NAVEL ORANGEWORM "TOXIC MUMMY" LURE AND SEX PHEROMONE

Project No. 85-A6-Navel Orangeworm, Mite and Insect Research

Progress Report

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OBJECTIVES

(1) Develop an attractant for egg-laying female moths by chemically micmicking a mummy nut and combine the attractant with an insecticide to kill visiting females. (2) To isolate and identify secondary pheromone components essential for optimal male sex pheromone response.

INTERPRETIVE SUMMARY

(1) On a new project this year, substantial progress has been made in the development of an "attracticide" for NOW females utilizing almond odors. Using a wind-tunnel bioassay, as well as field trapping, we have determined that: a) crude almond oil (CAO) is about 10X more attractive than presscake (PC) on a per weight basis, b) the combination of PC and CAO is no more attractive than CAO alone, and c) attraction to CAO is about 2X greater than to NOW-infested mummy nuts and 6.5X greater than to non-infested mummies. Screening of a number of pyrethroid insecticides for repellency when combined with CAO indicated that Pydrin does not reduce attraction to CAO even when present at a 2.5% formulation. Toxicity studies demonstrated that females allowed to fly upwind and land on this bait experience 90% mortality within 24 hr, with significant sub-lethal effects expected in the surviving females.

Our understanding of the behaviorally active constituents in CAO has also been significantly advanced this year. Fractionation of CAO using high-performance liquid chromatography, infrared spectrometry, and gas chromatography-mass spectrometry, along with chemical reaction techniques and wind-tunnel bioassay have demonstrated that primary upwind flight to CAO is due to long-chain fatty acids (palmitic, linoleic, oleic, and stearic acids). Treatment of CAO with diazomethane to destroy free fatty acids results in almost complete loss of upwind-flight response. Long-range female attraction to a mixture of synthetic fatty acids is comparable to that to CAO, although landing and oviposition on the synthetics is significantly lower. Future work will focus on the stimuli needed for these short-range behaviors as well as developing a chemical delivery system for the toxic mummy bait for use in the field.

(2) Considerable effort continues to be waged in elucidating the complete female pheromone of NOW. Pheromone from many thousands of females has been collected by Dr. Li, purified by chromatographic methods by Dr. Phelan, and shipped to Dr. Klun for analysis by gas chromatography and mass spectrometry. Our efforts have been hampered by problems with chemical lability, an unusually complex pheromonal blend, and high variability in levels of male response; however, using a new GC configuration, Dr. Klun recently has successfully maintained activity after GC fractionation of the suspected hydrocarbons.

EXPERIMENTAL PROCEDURE

Wind-tunnel bioassay

Earlier work by one of us (TCB) indicated that almond by-products could evoke in gravid females discrete behavioral response in the wind tunnel. During the past year, these observations were built upon and a reliable and reproducible bioassay was developed for the laboratory. Male and female NOW were maintained together on a 16L:8D light cycle. Two hours prior to "lights off," mated 2-4-day-old females were removed from the colony and held in small cylindrical cages (10 ♀♀/cage). These females were placed in the wind-tunnel room under simulated twilight conditions. During testing, a candidate attractant was applied to a 5.5 cm filter paper circle and placed at the upwind end of the 2.5 m-long wind tunnel. A cage of females was then placed 2 m downwind of the material and females were allowed to take off. Each cage of females was held in the tunnel for 5 min and the number of females responding by taking flight, orienting upwind, and landing on the material during this time was recorded. This procedure was replicated for each treatment using a randomized complete-block design.

Field trapping

Results from the wind tunnel were corroborated by field trapping. During the period between April 17 to May 29, the materials were assessed for their ability to lure female NOW using Pherocon 1C traps placed in almond orchards. Trapping took place in the orchards of the Bidart Bros. and the Clement ranch. Trap capture was assessed on a weekly basis and treatments were also replaced and re-randomized weekly.

