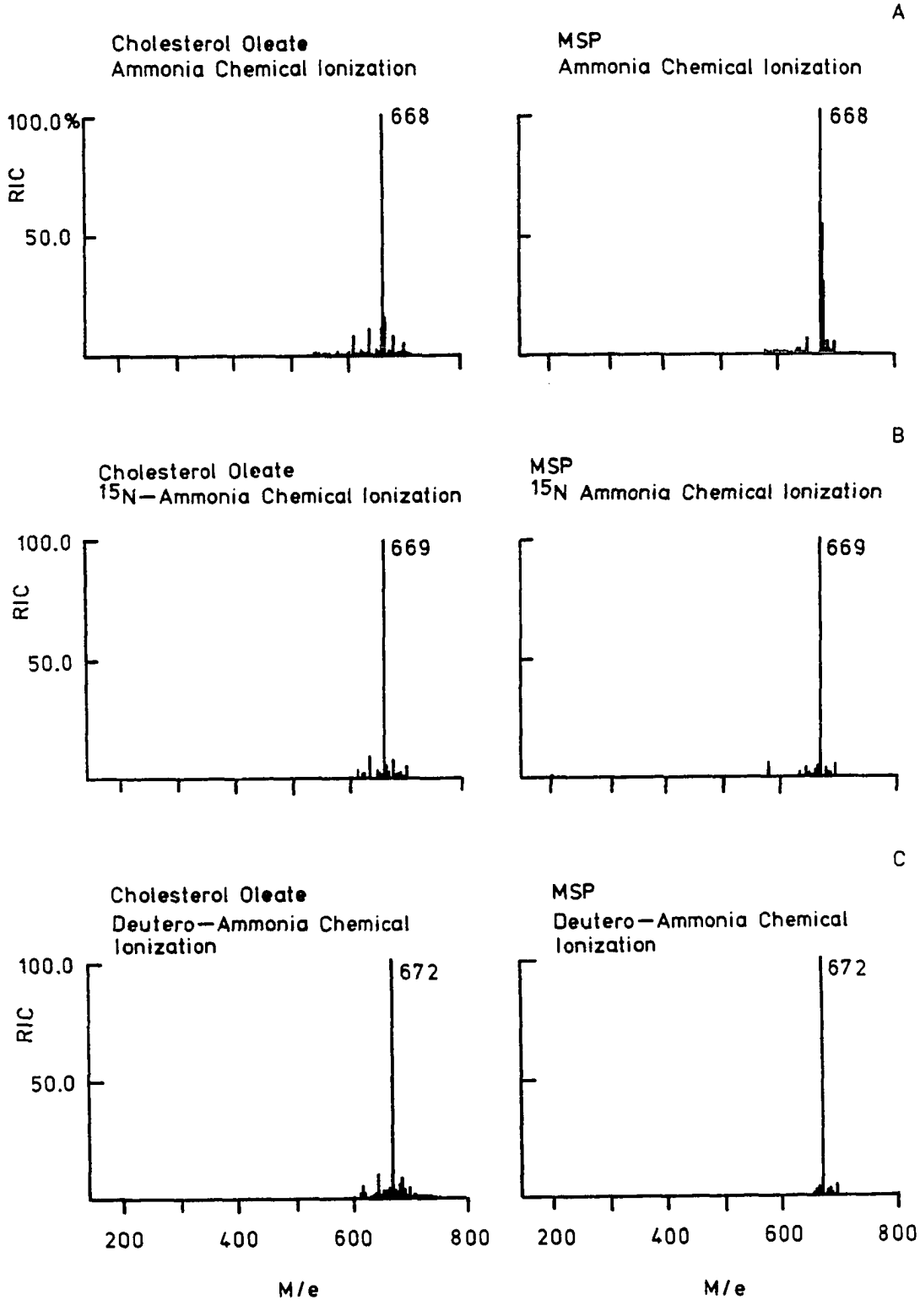
results Fig. 18 for mass spectra comparisons as well as Table 12 for a complete summary of all physical data.

The sterol fraction of the saponification products contained a major (approximately 90%) component and a minor (approximately 10%) component. The Rt of the cholesterol component (9.53 minutes) produced on the saponification of cholesterol oleate was the same as that for the sterol component produced on the saponification of MSP (Fig. 19). A comparison of the EI spectra confirmed that MSP is composed partially of cholesterol (Fig. 20). EI-GC-MS analysis and GC retention time analysis identified the 10% sterol component as being cholestanol. See Table 12 for physical data.

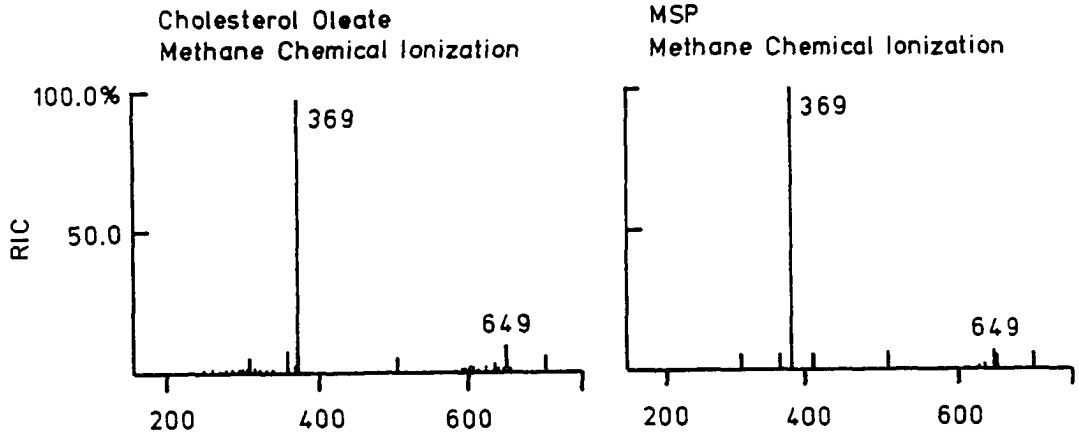
The retention time of the fatty acid methyl ester fraction of the saponification products of MSP and cholesterol oleate were the same (26·3 minutes) (Fig. 21). EI-GC-MS confirmed the presence of oleic acid (Fig. 22). The double bond geometry of the fatty acid moiety was confirmed as cis by comparison of methyl oleate (cis) and methyl elaidate (trans) GC retention times with that of the MSP fatty acid methyl ester. The methyl oleate peak (peak #1) was greatly increased in size in chromatogram B compared with chromatogram A when MSP fatty acid methyl ester moiety was co-injected with the authentic standards (Fig. 23). See Table 12 for physical data.

These results indicate that cholesterol oleate (Fig. 24) was the dominant component of MSP (90%).

Figure 18. Comparison of MSP molecule and cholesterol oleate fragmentation mass spectra. (a) Ammonia chemical ionization, (b) ^{15}N-Ammonia chemical ionization, (c) Deutero-ammonia chemical ionization, (d) Methane chemical ionization and (e) Electron ionization.



D



E

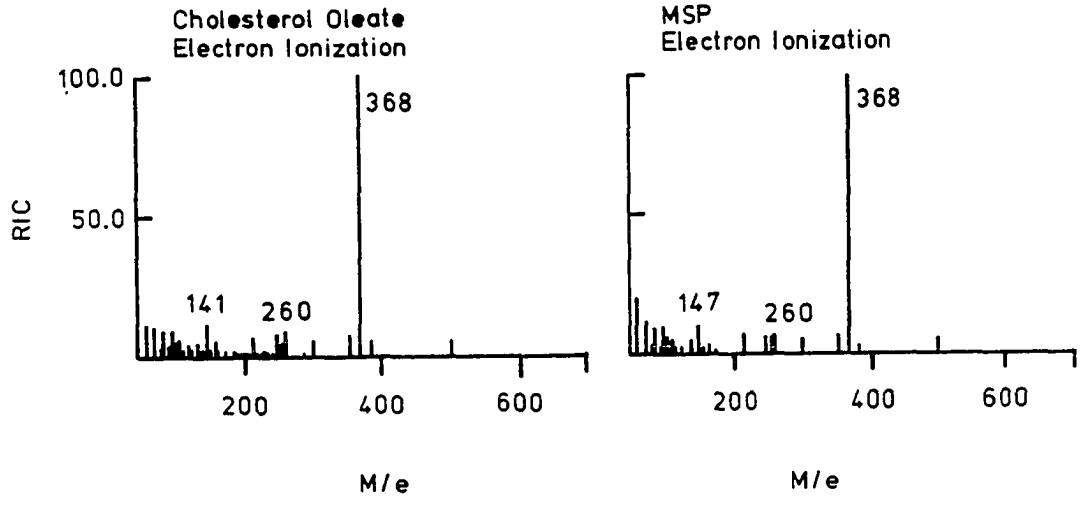


Figure 19. Comparison of Gas-chromatographic chromatograms of the sterol fractions of the saponification products of MSP (a) and Cholesterol oleate (b).

