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ISOLATION OF LIPIDS AND SEX PHEROMONE FROM HARD TICKS

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ABSTRACT

Y. S. Chow, F. M. Lu, C. T. Peng and P. C. Cheng (1972) *Isolation of Lipids and Sex Pheromone from Hard Ticks*, Bull. Inst. Zool., Academia Sinica **11**(1): 1-8. The following lipids were isolated from the females of brown dog tick, *Rhipicephalus sanguineus* (Latreille) and tropical cattle tick, *Boophilus microplus* (Canestrini): 4-methyl-nonane, methyl esters of fatty acids, triglycerides, free fatty acids and cholesterol. The sex pheromones of the two species of ticks had identical gas chromatographic properties regardless of whether they were analyzed on polar or nonpolar columns. It is a weak acid or phenolic compound closely related to thymol.

It has been clearly shown that females of the lone-star tick, *Amblyomma americanum* (Linnaeus), the Gulf Coast tick, *A. maculatum* Koch and the American dog tick, *Dermacentor variabilis* (Say) produce sex pheromones to attract males to them just like insects do[1]. Although Gladney[6] reported that, when using bovine as the host, 25% of the experimental females of the Gulf Coast ticks were attracted to males, the results obtained in this preliminary study lead us to believe that the pheromone produced by the females of brown dog tick, *Rhipicephalus sanguineus* (Latreille) and tropical cattle tick, *Boophilus microplus* (Canestrini) found on Taiwan cattle was a substance identical to that previously found in the lone-star tick.

MATERIALS AND METHODS

Partially engorged females of the tropical cattle tick, *Boophilus microplus* (Canestrini) were homogenized and extracted with methylene chloride according to the method of Berger *et al.*[1]. Crude oily residue after evaporation of the solvent was fractionated on a column packed with 200 g of 100-200 mesh silicic acid. Elution was carried out with hexane and hexane containing increasing amounts of ethyl ether. 9 fractions were obtained and for checking the results of column chromatography, fractions 1, 3, 5 and 7 (Table 1) were spotted on thin layer chromatographic (TLC) plates according to Chow & Liang[3] except that the solvent system used was n-hexane/ ethyl ether/ acetic acid (90:10:1). The major spots separated on a TLC plate were examined with a Perkin Elmer IR spectrophotometer. Three fractions (F4, F5 and F6) showing sex pheromone activity were combined and mixed with an equal amount of 5% aqueous sodium hydroxide. The aqueous layer was

washed with n-hexane and the hexane layer discarded. The aqueous solution was acidified by saturating with carbon dioxide or by adding phosphoric acid to neutrality. Then the pheromone was partitioned back into hexane layer again. The hexane solution was injected into a gas chromatograph (Hitachi model 063) equipped with a heated splitter outlet. The columns used were stainless steel columns of the size 1.9 meter in length and 4 mm in diameter packed with shimalite mesh 60-80 coated with 12% diethylene glycol succinate (DEGS), 1% neopentyl glycol succinate (NPGS) or 25% silicone D. C. 200. Virgin male ticks were attached on a rabbit host for 7 days and then placed beside the chromatograph to receive the column elute emerging from the splitter outlet. Usually the attached males were quiet on the rabbit skin and not disturbed by the movement of small amount of air. When the pheromone come out of the splitter outlet, the male ticks showed olfactory response by waving their legs fast and detaching from the rabbit host. The major peaks of the active fraction were also studied by using a gas chromatograph-mass spectrograph (Hitachi-Model RMU-6GC) for the determination of molecular weight.

The extraction and analysis of female brown dog tick *R. sanguineus* were done in the identical manner.