Chemical procedures

To identify the behaviorally active constituents of crude almond oil (CAO), this material was submitted to a series of analyses. CAO was first fractionated using normal phase HPLC. The behaviorally active fractions were then combined and analyzed by GC, FT-IR, and GC/MS. FT-IR analyses indicated the presence of long-chain fatty acids, so these were derivatized using diazomethane before analyzing by GC/MS. The position of the double bonds was determined by first treating with 3-chloroperbenzoic acid to form epoxide derivatives. These were then submitted to GC/MS for analysis of key fragments.

RESULTS AND DISCUSSION

Toxic mummy

Products of almond processing, crude almond oil (CAO), presscake (PC), and refined or sweet almond oil (SAO), were bioassayed as potential NOW female lures for the toxic mummy strategy. We found that on a per-weight basis, CAO was a much more potent attractant than PC (Figure 1). Maximum response to CAO was evoked by 50-250 mg, while 500-1000 mg of PC was necessary for a comparable level of response. Furthermore, the combination of PC and CAO in a 10:1 ratio as presently used in egg traps did not increase the level of response over that to CAO alone (Figure 2). Figure 2 also demonstrates that response to SAO was very minimal.

The specificity of the response to CAO with regard to sex and mating status is demonstrated in Figure 3. This points to a very dramatic shift in behavior due to the mating process, and indicates that if used in an attracticide formulation, only gravid females will be targeted. Since the use of the toxic mummy is intended for spring NOW populations, overwintering mummy nuts represent the primary competition for attraction of females and thus become a benchmark for comparing attraction levels. Wind-tunnel results suggest that CAO is indeed very competitive with mummies, evoking a response 2.5X greater than that of mummies infested with NOW larvae and 6.5X greater than that of non-infested mummies (Figure 4). Thus, the toxic mummy should be competitive when used in orchards with good sanitation bearing only 1-2 nuts/ tree. The higher level of response to infested mummies as compared to non-infested mummies confirms the earlier field-trapping results of Keith Andrews and Martin Barnes.

As a final measure of the relative attraction to almonds and by-products, field trapping was carried out. Although only somewhat low numbers of females were captured, the relative pattern was consistent with the wind-tunnel results (Figure 5). Thus 50 mg CAO far out-performed PC, was just as good as the CAO/PC combination, and was better than larvae-infested mummies, although not significantly so. One interesting finding was the inverse relationship between CAO quantity and trap catch. This may be due to a premature in-flight