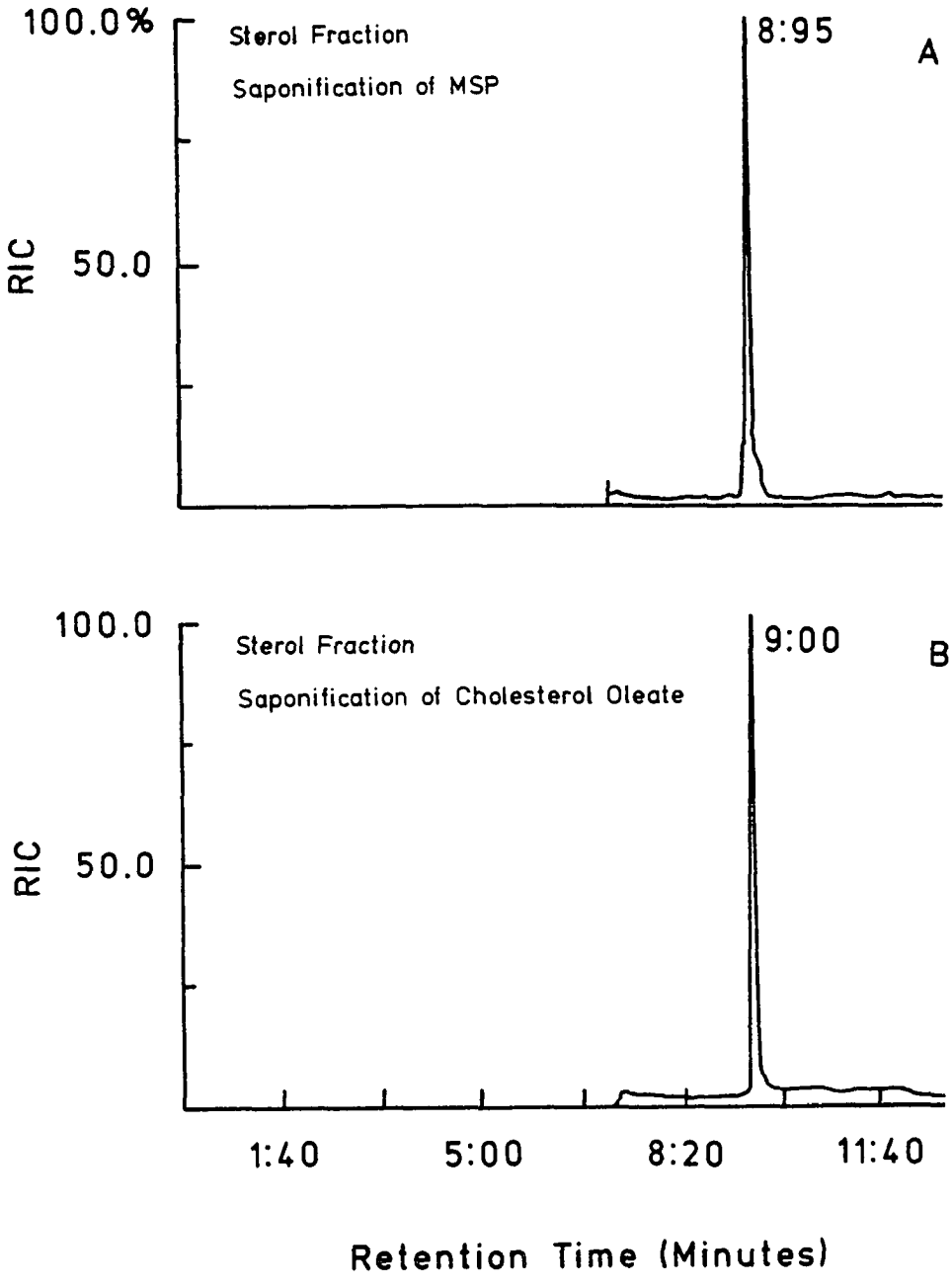


Figure 20. Comparison of Electron Impact mass spectra of the sterol fractions of the saponification products of MSP (A) and cholesterol oleate (B).

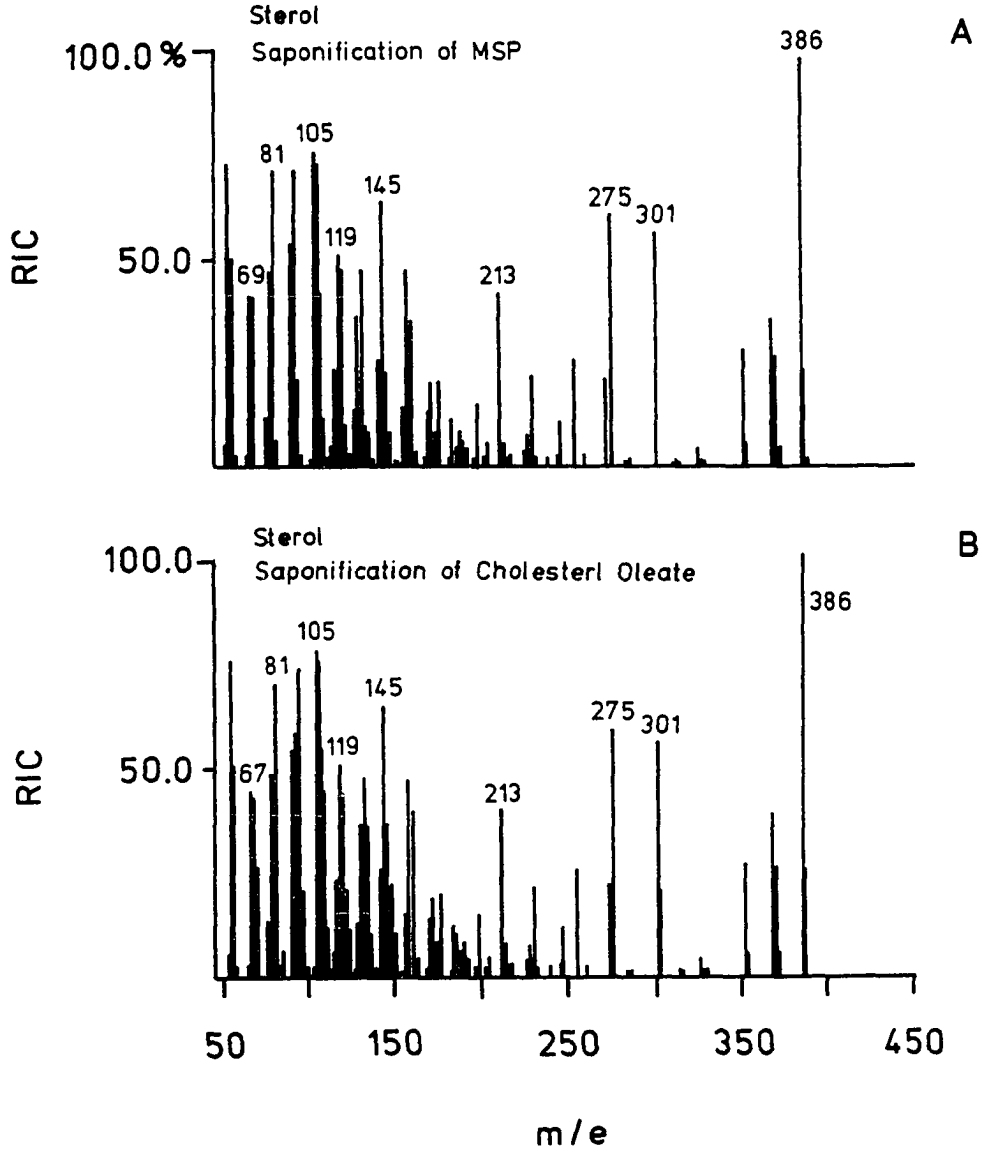


Figure 21. Comparison of Gas-chromatographic chromatograms of the fatty acid methyl ester fractions of the saponification products of MSP (A) and Cholesterol oleate (B).

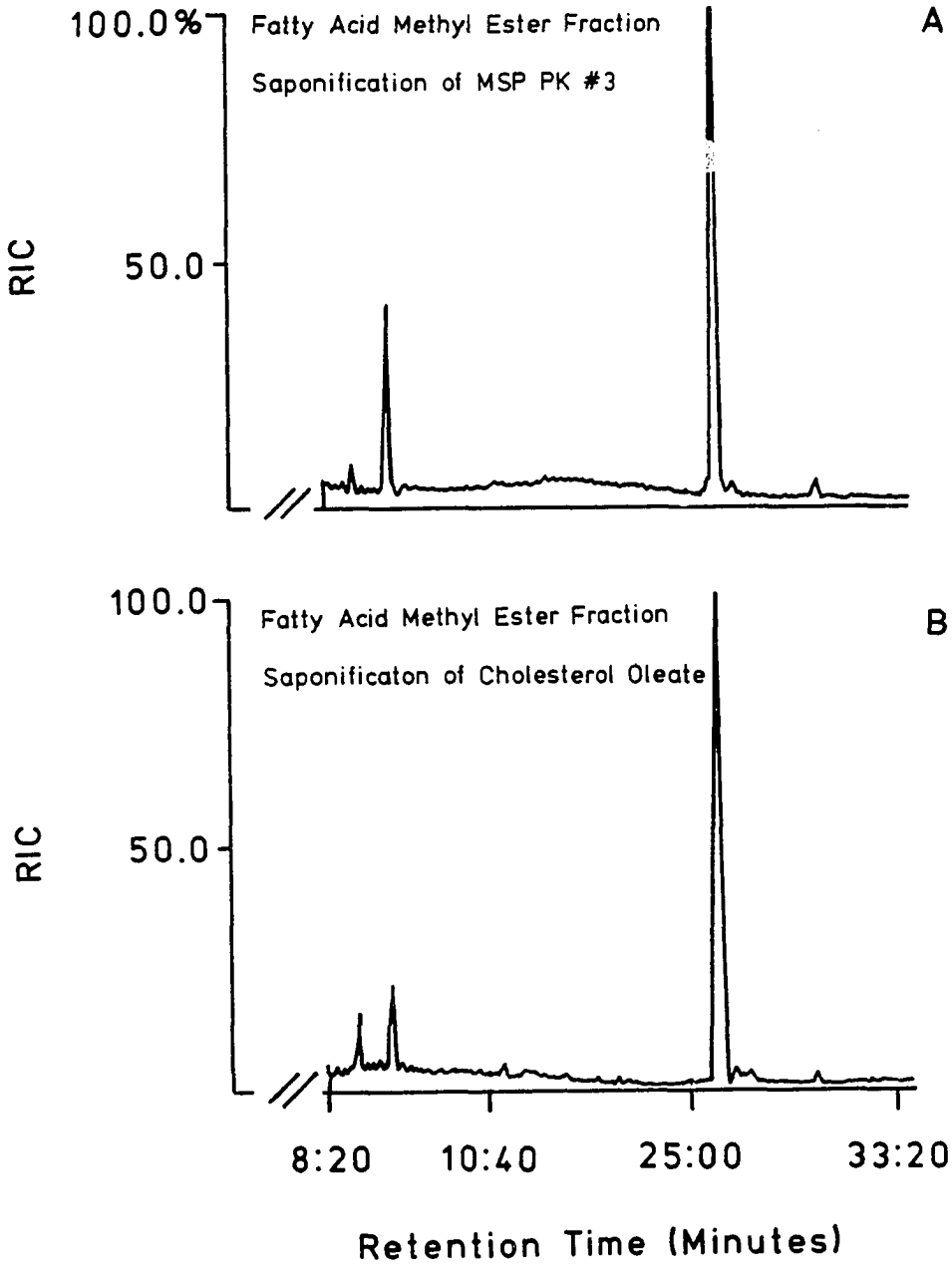


Figure 22. Comparison of Electron Impact mass spectra of the fatty acid methyl ester fractions of the saponification products of MSP (A) and cholesterol oleate (B).