RESULTS

From 231.56 g of female tropical cattle ticks, *B. microplus* (Canestrini), 4.34 g of a crude oily residue was obtained. The recoveries of fractions from the silicic acid column were shown in Table 1. There were 9 fractions, F1 to F9. In TLC and IR study, the chromatogram of the crude oil preparations & fractions from silicic acid column were shown in Fig. 1. Fractions 8 and 9 were solids. Fractions 2, 4 and 6 not different very much from 1, 3, and 5, respectively, were not shown in Fig. 1. The fraction 1 had a large R_f value corresponding to hydrocarbon and showed strong absorption bands at 2,985 cm^{-1}, 1,455 cm^{-1} and 1,380 cm^{-1}, and a finger print identical to 4-methyl-nonane. Fraction 3 showed strong sharp bands at 2,970 cm^{-1}, 1,735 cm^{-1}, 1,454 cm^{-1}, 1,235 cm^{-1}, 1,178 cm^{-1} and 1,035 cm^{-1} and a weak one at 732 cm^{-1}. These spectra

TABLE I

Lipid Fractions Separated on a Silicic Acid Column

Fractions	Solvent	Residue weight (g)	Appearance	Recovery, % of total input
F_1	n-hexane	0.34	yellow oil	7.0
F_2	n-hexane with 1% diethyl ether	0.14	yellow oil	3.0
F_3	n-hexane with 2% diethyl ether	0.89	yellow oil	22.0
F_4	n-hexane with 4% diethyl ether	0.36	yellow oil	8.2
F_5	n-hexane with 8% diethyl ether	0.63	yellow oil with white powder ppt	14.5
F_6	n-hexane with 10% diethyl ether	0.34	yellow oil with white powder ppt	7.0
F_7	n-hexane with 20% diethyl ether	0.21	yellow oil with white scale	5.0
F_8	n-hexane with 50% diethyl ether	0.16	needle crystal	3.6
F_9	100% diethyl ether	0.09	yollow powder	2.0
Total				72.3

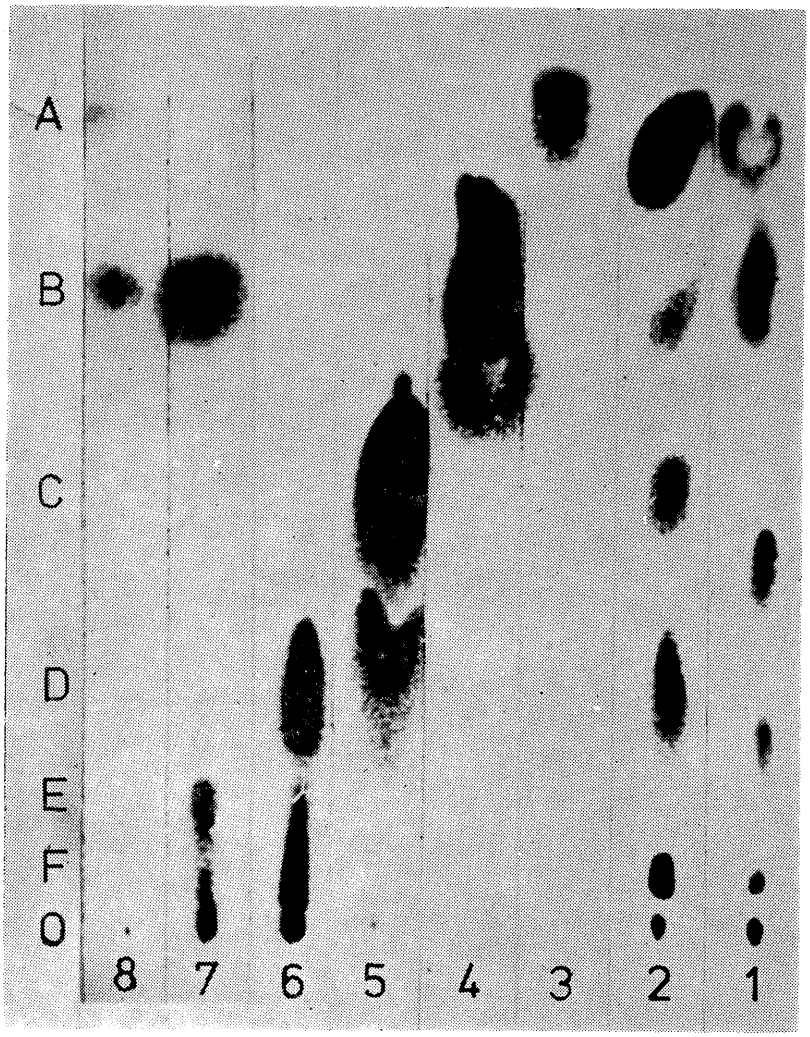

Fig. 1. Thin layer chromatograms of the extracts of females of 2 hard tick species. Spots were visulized using the method described by Chow and Liang[8].