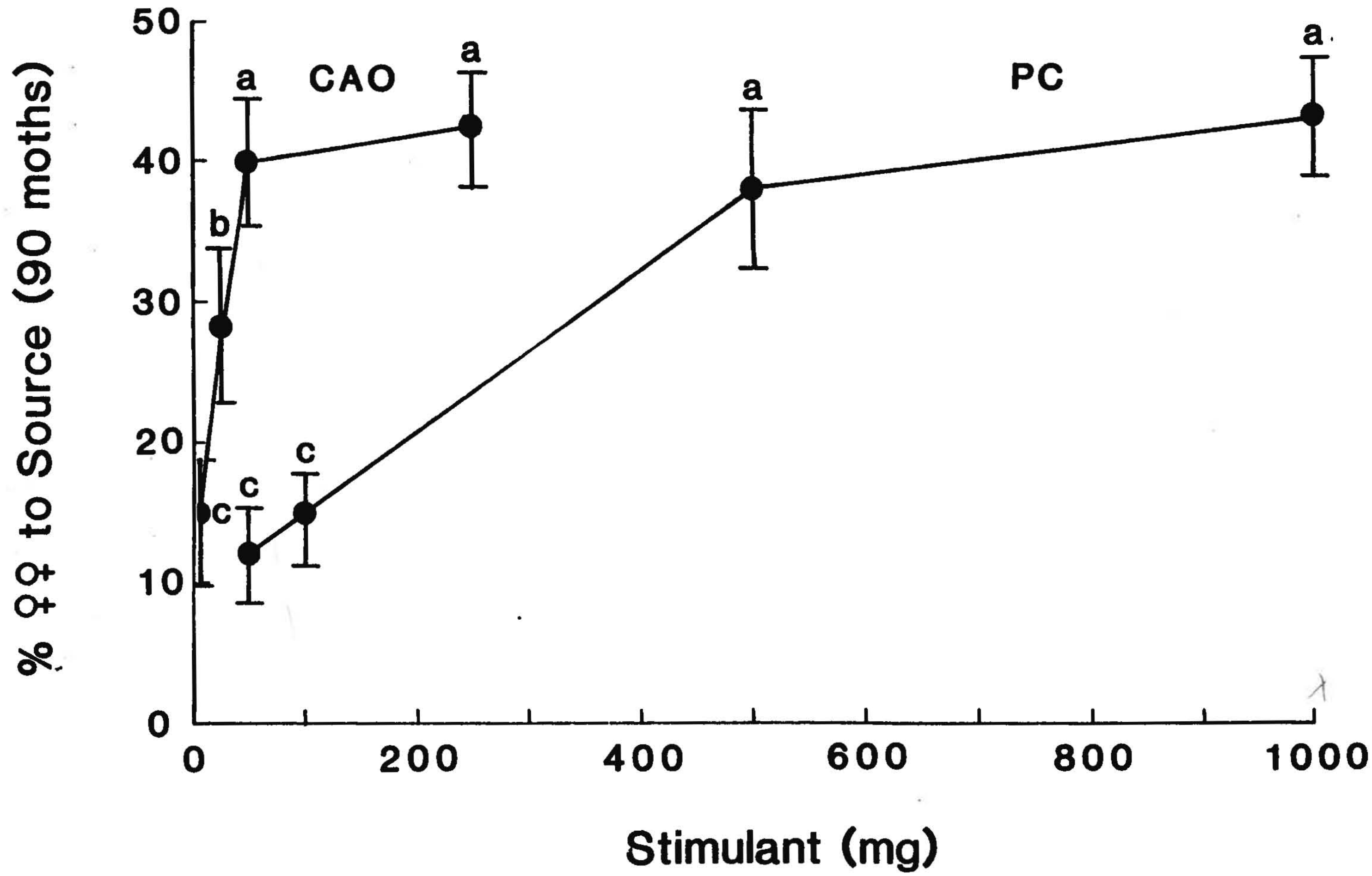


Figure 1. Response of NOW ♀♀ to various quantities of crude almond oil (CAO) and almond presscake (PC) in the wind tunnel.