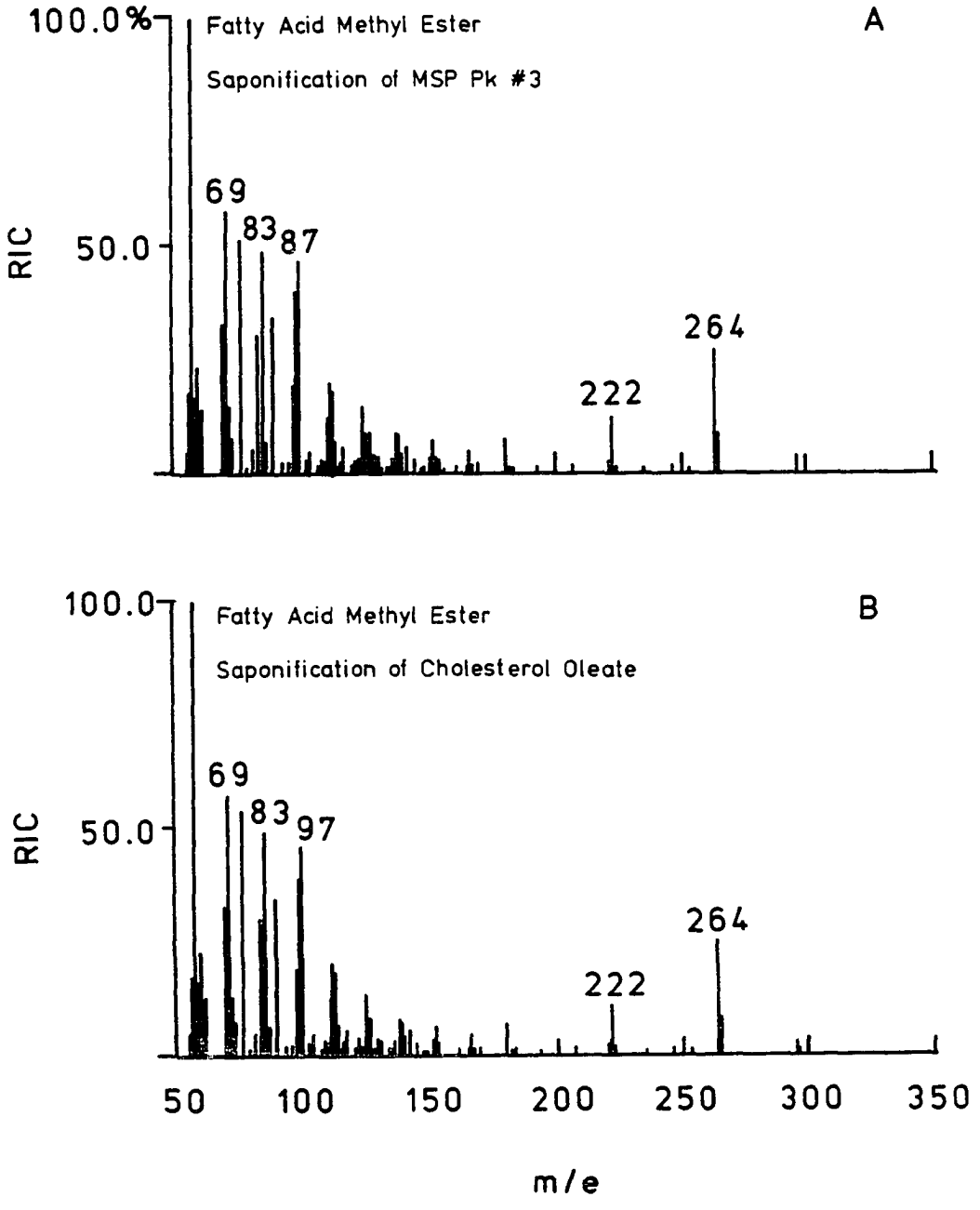


Figure 23. Comparison of retention times of methyl oleate (trans double bond, peak #1) and methyl elaidate (cis double bond, peak #2) (a). Comparison of retention times of methyl oleate, methyl elaidate and the fatty acid methyl ester product of the saponification of MSP.

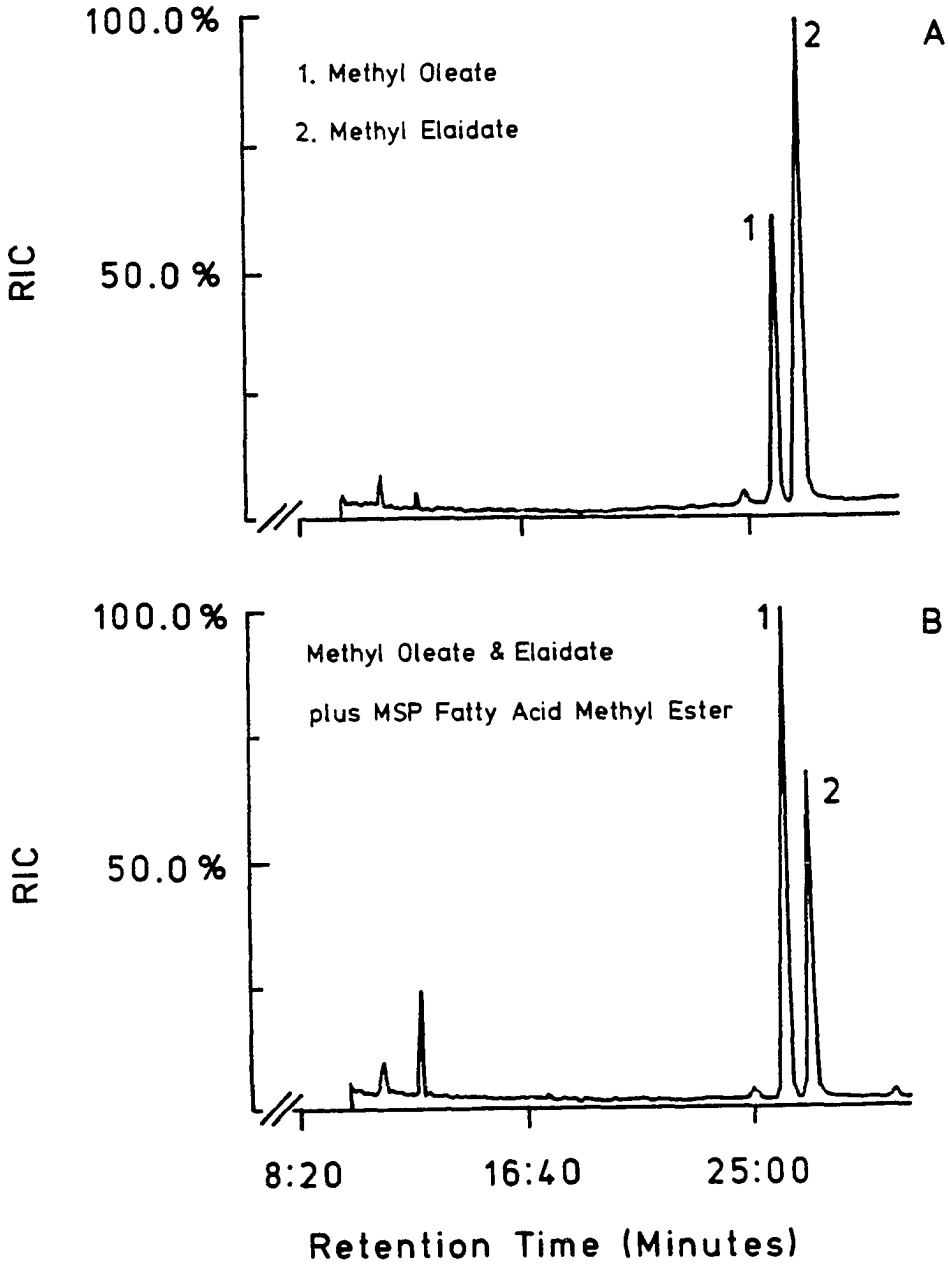
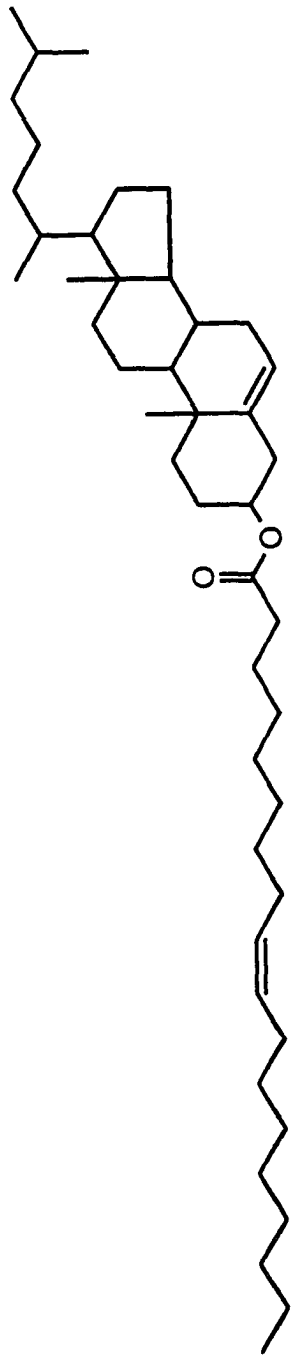


Figure 24. Diagram of cholesterol oleate, Mounting Sex Pheromone of *Dermacentor variabilis*.



CHOLESTEROL OLEATE

Chapter four: DISCUSSION

The results of the biological investigation clearly indicate the presence of a pheromone on the cuticular surface of *Dermacentor variabilis*, *D. andersoni*, *Amblyomma americanum* and *A. maculatum*. The pheromone has been termed the Mounting Sex Pheromone (MSP) (Hamilton and Sonenshine, 1988) because it is responsible for releasing the mounting behavior i.e. steps five through eight of the sequence of behavior described in the introduction. Size of female as well as the texture of the female cuticle were shown to be unimportant in stimulating male mating activity for *D. variabilis* and *D. andersoni*. The pheromone was isolated and identified as being cholesterol oleate for *D. variabilis*. The presence of sterol esters in *D. andersoni*, *A. americanum*, *A. maculatum* and *Hyalomma dromedarii* as well as *D. variabilis* was confirmed by TLC. The cross reactivity between these species suggests that sterol esters (either different concentrations, or different esters or a combination of both) act as the MSP for each of these species. This is the first report of a sterol ester as a sex pheromone in any arthropod. In this communication system a hierarchy of chemical signals directs and controls male mating behavior with no other non-chemical stimuli significantly modulating or synergyzing the male response. The MSP is second in this hierarchy of three pheromones, each pheromone releasing an increasingly more specific series of mating behaviors. This mating system may be unique among arthropods; it certainly differs substantially from known insect chemical systems which invariably rely on both non-chemical

stimuli as well as chemical stimuli (Matthews and Matthews, 1978). This uniqueness attests to the evolutionary independence of this group of arthropods from the Insecta.

Classification of MSP as a Sex Pheromone.

MSP can be correctly described as a sex pheromone based on the following arguement. Rutowski (1981) defined a pheromone as, "a compound (or compounds) that function in a unique set of organismic interactions, namely those involving communication. Communicatory interactions are those in which the behavior or signal of one animal (the sender) alters the behavior or physiology of another animal (the receiver) in a way that promotes the fitness or reproductive success of both". A sex pheromone is involved in sexual communication and MSP can be seen to mediate the mounting phase of ixodid tick courtship behavior, which was described by Sonenshine, 1985. Following their attraction and excitation by 2,6-DCP, males encountering the pheromone mount the females and apply their legs and mouthparts against the female body surface so that the male body is in direct contact with the female. While still in contact, the male moves to the female's venter and probes until the gonopore is located. Experiments performed demonstrated that, 1/ the pheromone can be removed from the female, disrupting male mating behavior, 2/ the male mating response can then be restored by placing extract made from the female tick cuticle on 'cleaned' females (i.e. females which have previously been cleaned in organic solvent to remove the active compound) and 3/ the male mating response can be transferred to inanimate objects by placing the extract containing

the pheromone on them. If this compound is absent from the female, the male tick is unable to continue the mating process initiated by its own physiological state and/or the presence of 2,6-DCP, the ubiquitous SAP. In addition the responses of *D. variabilis* males to cholesterol oleate followed a dose response curve typical of responses to sex pheromones (Vinson *et al.*, 1975; Hendry *et al.*, 1973).