(1) 2.0 μl of crude oil of *Rhipicephalus sanguineus*.

(2) 2.0 μl of crude oil of *Boophilus microplus*.

(3), (4), (5) and (6) are fractions 1, 3, 5 and 7 (Table 1) of *B. microplus* respectively.

(7) A mixture of synthetic methyl esters of homologus fatty acids.

(8) Methyl stearate alone.

A, B, D, E and F were 4-methyl-nonane, fatty acid methyl esters, triglyceride, free fatty acid, and cholesterol, respectively. C was an unidentified material and O, the origin of the chromatogram.

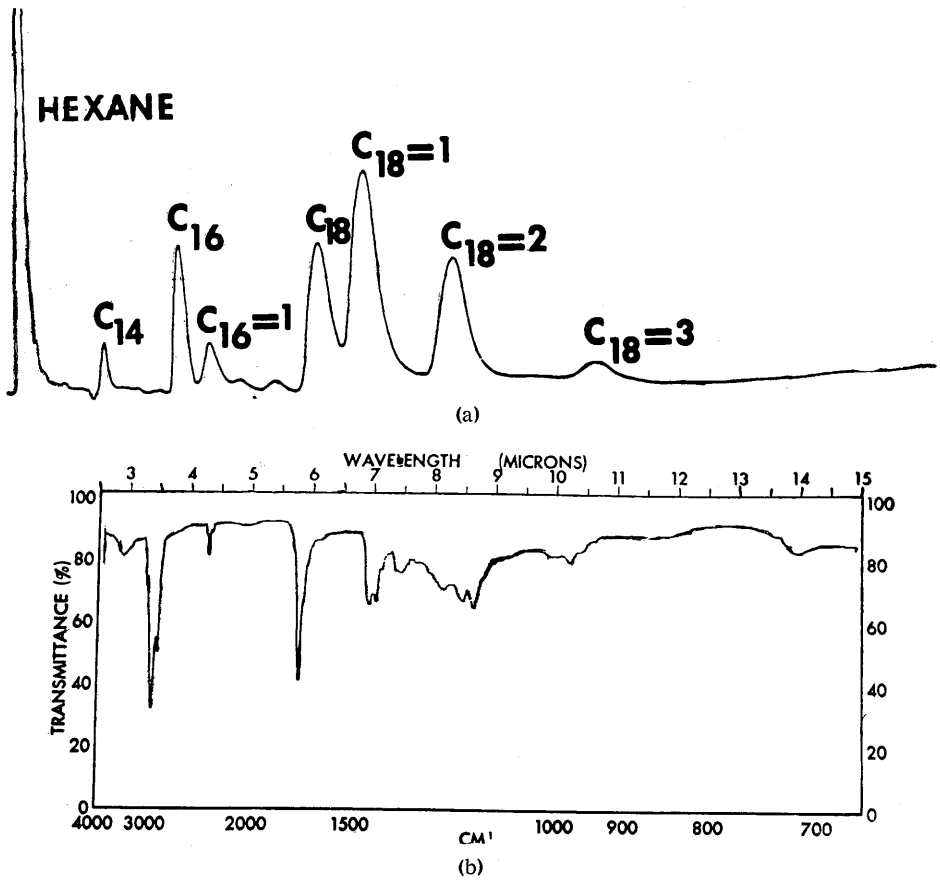

Fig. 2a. Gas-liquid chromatogram of the esters (F3) of tropical cattle tick, *B. microplus*.
2b. Infrared spectra of the esters (F3) of tropical cattle tick, *B. microplus*.

were identical to those of our synthetic fatty acid methyl esters according to the method of Stoffel, *et al.*[6], and the data of Yinon, *et al.*[7]. The gas-liquid chromatogram and IR spectra of this fraction (F3) were shown in Fig. 2. The main esters identified were methyl esters of myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids. Other spots except 4-methyl-nonane and fatty acid methyl esters studied were corresponding to triglyceride, free fatty acid and cholesterol.