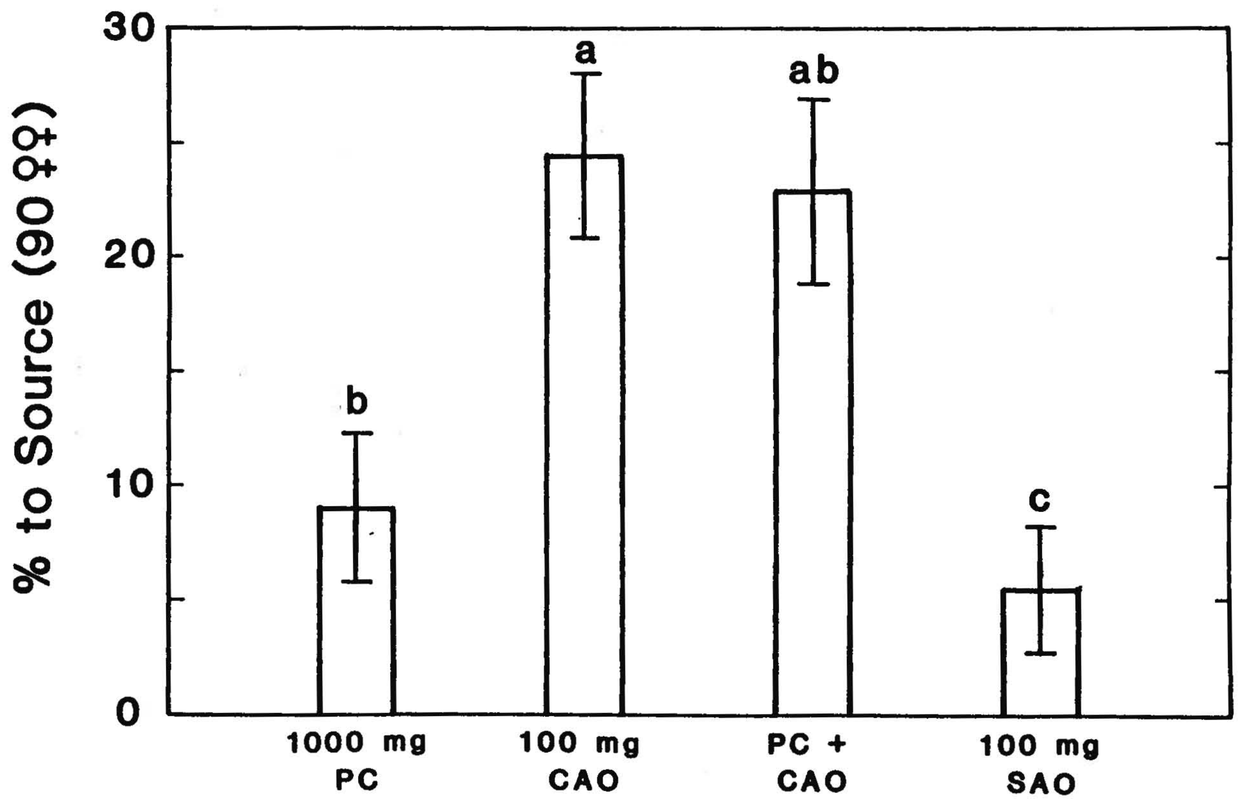


Figure 2. Response of NOW ♀♀ to almond presscake (PC), crude almond oil (CAO), combined PC and CAO (1100 mg), and sweet almond oil (SAO) in the wind tunnel.

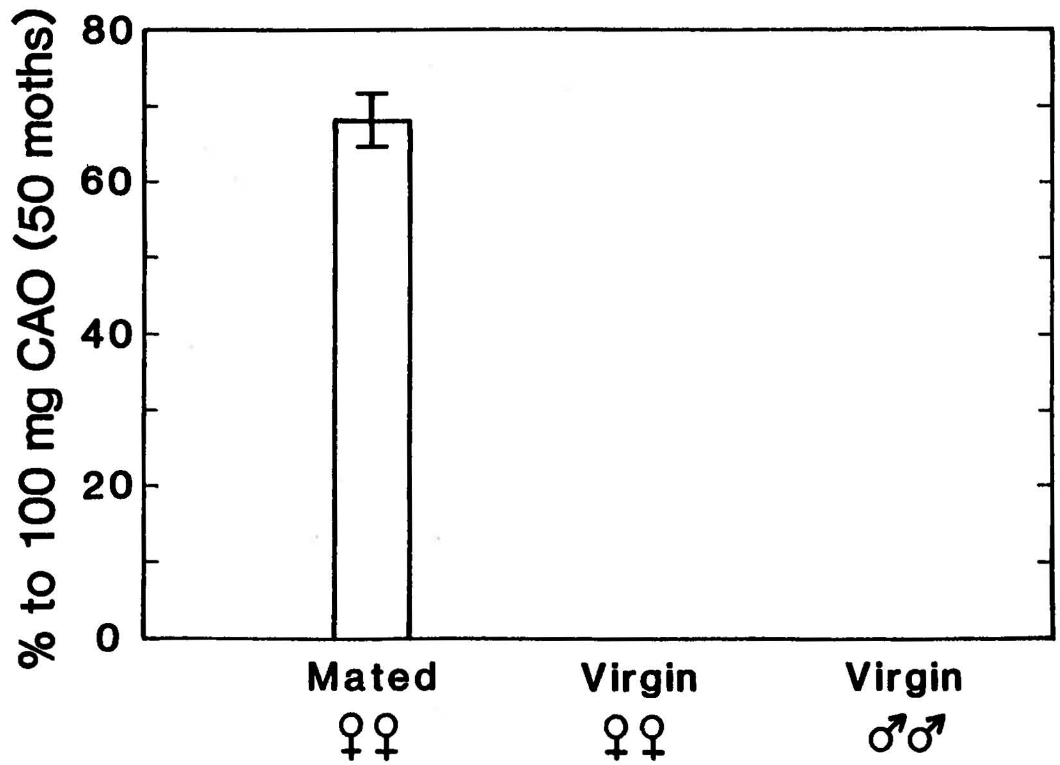


Figure 3. Response of mated and virgin females and virgin males to 100 mg CAO in the wind tunnel.