Classification of MSP as a Cuticular Contact Sex Pheromone.

Sex pheromones can be classified by their origin in or on the emitter organism i.e. glandular, cuticular or other e.g. frass. They can also be classified by their relative volatility, e.g. 2,6-DCP of the ixodidae, which is highly volatile, to non-volatile compounds, e.g. the long chain methyl branched alkanes of the Diptera. Volatile pheromones typically have a glandular origin i.e. they are produced in specific modified groups of epidermal cells and access the cuticle surface via specialized ducts which may or may not be under CNS control (Bradshaw *et al.*, 1983). Examples of these types of glands are the Lepidopteran abdominal sex pheromone glands. In the ixodidae volatile sex pheromone (2,6-DCP) is produced and released from the foveal gland situated on the dorsum.

In contrast to contact cuticular sex pheromones, volatile pheromones allow individuals to locate a mate over relatively very large distances (Matthews and Matthews, 1978). The Lepidoptera, which produce a vast array of volatile sex pheromones (Baker and Bradshaw, 1981; Baker and Evans, 1979), are an excellent example of this ability. Spiders are also known to produce volatile sex attractant pheromones, Watson (1986), Tietjen and Rovner (1982). Lepidopteran volatile sex

pheromones are typically C:10 to C:18 alcohols and esters with varying degrees of unsaturation (Baker and Bradshaw, 1981). Males orientate towards the female by flying up the concentration gradient.

Volatile sex pheromones are typically a blend of chemicals containing a major (> 90 %) component and minor (< 10 %) components. The male is believed to orientate by responding to either the dominant component of the mixture (Bradshaw *et al.*, 1983), or as also has been suggested by Linn *et al.*, (1987), to the complete pheromone mix. This ability to detect pheromones at great length from the female would provide a selective advantage to highly mobile species, e.g. flying insects. The ixodidae have a limited range in which to search for a potential mate, namely the host, hence perhaps the reduced reliance on volatile sex pheromones and dependance on the non-volatile contact sex pheromones to identify the location of the female.

Cuticular sex pheromones are non volatile or have a very low volatility. These pheromones are synthesized within the cuticle epidermal cells or associated cells and located predominantly on the cuticular surface. They are not uncommon among the Insecta. They have also been termed cuticular aphrodisiacs, indicating a role in mate recognition after the pair have been brought together as part of a complex pattern of courtship behavior (Birch, 1974). Principally these compounds are long chain (C:21 - C:40) hydrocarbons, (alkanes, methyl branched alkanes, olefins etc). Their low volatility assumes a long term efficiency for these molecules (Peschke, 1986). Only the non-volatile compounds or compounds which are perceived when they are contacted on the cuticle can be correctly described as

contact pheromones as the 'receiver' organism must contact the compound in order to perceive it. The effect of these compounds is invariably enhanced by other sex recognition cues such as vision, tactile stimuli and interactive behavioral patterns. Due to the relatively high molecular weight, consequent low volatility and concomitant cuticular position, cholesterol oleate can be seen to be a contact cuticular sex pheromone.

Cuticular pheromones have also been demonstrated in other arthropod groups. In the Tetranychidae they act as an arrestant resulting in the male guarding an immature female until she moults to the mature adult stage, Cone (1979). Suter *et al.*, (1987), demonstrated the presence of an unidentified pheromone in cuticular surface extracts of the female bowl and doily spiders (*Frontinella pyramitela*). This pheromone enables the male spider to recognize a conspecific female for mating purposes. The presence of a cuticular contact sex pheromone produced by the dermal androconial glands of the Scutellerid (Heteroptera) *Tectocoris diopthalmus* has also been suggested (Knight *et al.*, 1985).

The Role of Sterols, Sterol Esters and Associated Compounds as Pheromones.

Sterol esters occur widely in nature where they are formed by the conjugation of various sterol and fatty acid moieties. They have been reported in fungi, algae, plants, invertebrates and vertebrate animals (Nes and M'Kean, 1977). In mammals, cholesterol esters are major constituents of plasma low-density lipoproteins and they are an important transport form of cholesterol.

The discovery of cholesterol oleate as the MSP of *Dermacentor variabilis* was most unexpected. Cholesterol oleate and other sterol esters secreted or deposited onto the tick cuticular surface have previously been regarded merely as waste products of feeding (Cherry, 1969a). In the present study other sterol esters induced mounting behavior in some *D. variabilis* males [(i.e. weak positive responses) especially when used in higher concentrations (10 μg) than they are normally found on the female cuticle surface]. However only cholesterol oleate induced responses that were indistinguishable from the natural extract (MSP). The TLC chemical analysis demonstrated that this class of compounds occurs in other species of hard ticks, and the results presented above indicate that males of different species also respond to extracts containing this class of compounds. Although responses varied among species, significant interspecific activity was detected, suggesting that mounting behavior is widespread in the Ixodidae and that sterol esters are responsible for this activity.

Sterols and sterol esters have not been found to serve as sex pheromones or pheromones in any other arthropod although they have been found to serve as sex pheromones in endoparasitic trematodes. Metacercaria of *Amblosoma suwaense* are attracted to worms secreting sterol esters and pair in the presence of these compounds (Fried and Robinson, 1981). In several other trematode species, free sterols rather than sterol esters acted as sex pheromones (Haseb and Fried, 1988).

A sterol derived compound 5ß-cholestane-3,24-dione (Crump *et al.*, 1987) has been shown to be a trail following pheromone in the eastern tent caterpiller, *Malacosoma americanum* (Fitzgerald and Edgerly, 1982).

The presence of sterols and sterol esters has been reported in the cuticular extracts of many insects where they are present as minor constituents. None of them have been reported to have biological activity. Sterol esters have been shown to account for 3 % of the cuticular lipid of the weevil, *Ceutorrhynchus assimilis* (Richter and Krain, 1980). Fatty acid constituents of the esters range from C:12 to C:26, the most abundant being 18:1 (30 %), 18:2 (13 %) and 18:3 (19 %). At most, sterols account for 6 % of the cuticular lipid of the adult fleshfly, *Sarcophaga bullata* (Jackson *et al.*, 1974). In the larva, pupa and adult of the beetle, *Epilachna varivestis* (Danehower and Bordner, 1984) sterols account for 5.8, 5.0 and 0.4 % respectively of the lipids.

The Role of Size, Tactile Stimuli and Motion in Mate Recognition.

The experiments above also established that no other factors are as important in eliciting the male mating response in *D. variabilis* and *D. andersoni* as the MSP. The removal of the cuticular ridges did not disrupt male mating activity. Males did not respond significantly differently to decoys similar in size to that of a conspecific mate. This is an unusual finding as in several insect species studied, size of female is extremely important to the male in mate recognition. The male Tsetse (*G. m. morsitans*) is highly sensitive to differences in size of the female and can detect small differences in the radius (0·5 mm) of glass spheres coated with the female produced pheromone. The cuticle of the ixodidae and *Glossina* spp. are similar in that both cuticles undergo a significant amount of stretching due to blood feeding. However, female ticks are attractive throughout the period of blood engorgement, the period

of most rapid growth, therefore the male must be responsive to a wide range of different sized females as potential mates. Alternatively in *G. m. morsitans* mating takes place before feeding (feeding is a relatively fast procedure lasting only a few minutes). The female size is therefore relatively stable, and males do not have a wide range of potential sizes of female to respond to. Thus this might explain the enhanced sensitivity of male *G. m. morsitans* to mate size when compared to that shown by male ixodidae. The ability of arthropods to detect the size of the substrate with which they are in contact appears to be widespread in the Insecta. Females of the parasitic wasp (*Trichogramma* spp.) are able to determine the number of eggs to deposit by the length of time taken to make a single transit across the host surface (Schmidt and Smith, 1987). This is in turn determined by the radius of curvature of the substrate. The means by which male *G. m. morsitans* are able to determine female size remains to be elucidated. Tsetse flies typically also mate in the air and consequently female movement has also been found to be an important stimulatory factor for male *G. m. morsitans*. Movement of the female is unimportant in most species of Ixodidae that have been studied. This is undoubtedly due to the sessile behavior of the female whilst attached and feeding during which time mating takes place. Thus it can be seen that the ixodid Mounting Sex Pheromone is indeed unique among arthropod cuticular sex pheromones.

Detailed Comparison of the *D. variabilis* Contact Sex Pheromone Mediated Mating System With Those of the Insecta.

The housefly and the tsetse fly have frequently been cited as examples of insect mating systems which depend on the presence of cuticular contact sex pheromones for successful mating. In these insects, cuticular hydrocarbons, esters, and ketones may have sex pheromonal properties (Howard and Blomquist, 1982). These examples and the Rove Beetle example described in detail below are significantly different from the tick model as they also require visual and tactile stimuli, which are of equal importance to the chemical stimulus, to ensure successful mating.

Mating in *Musca domestica*, the house fly, has been shown to be dependant on a combination of; (1) a volatile sex attractant pheromone, (2) physical and (3) visual stimuli. Contrary to previous reports, no true contact sex pheromone exists (Adams and Holt, 1987; La-France *et al.*, 1989). In another example, i.e. the Tsetse fly *Glossina morsitans morsitans* model, a contact sex pheromone has been shown to exist although it also operates in concert with other factors such as visual and tactile stimuli. Elimination of any of these factors renders the entire system virtually inoperative. The tick model, does not appear to require tactile or visual stimuli. Rather, a chemically mediated sequence of behavior directs the mating process. In Ixodid ticks, males are attracted by the volatile sex attractant pheromone, make contact with the female, and execute a preprogrammed sequence of behaviors which are released by the MSP. As demonstrated above, the physical, e.g. tactile or visual components of these behaviors are either non existent, or, if present, of very little

significance.