The sex pheromone fractions (F4, 5, and 6) obtained after acidifying the sodium hydroxide solution with carbon dioxide was less than 0.4 ml. This oily substance was diluted with 5 ml *n*-hexane for GLC injection. The column elute was bioassayed with male ticks attached on rabbit host, and the retention time of the sex pheromone was shown by an arrow in Fig. 3. In polar columns, the retention time of the sex pheromone in a DEGS column was longer than that of the indicator thymol, but in a NPGS column, it was shorter than that of thymol. In a nonpolar column, silicone D. C. 200, the retention time of pheromone was also shorter than that of thymol. The retention time of the sex pheromone in comparison with those of some reference compounds were presented in Table II.

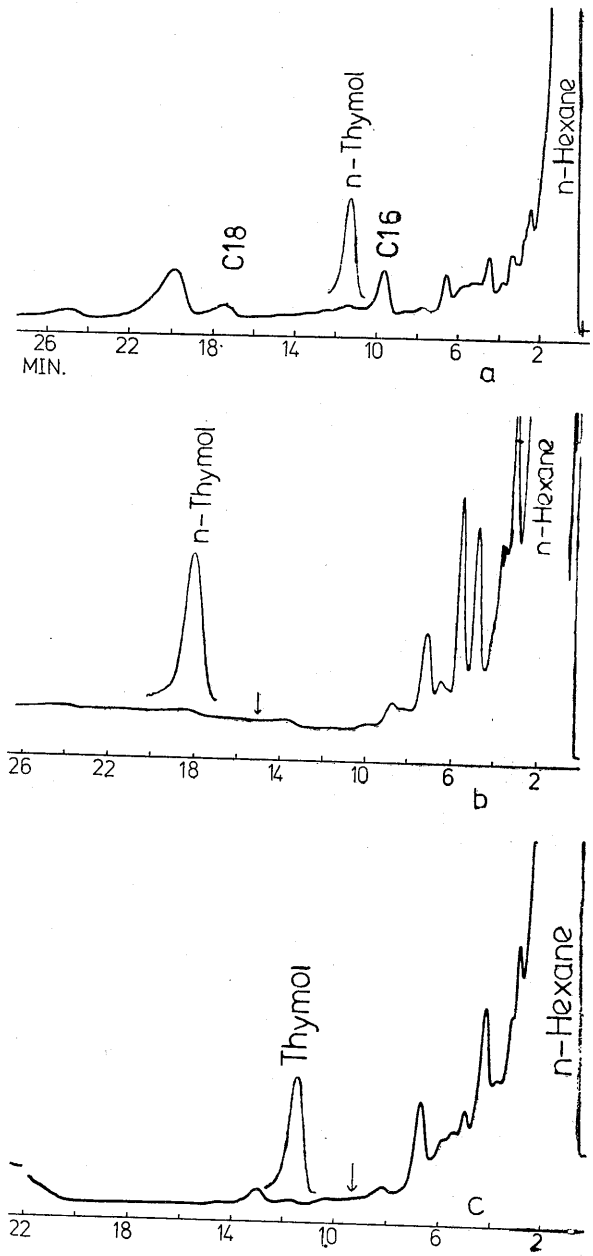

Fig. 3. Gas chromatogram of the sex pheromone containing fraction showing the retention times (arrows) of the pheromone in 3 different columns:
a. DEGS at 180°C.
b. Silicone D.C. 200 at 160°C.
c. NPGS at 140°C.
Min: Retention time in minute is counted form injection.