Early work on the housefly (*Musca domestica* Meigen) cuticular sex pheromone indicated that this pheromone consisted of several components; (1) an alkene, (Z)-9-tricosene, (2) an epoxide, (Z)-9,10-epoxytricosane and (3) a ketone, (Z)-14-tricosene-10-one, as well as other methyl branched alkanes. (Z)-9-Tricosene (C:23) was shown to induce male mating strike behavior (Carlson *et al.*, 1971; Rogoff *et al.*, 1973; Adams and Holt, 1987). Strike activity involves male orientation to the female, jumping and landing on the female (Tobin and Stoffolano, 1973). The methyl alkanes (Uebel *et al.*, 1976), epoxide and ketone (Uebel *et al.*, 1978), were reported to enhance the activity of (Z)-9-tricosene thereby increasing strike activity. A very detailed analysis of *Musca domestica* mating activity undertaken by Adams and Holt (1987), showed that previous studies were biologically meaningless due to the excessive concentrations (30 - 60 times physiological concentrations), of these components used in the previous studies. These results have been recently confirmed by La-France *et al.*, (1989). They concluded from their own work that male mating strike behavior is influenced by (Z)-9-tricosene only. The other components enhanced strike activity only on pseudo flies. When the male fly actually encounters a real fly other more dominant factors (presumably non-chemical) come into play to enhance the activity of the (Z)-9-tricosene. It should also be noted that in tests with (Z)-9-tricosene only 30 % of males were attracted to a synthetic pheromone source (Carlson *et al.*, 1971; Mayer and James, 1971; Voaden *et al.*, 1972). These results were not significantly different from tests with 500 trapped virgin females (Mayer and Thaggard, 1966). Richter *et al.*, (1976),

proposed that (Z)-9-tricosene served as a release mechanism for responsiveness to optical cues, thus leading to mating strikes.

A considerable amount of work has been performed to investigate the biology and chemistry of African Tsetse fly cuticular contact sex pheromones. The presence of a contact cuticular sex pheromone, 15,19,23-trimethylheptatriacontane (C:40) on *Glossina morsitans morsitans* was first described by Langley *et al.*, (1975). The compound was isolated, identified and synthesized by Carlson *et al.*, (1978) who also showed that similar compounds, 15,19-dimethylheptatriacontane and 17,21-dimethylheptatriacontane (C:39) were non-stimulatory. The active component was found to be present at concentrations of about $4 \cdot 2$ μg per female and $0 \cdot 6$ μg per male. This contact pheromone was shown by Carlson, *et al.*, (1978), Huyton *et al.*, (1980a) and Langley and Hall (1984), only to be stimulatory on actual female Tsetse flies. It was not effective on decoys at physiological concentrations. Huyton *et al.*, (1980b), concluded that 15,19,23-trimethylheptatriacontane must be presented "on a surrogate female bearing some of the configurational characteristics of a female fly,..males...are less responsive to an object which departs significantly in size and shape from a female fly". These results indicate that the presence of the Tsetse fly contact pheromone alone does not account for all of the male mating activity. Males are attracted to the female by visual stimuli, contact is made between the sexes and then the non-volatile sex pheromones become important in reinforcing mate selection and releasing further mating activity. It also appears that motion, at least in the field, is required to allow the male to attempt copulation, Langley *et al.*, 1982. Indeed, these authors concluded that decoys which visually, vaguely resembled

real females and which were coated with pheromone were largely ineffective in attracting male tsetse flies. Work by Langley *et al.*, (1982), Offor *et al.*, (1981), and Nelson *et al.*, (1988), has demonstrated methylalkanes (which they suggest also have contact sex pheromonal activity) in a range of other species of *Glossina*. Langley *et al.*, (1982), also provided evidence that male mating success may be due in some part to female behavioral maturation.

Peschke (1978), demonstrated the presence of a hydrocarbon sex pheromone on the cuticular surface of the female Rove Beetle (Aleochara curtula). This pheromone which has several active components (Z)-7-heneicosene (C:21) and (Z)-7-tricosene (C:23), induces grasping of the female genitalia with the male genital parameres (Peschke, 1986; Peschke and Metzler, 1987). This cuticular pheromone was detected by males only over very short distances of about 2.2 mm. by olfactory mechanisms (Peschke, 1983). This model appears to be less dependant on non-chemical stimuli than either the housefly or tsetse fly examples. It would appear that only the ixodids among many other arthropods are solely dependant on chemical cues for mating success.

Production of MSP.

The site of production of cholesterol oleate is unknown. Indeed it is not known if it is produced by the esterification of dietary derived cholesterol or if it is sequestered intact from the blood meal.

Work performed on the metabolism of insect cuticular sex pheromones and cuticular lipids indicates that the low volatility compounds are produced in the

oenocytes and these cells which are also present in the ixodidae represent a possible site of MSP production. These are epidermal cells modified by the addition of smooth endoplasmic reticulum for lipid production (Lockey, 1988). Evidence was provided by Katase and Chino (1984), of oenocytes synthesizing the three main classes of hydrocarbons occurring in cuticular lipids, namely n-alkanes, methylalkanes and olefins. There is no direct evidence linking oenocytes to the synthesis of the polar constituents of cuticular lipids, such as fatty acids, alcohols and esters (Romer, 1980). However oenocytes (Blomquist *et al.*, 1980) as well as the epidermal associated fat body (Piek, 1964) have been suggested to be the sites of synthesis of these compounds.

The function of the ixodid epidermal cell layer is not as clearly understood as in the Insecta. Tick cuticle structure is however very similar to that of insect cuticle. The main difference between them being the ability of the female tick alloscutum for rapid growth and expansion during engorgement, a process known as neosomy (Audy *et al.*, 1972). There are many minor structural differences between tick and insect cuticle and, these are reviewed by Hackman and Filshie, (1982). The epidermal cell layer secretes the chitin-protein material of the cuticle and also those lipids associated with the cuticle. The epidermal cell layer is connected with the epicuticular wax layer via a system of microscopically fine pore canals and wax canals. Dermal glands also lying within the epidermal layer are connected with the epicuticle via ducts. Lees (1947), found that in ixodids the dermal glands secrete a thick yellow grease which slowly spreads over the cuticle surface or collects around the duct openings. Other workers have noted secretions in other species of ticks of

differing color and fluidity. If cholesterol oleate is synthesized by the tick it may well be that the dermal glands are a likely site of this synthesis. Although insects lack a cholesterogenic pathway (Blum, 1987) they do have biochemical pathways to modify dietary derived sterols. Cholesterol is the precursor of ecdysone and other similar molecules. Insects have also been shown capable of modifying cholesterol for exogenous use (Schildknecht, 1970). For example the prothoracic glands of the dytiscid beetle *Acilius sulcatus* are able to produce a wide range of steroids, which it then uses in its defensive gland secretions (Meinwald, *et al.*, 1987).

The female soft tick *Ornithodoros moubata* has been shown by Connat *et al.*, (1986), to be able to inactivate ingested ecdysteroids by conjugation with long chain fatty acids. Kuthiala and Ritter (1988a,b) demonstrated the ability of *Heliothis zea* to take up dietary cholesterol and cholestanol and store it in various tissue organs in the esterified form.

MSP Perception.

The site of MSP perception is at present unknown. It is most likely however that because of the non-volatility of the molecule (mw 650 Daltons) the receptor will be shown to be a gustatory type receptor. Contact sex pheromone perception has been studied in *M. domestica* and *G. m. morsitans*. Schlein *et al.*, (1981), suggested that the tibial receptors present on the forelegs rather than the hindlegs were involved in the perception of cuticular pheromones. These receptors are probably saucer-like paired sensilla with one orifice located on both sides of the proximal part of the tibia and resemble the tarsal organs of spiders (Dumpert, 1978). The

structural similarity of those high molecular weight compounds in the Insecta which have been shown to have contact sex pheromone activity (Carlson *et al.*, 1971; Rogoff *et al.*, 1973; Bartelt and Jackson, 1984; Jallon, 1984; Adams and Holt, 1987 and Peschke and Metzler, 1987) indicates perhaps parsimony in this type of receptor evolution. These compounds are typically methyl branched alkanes or mono or di unsaturated alkenes. Receptor fields analogous to these have not been demonstrated in the ixodidae. Relatively little is known about the mechanisms by which ticks recognize pheromonal chemicals in comparison to the Insecta (Sonenshine *et al.*, 1982). However ticks possess a wide range of sensory organs including chemoreceptors (olfactory and gustatory) as well as combined mechano-chemosensory receptors (Hess and Vlimant, 1986). Of the known receptors those present on the tick forelegs are most likely to be involved in MSP perception although the possibility of as yet undiscovered gustatory receptors being the site of MSP perception can not be discounted.

Several possible sites exist for the location of the MSP receptor. The first of these is Haller's organ located on the dorsal surface of the tarsi of leg 1. Haller's organ consists of an anterior trough with six or seven setiform sensilla and a posterior capsule with both setiform and pleomorphic sensilla. The anterior trough in most ixodid species has at least four types of setiform sensilla, including a large broadly based multiporose seta. In addition, a group of setae on a ridge or hump, anterior to the trough contain one or more multi porose setae (Sonenshine, 1987). This is the sensillum type that serves to detect the SAP, 2,6-DCP (Haggart and Davis, 1981; Waladde, 1982). Waladde and Rice (1982), observed that *A. variegatum*

and *Rhipicephalus appendiculatus* respond to 2,6-DCP in amounts as low as 0·4 $ng \cdot \mu l^{-1}$ which is quite remarkable given that neither species actually produces this chemical. It also indicates that although a sensilla may respond to certain chemical stimuli it does not indicate that a biological role for that chemical should be taken for granted, or that the sensilla that is responding is the one that responds normally or is connected to the CNS. Tip pore sensilla elsewhere on the tip of the digit serve as gustatory receptors.

Another sensory organ which may perceive the MSP is the palpal sensory organ. The palpal sensory organ is located on the terminal segment of the palp and possesses gustatory chemoreceptor sensilla. The cheliceral digits which have been shown by Moorhouse (1966) and Sonenshine *et al.*, (1985) to be important in the perception of the GSP also possess gustatory receptors. It is unlikely that either the palpal or cheliceral receptors play a significant role in the perception of MSP because even when these receptor fields are ablated or removed, males are still able to recognize and mount conspecific females (Feldham-Muhsam and Borut, 1971; Sonenshine *et al.*, 1985).

Other Possible Roles for Sterol Esters and Other Ixodid Non-polar Cuticular Lipids.

Cholesterol has been identified among the lipids of *Boophilus microplus*, Cherry (1969b). Cherry (1976), showed that cholesterol and its esters were the only steroid materials present in the tissue lipids of the adult female Cattle Tick and their eggs. Gilby (1957) concluded that major components of the *B. microplus* cuticular lipid fraction were saturated acids and alcohols (about C:30) largely

combined as esters. He concluded that free acids, alcohols and short chain and unsaturated compounds were present only in small amounts. Fieser and Fieser (1959), and Atkinson and Binnington (1973), identified 2,4,6-cholestatriene in the surface lipids from eggs of *B. microplus* and from hexane washings of five other species of ixodid eggs. These lipids are produced by the female and coated on the eggs during egg laying via a specialized organ. Atkinson and Gilby (1970), suggested the presence of a phenolic anti-oxidant (2,6-DCP ?) to protect unsaturated lipids.

McCamish *et al.*, (1977) identified the presence of an unbranched n-alkane series, C:13 to C:32, and esters composed of branched-chain acids C:14 to C:19 and n-alcohols C:26 to C:36 in *B. microplus*. Recent work by Hunt (1986), has demonstrated the presence of branched alkanes and alkenes in extracts made from three species of *Amblyomma*. Hunt noted the small number of n-alkanes present in the *Amblyomma* spp. unlike the Insecta were n-alkanes are preeminent. The *Amblyomma* species also had an excess number of alkenes compared to the insects.

The work presented above indicates the presence of a series of n-alkanes from C:21 to C:35 in *D. variabilis*. Wax esters, fatty acid methyl esters and free fatty acids as well as hydrocarbons, sterol esters and cholesterol were also shown to be present in the five species of ticks examined.

The function of cuticular lipids in Ixodidae is believed to be; (1) waterproofing, (2) protection from physiochemical hazards of the environment and (3) act as a repository of non-utilizable lipid [Cherry (1969b) and McCamish *et al.*, (1977)]. Cuticular lipids have been shown to increase 3 fold in amount after the female drops off the host, Cherry (1969b). Davis (1974) believes that this increase in lipid

requirement may be related to an increased water retention requirement when the tick is exposed to a much more desiccating environment than on the host. Davis also believes that the lipid concentration and composition of adult ticks is continuously changing reflecting the different environments in which the tick is present unlike the insects where lipid concentration and composition is remarkably constant over time.

It appears from the results provided that MSP does not provide species specificity. It is also clear that genera specificity is limited. It may be that differences in male response to the different species and genera tested is simply in response to varying concentration of cholesterol oleate. Alternatively it may be due to the presence of different sterol esters on the female body surface. It seems most likely that these sterol esters are derived from the diet, it has been reported (Hamilton *et al*, 1989) that sterol esters increase in concentration during feeding. The male may well be responding to these sterol esters as an indication of a feeding female and thus a viable mate. The predominant sterol ester on the female body surface would be a function of the ticks normal host blood chemistry. Presumably males have evolved through time to respond to this optimal concentration or type of sterol ester, when conditions are not optimal, male response is reduced.

A Note on the HPLC and GC Analysis of Sterol Esters.

The relatively low volatility of sterol esters, has until recently tended to preclude the analysis of intact sterol esters by gas chromatography, (Evershed *et al.*, 1987). The most common approach has been saponification with subsequent separation and GC

analysis of the components of the released sterols and fatty acid components after methyl esterification (Gibbons *et al.*, 1982; Lusby, *et al.*, 1987). HPLC has been used to separate individual components of mixtures of sterol esters. Duncan *et al.*, (1979) and Carroll and Rudel, (1981), used three reverse phase HPLC columns joined in series to achieve adequate separations of individual components. Several problems exist with using HPLC to separate sterol esters, there can be a lack of resolution (Carroll and Rudel, 1981) although this was not a problem encountered in this study due to the relative simplicity of the refined tick extract. In addition the relatively poor detection limits due to the lack of a strong chromophore means that 10 - 50 μg of each component is generally required for analysis (Carroll and Rudel, 1981). In the HPLC analysis 0·5 μg of cholesterol linolenate (C27, 18:3) was observable and five times that amount of cholesterol oleate (C27, 18:1) was required to produce a peak. Resolution of sterol esters of the same carbon number but possessing differing degrees of unsaturation is not satisfactory by GC (Evershed, *et al.*, 1987). This observation was confirmed by the GC analysis undertaken in this study although the identification of sterol C:18 fatty acid compounds added significance to the HPLC identification. However these compounds can be separated according to their degree of unsaturation by argentation column chromatography or argentation TLC (Evershed, *et al.*, 1987). Alternatively Kovacs, *et al.*, (1986) have described a TLC system for analysis of crude extracts without resort to HPLC or column chromatography. This method is however only suitable for a preliminary type of analysis.

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APPENDIX A

TABLE A1 Results of thin layer chromatographic separations of lipid extracts from five species of ixodid ticks and standards (IB_2 plate, Skipski solvent system).

	Compound classes and Rf values					
species	hydro-carbons	hydro-carbons	sterol-esters	FAMEs	fatty acids	chole-sterol
D. variabilis	0.97	—	**0.84***	0.70	**0.65**	**0.37**
D. andersoni	0.99	0.96	**0.84**	0.73	**0.67**	**0.37**
A. americanum	0.99	0.97	**0.84**	—	—	**0.37**
A. maculatum	—	—	**0.84**	0.74	**0.65**	**0.37**
H. dromedarii	0.99	0.97	**0.84**	0.73	**0.67**	**0.37**
Sterol ester[1]	—	—	**0.84**	—	—	0.37
Cholesterol	—	—	—	—	—	**0.37**
Hydrocarbon[2]	0.99	—	—	—	—	—
NPLM[3]	—	—	**0.85**	0.73	**0.65**	**0.37**
Lipid Stds[4]	—	—	—	0.74	—	—
DV ♀ ext[5]	—	—	**0.84**	—	**0.64**	**0.38**
Fatty acid	—	—	—	—	**0.64**	—

1 Sterol ester (cholesterol oleate) 50 μg.
2 n-tetradecane and n-tetradecene (70 μg of each).
3 Non-polar lipid mix; colesterol oleate, methyl oleate, triolein, oleic acid and cholesterol (70 μg).
4 Lipid Standards (FAMEs); linoleic, linolenic oleic, palmitic and stearic fatty acid methyl esters (70 μg).
5 *DV* ♀ cuticle extract made in a soxhlet extractor (100 μg).
* Those values in bold type represent the most intensely charring spots.

TABLE A2 Results of thin layer chromatographic separations of lipid extracts from five species of ixodid ticks and standards (IB-2 plate, Mangold solvent system).

	Compound classes and Rf values					
species	hydro-carbons	sterol-esters	FAMEs	fatty acids	unknown	chole-sterol
D. variabilis	—	**0.60***	0.46	**0.32**	0.12	**0.08**
D. andersoni	0.99	**0.64**	0.45	**0.33**	—	**0.08**
A. americanum	0.99	**0.65**	0.45	0.28	0.12	**0.08**
A. maculatum	—	**0.63**	0.45	**0.33**	0.12	**0.08**
H. dromedarii	0.99	**0.64**	0.45	**0.30**	0.12	**0.08**
Sterol ester[1]	—	**0.60**	—	—	—	**0.08**
Cholesterol	—	—	—	—	—	**0.08**
Hydrocarbon[2]	—	—	—	—	—	—
NPLM[3]	—	**0.63**	0.45	**0.32**	—	**0.08**
Lipid Stds[4]	—	—	**0.48**	—	0.15	—
DV ♀ ext[5]	—	0.60	—	**0.39**	0.15	**0.08**
Fatty acid	—	—	—	**0.33**	—	—

1 Sterol ester (cholesterol oleate) 50 μg.
2 n-tetradecane and n-tetradecene (70 μg of each).
3 Non-polar lipid mix; colesterol oleate, methyl oleate, triolein, oleic acid and cholesterol (70 μg).
4 Lipid Standards (FAMEs); linoleic, linolenic oleic, palmitic and stearic fatty acid methyl esters (70 μg).
5 *DV* ♀ cuticle extract made in a soxhlet extractor (100 μg).
* Those values in bold type represent the most intensely charring spots.

TABLE A3 Results of thin layer chromatographic separations of lipid extracts from five species of ixodid ticks and standards (IB-2 plate, Non polar solvent system).

	Compound classes and R*f* values					
species	hydro-carbons	hydro-carbons	sterol-esters	FAMEs	fatty acids	chole-sterol
D. variabilis	—	—	**0.56***	0.41	**0.34**	**0.13**
D. andersoni	0.79	0.75	**0.55**	—	**0.31**	**0.13**
A. americanum	0.79	0.75	**0.56**	—	0.27	**0.12**
A. maculatum	—	0.75	**0.56**	—	**0.31**	**0.12**
H. dromedarii	0.79	0.75	**0.55**	0.37	**0.31**	**0.12**
Sterol ester[1]	—	—	**0.50**	—	—	0.12
Cholesterol	—	0.74	—	—	—	**0.12**
Hydrocarbon[2]	—	**0.70**	—	—	—	—
NPLM[3]	—	—	**0.54**	**0.39**	**0.31**	**0.12**
Lipid Stds[4]	—	—	—	**0.43**	—	0.13
DV ♀ ext[5]	—	—	**0.54**	**0.40**	**0.35**	**0.12**
Fatty acid	—	—	—	—	**0.35**	—

1 Sterol ester (cholesterol oleate) 50 μg.
2 n-tetradecane and n-tetradecene (70 μg of each).
3 Non-polar lipid mix; colesterol oleate, methyl oleate, triolein, oleic acid and cholesterol (70 μg).
4 Lipid Standards (FAMEs); linoleic, linolenic oleic, palmitic and stearic fatty acid methyl esters (70 μg).
5 *DV* ♀ cuticle extract made in a soxhlet extractor (100 μg).
* Those values in bold type represent the most intensely charring spots.

TABLE A4 Results of thin layer chromatographic separations of lipid extracts from five species of ixodid ticks and standards (HPTLC plate, Non polar solvent system).

	Compound classes and Rf values					
species	hydro-carbons	sterol-esters	FAMEs	fatty acids	unknown	chole-sterol
D. variabilis	—	**0.54***	0.48	0.36	**0.11**	**0.08**
D. andersoni	—	**0.54**	—	0.36	**0.11**	**0.08**
A. americanum	0.74	**0.54**	—	0.36	—	**0.08**
A. maculatum	—	**0.54**	0.48	0.36	**0.11**	**0.08**
H. dromedarii	0.73	**0.53**	—	0.36	**0.11**	**0.08**
Sterol ester[1]	—	**0.52**	—	—	—	0.08
Cholesterol	—	—	—	—	—	**0.08**
Hydrocarbon[2]	0.73	—	—	—	—	—
NPLM[3]	—	**0.54**	**0.48**	**0.36**	—	**0.08**
Lipid Stds[4]	—	—	**0.48**	—	—	—
Fatty acid	—	—	—	**0.36**	—	—

1 Sterol ester (cholesterol oleate) 50 μg.
2 n-tetradecane and n-tetradecene (70 μg of each).
3 Non-polar lipid mix; colesterol oleate, methyl oleate, triolein, oleic acid and cholesterol (70 μg).
4 Lipid Standards (FAMEs); linoleic, linolenic oleic, palmitic and stearic fatty acid methyl esters (70 μg).
5 *DV* ♀ cuticle extract made in a soxhlet extractor (100 μg).
* Those values in bold type represent the most intensely charring spots.

APPENDIX B

Figure B1. Gas-chromatographic analyses of acid/base/neutral separation of hexane fraction of *D. variabilis* extract. (a) neutrals, (b) acids and (c) bases.