TABLE II

Retention Time (Minutes)* of Sex Pheromone on Gas Chromatographic Analysis

Column	Temp. (°C)	Methyl palmitate	Methyl stearate	Thymol	Sex pheromone
12% DEGS	180	9.5	17.4	11.2	12.4
1% NPGS	130	—	—	12.4	11.0
	140	—	—	11.0	9.5
	170	8.2	18.0	4.0	4.2
25% Silicone D.C. 200	160	—	—	17.8	14.8
	230	30.3	56.0	—	—

* Calculated by taking the solvent peak n-hexane as zero time.

In an attempt to gain some knowledge of the relative volatility and possible range of structure of the pheromone, its retention time was also compared with that of certain known compounds. It fell between myristyl acetate and palmityl acetate or nonanoic acid and decanoic acid on DEGS column, and between octyl alcohol and decyl alcohol and shorter than 2-methoxy-4-methyl-phenol on D. C. 200 column. These data were identical to that of previously reported by Berger *et al.*[1]. The major peaks emerging before and after the sex pheromone from a DEGS column were analyzed by a mass spectrograph; the results are shown in Fig. 4. The parent peaks gave mass numbers of 270 and 298, corresponding to the methyl esters of palmitic and stearic acids. These results confirmed our IR and GLC data. The mass spectrographic analysis of the sex pheromone has not been possible because of its very low concentration in the active fraction.

The chromatographic behavior of the sex pheromone from brown dog tick, *R. sanguineus* was identical to that of tropical cattle tick.

DISCUSSIONS

Highly potent chemicals in the insect communication system belonging to the pheromone group have been described by Bossert and Wilson[2]. Among the pheromones, the specificity of sex pheromone was the highest and the threshold concentration the lowest. For example, according to Law and Regnier[4] a concentration of 10^{-12} μg/ml of female sex pheromone, trans-10-cis-12-hexadecadien-1-ol of *Bombyx mori* will produce 50% positive behavioral responses in male moth. This indicated that 1 or 2 molecules could elicit an olfactory potential. In the extract of female hard ticks, we found that a very potent sex pheromone which was dissolved in dilute sodium hydroxide solution (5%) and then partioned into benzene by neutralization of the alkali solution with carbon dioxide. So according to its solubility and pK_a value (close to pH 7-pH 8). It was a phenolic compound. This compound behaved similarly to thymol on both polar and nonpolar columns in GLC analysis. The gas chromatographic properties of the pheromone obtained in this study (Fig. 2) were identical with those obtained from other hard ticks, such as *Amblyomma americanum* (Linnaeus)[1]. Because the material was available in so small a quantity that its structural determination has not been possible.