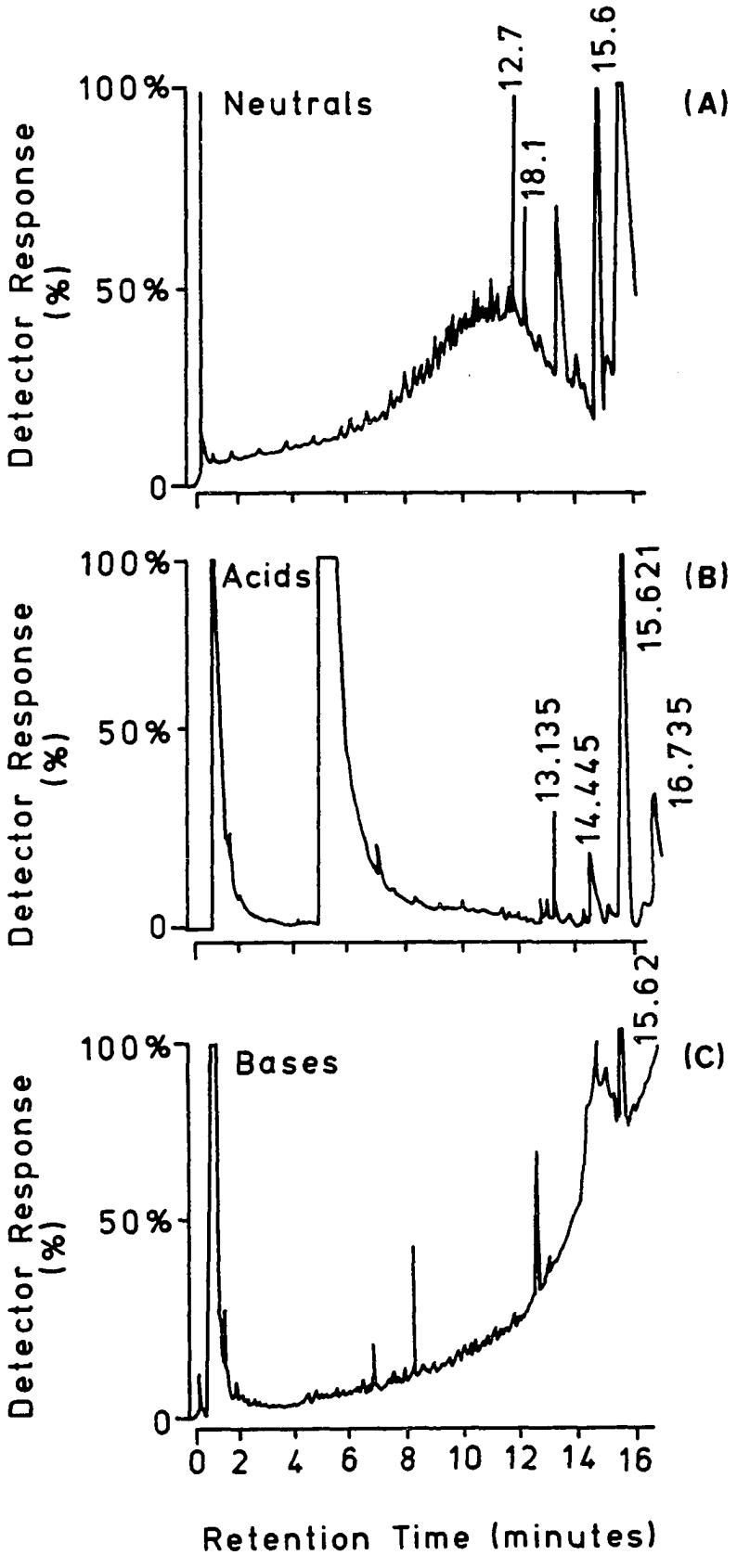
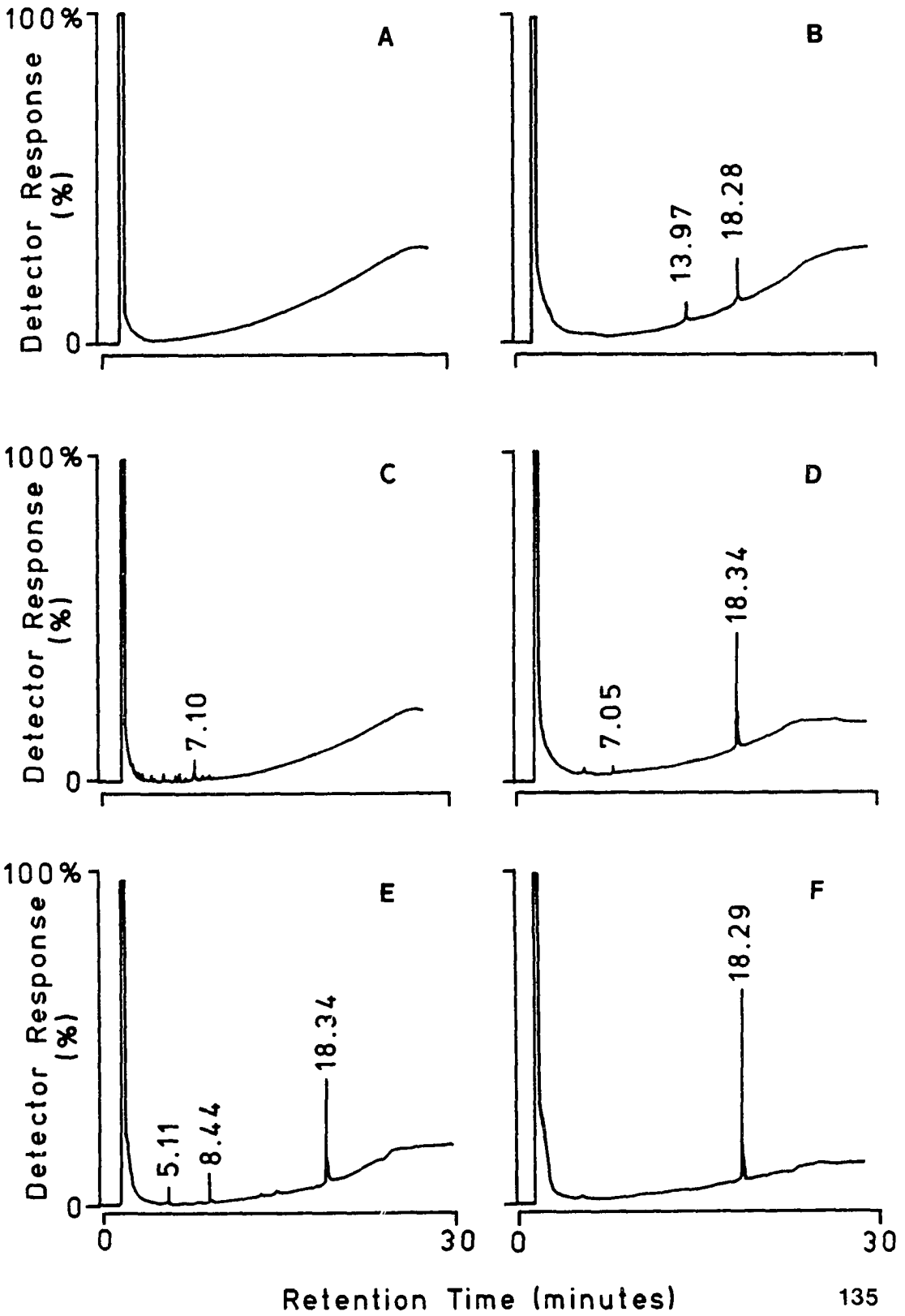


Figure B2. Gas-chromatographic analyses of eight fractions generated from Bio-Sil A column fractions (Non-methyl esterified, 180°C to 260°C). (a) **F1**, Column blank, 20 volumes eluted and collected before addition of biological material, concentrated; (b) **F2**, hexane 100 %; (c) **F3**, hexane:diethyl ether, 99:1; (d) **F4**, hexane:diethyl ether, 98:2; (e) **F5** hexane:diethyl ether, 95:5; (f) **F6**, hexane:diethyl ether 90:10; (g) **F7**, hexane:diethyl ether, 80:20; (h) **F8**, soxhlet extract of Bio-Sil A column after elution of extract.