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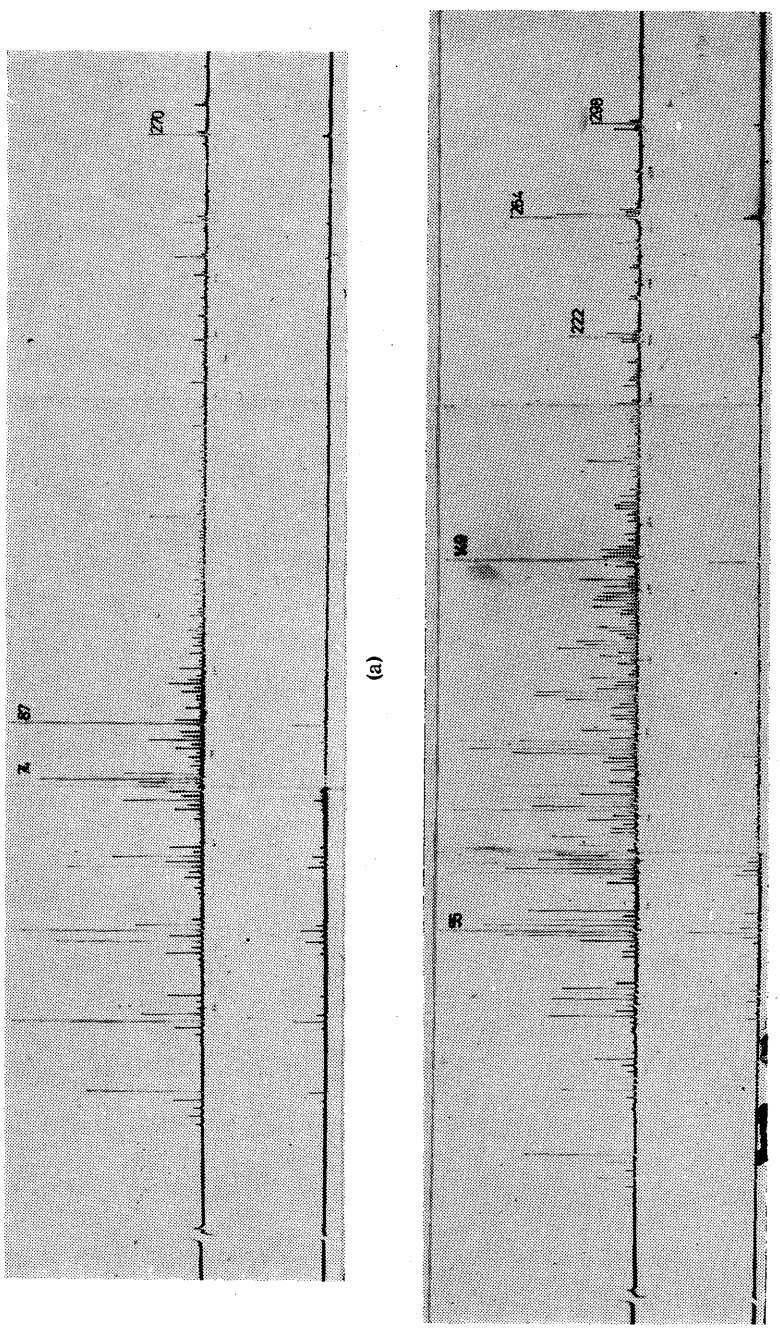

Fig. 4. Mass spectra of the major peaks which were eluted before (a) and after (b) the sex pheromone from DEGS column. Recovered with a Hitachi-Model RMU-6GC instrument equipped with a 2-element galvanometer.

Sato for his help in GC-Mass operation, and to Drs. J.C. Su and S.S. Jeng for their reading of the manuscript.

REFERENCE

1. Berger, R.S., J.C. Dukes and Y.S. Chow (1971) Demonstraction of a sex pheromone in three species of hard ticks. *J. Med. Ent.* **8**: 84-86.
2. Bossert, W.H. and E.O. Wlison (1963) The analysis of olfactory communication among animals. *J. Theoret. Biol.* **5**: 443-469.
3. Chow, Y.S. and H.M. Liang (1967) The lipid contents of the mouse skin. *Bull. Inst. Zool., Academia Sinica* **6**: 1-9.
4. Law, J.H. and F.E. Regnier (1971) Pheromones. *Annu. Rev. Biochem.* **40**: 533-548.
5. Gladney, W.J. (1971) Mate-seeking by female *Amblyomma maculatum* (Acarina: Ixodidae) on bovine. *Nature* **323**: 401-402.
6. Stoffel W., F. Chu and E.H. Ahrens, Jr. (1959) Analysis of long-chain fatty acids by gas-liquid chromatography. *Anal. Chem.* **31**: 307-308.
7. Yinon, U., A. Shulov and R. Ikan (1971) The olfactory responses of granary beetles towards natural and synthetic fatty acid esters. *J. Insect Physiol.* **17**: 1037-1049.

壁蝨類之油脂及性費洛蒙

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自褐蝨 *Rhipicephalus sanguineus* (Latreille) 及熱帶牛壁蝨 *Boophilus microplus* (Canestrini) 之雌體內可以抽出下列油酯類：4-甲基壬烷、甲基酯肪酯、三酸甘油脂、酯肪酸及胆固醇。此兩種壁蝨類之性費洛蒙以其溶解度及 pKa 值之特性證明爲弱酸或者酚類物質。以氣層分析儀分析時，甚近似麝香草酚。