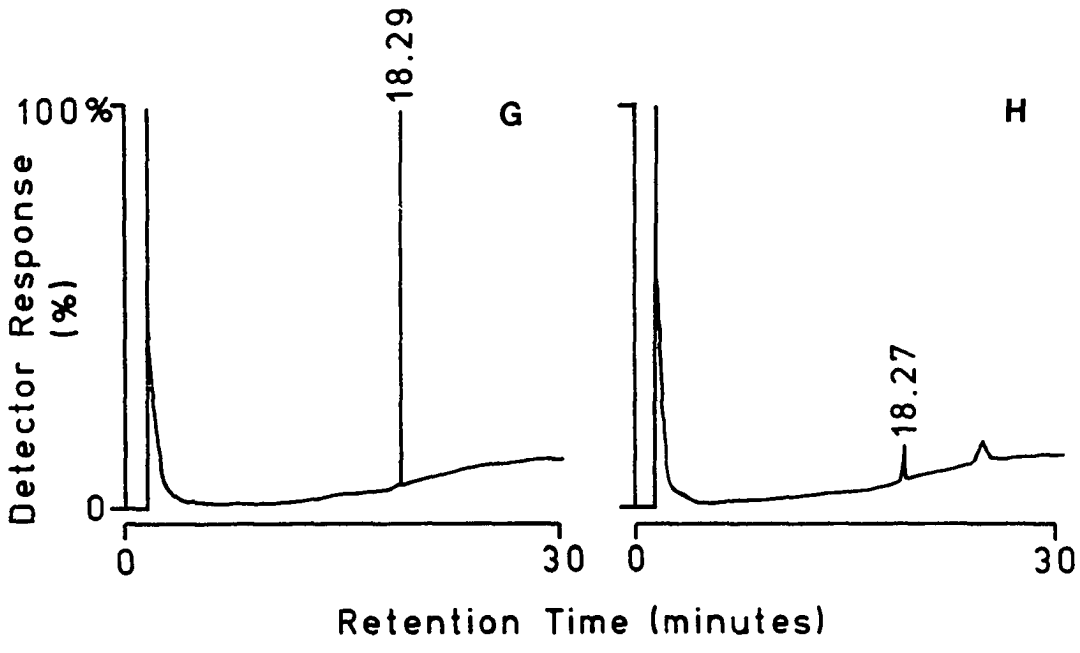
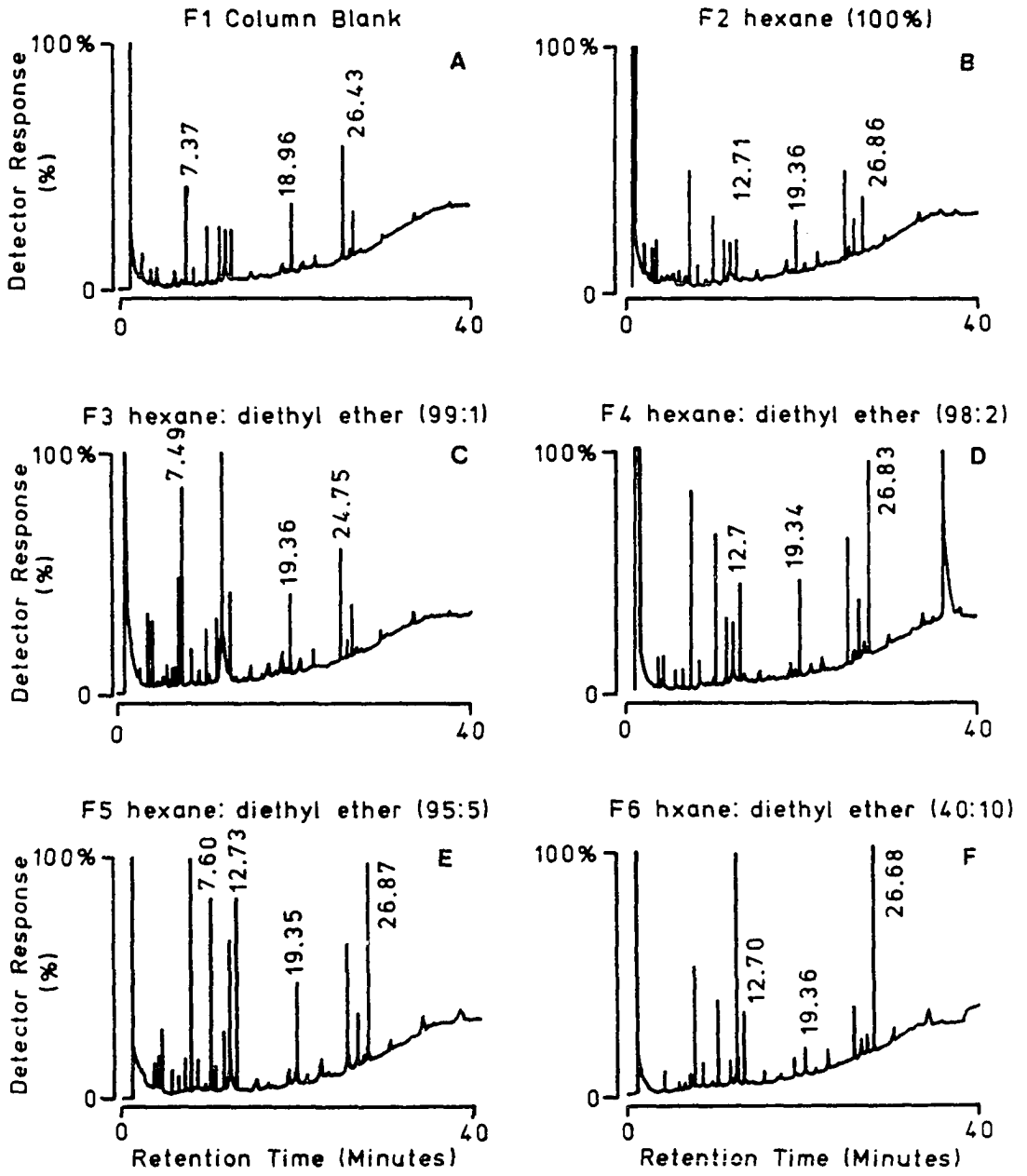
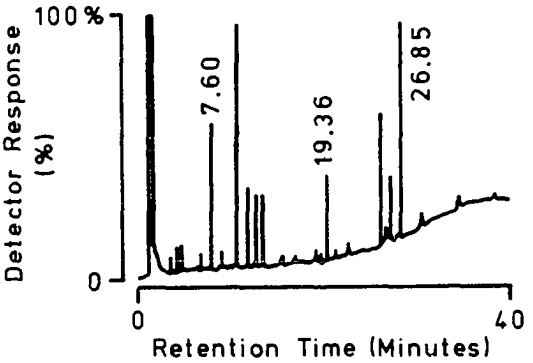


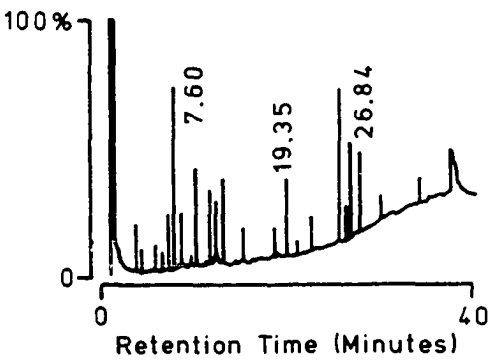
Figure B3. Gas-chromatographic analyses of eight fractions generated from Bio-Sil A column fractions (Methyl esterified, 180°C to 260°C). (a) **F1**, Column blank, 20 volumes eluted and collected before addition of biological material, concentrated; (b) **F2**, hexane 100 %; (c) **F3**, hexane:diethyl ether, 99:1; (d) **F4**, hexane:diethyl ether, 98:2; (e) **F5** hexane:diethyl ether, 95:5; (f) **F6**, hexane:diethyl ether 90:10; (g) **F7**, hexane:diethyl ether, 80:20; (h) **F8**, soxhlet extract of Bio-Sil A column after elution of extract.



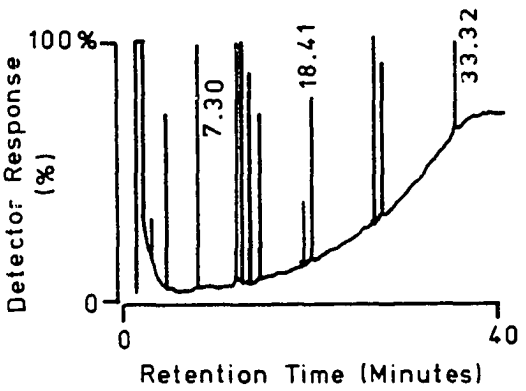
F7 hexane: diethyl ether (80:20)



F8 Column Extract



Rapeseed Oil Standard



APPENDIX C

LIST OF ABBREVIATIONS

AA	*Amblyomma americanum*
AM	*Amblyomma maculatum*
AUF	Absorbance Units Full Scale
bp	before present
CCHF	Crimean Congo Hemorrhagic Fever
CI	Chemical Ionization
DA	*Dermacentor andersoni*
2,6-DCP	2,6-Dichlorophenol
DFT	Dummy Female Tick
DV	*Dermacentor variabilis*
EI	Electron Ionization
eV	Electron Volts
FAME	Fatty Acid Methyl Ester
FE	Female Equivalent
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GSP	Genital Sex Pheromone
HPLC	High Pressure Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HD	*Hyalomma dromedarii*
IR	Infra Red Spectrometry
ME	Male Equivalents
MSP	Mounting Sex Pheromone
NMR	Nuclear Magnetic Resonance
P'	Polarity Index
RMSF	Rocky Mountain Spotted Fever
Rt	Retention Time
SEM	Scanning Electron Micrograph
TLC	Thin Layer Chromatography
UV	Ultra Violet

AUTOBIOGRAPHICAL STATEMENT.

Gordon was born on the 11th of February 1960 in Limavady, County Londonderry, Northern Ireland. Apart from a brief sojurn of 11 months in Australia he lived there until he went to university in Wales in 1978. He read Zoology and Chemistry at University College Cardiff and graduated with a B.Sc. (Honours) degree in 1982. He graduated with a Masters degree from the same university in 1985, his thesis was on the Pheromones and Allomones of an exotic West African plant bug (*Hotea gambiae*, Scutelleridae). In October 1985 he moved to Norfolk, Virginia, US and enrolled in the Biomedical Sciences PhD program of Old Dominion University and Eastern Virginia Medical School. He graduated from the program in August 1989.

Gordon has eight articles currently published or in press, these are listed below along with two patent applications submitted to the US Patent Office.

Gough, A.J.E., Hamilton, J.G.C., Games, D.E., and Staddon,B.W. (1985). Multichemical defense of the plant bug *Hotea gambiae* (Westwood) (Heteroptera: Scutelleridae), sesquiterpenoids from abdominal gland in larvae. *J. Chem. Ecol.* **11(3)**, 343-352.

Hamilton, J.G.C., Gough, A.J.E., Staddon, B.W., and Games, D.E. (1985). Multichemical defense of the plant bug *Hotea gambiae* (Westwood) (Heteroptera: Scutelleridae), (E)-2-hexenol from abdominal gland in male adults. *J. Chem. Ecol.* **11(10)**, 1399-1409.

Hamilton, J.G.C. and Sonenshine, D.E. (1987). Evidence for the occurence of a mounting sex pheromone on the body surface of female *Dermacentor variabilis* (Say) and *Dermacentor andersoni* (Stiles) (Acari: Ixodoidea). *J. Chem. Ecol.* **14(1)**, 401-410.

Sonenshine, D.E., Allan, S.A., Hamilton., J.G.C., Taylor, D., Phillips, J.S., and Silverstein, R.M. (1988). *Complex multicomponent pheromone systems regulating mate recognition and species discrimination in Ixodid ticks.* Proceedings of the XVIII

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Sonenshine, D.E., Taylor, D., Phillips, J.S., Hamilton, J.G.C., and Allan, S.A. (1989). *Sex Pheromones in Ixodid Ticks: Identification and Role in Species Discrimination.* Proceedings 5th. International Congress of Invertebrate Reproduction.

Hamilton, J.G.C., and Sonenshine, D.E. (1989) Pheromone baited decoys for control of ticks on animal hosts. (In preparation).

1986 *'A description of a biorational tick control system: Ixodex'* Co-inventors Sonenshine, D.E. and Hamilton, J.G.C. (Patent Pending).

1988 *'The use of a novel group of naturally occuring compounds as the synthetic mounting sex pheromone of Ixodidae.* Co-inventors Hamilton, J.G.C., Sonenshine, D.E., and Lusby, W.L. (Patent Pending).

Gordon was employed as a Pre-Doctoral Research Assistant from 1985 to May 1989, he was then employed as a Post-Doctoral Research Assistant in the same labotatory until September 1989 when he moved to Liverpool, England to commence a Wellcome Foundation Senior Post-Doctoral Fellowship at the Liverpool School of Tropical Medicine.

He recieved an Elizabeth Tuckerman Scholarship in 1986, and an International Student Award to attend the XIth. Annual American Association for the Advancement of Science (AAAS) Colloquium on Research and Development and Public Policy, held in Washington, DC. He is a Fellow of the Royal Entomological Society, a Member of the American Institute of Chemists, and an Associate Member of Sigma Xi.