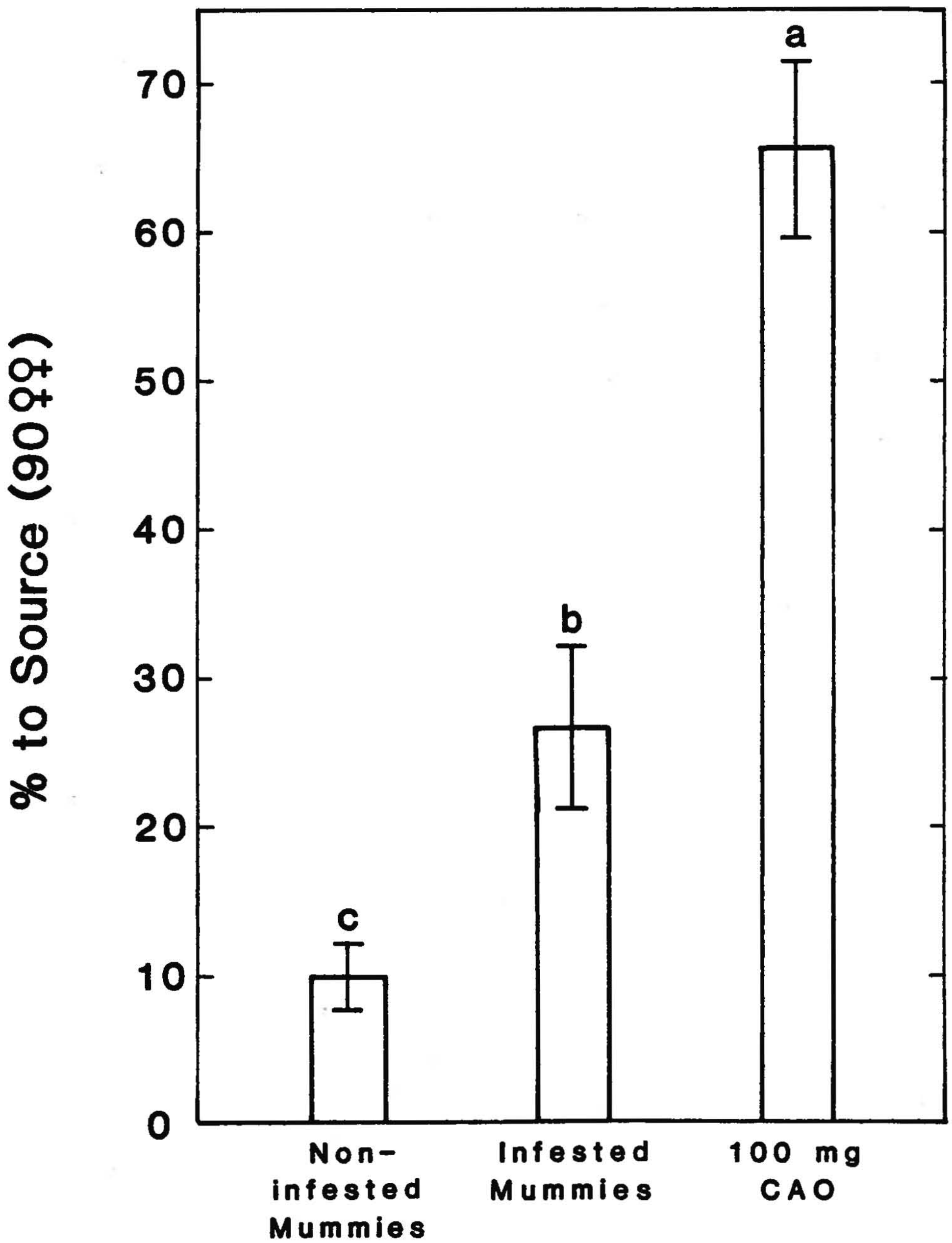


Figure 4. Response of NOW ♀♀ to non-infested and NOW larvae-infested mummy nuts and CAO in the wind tunnel.

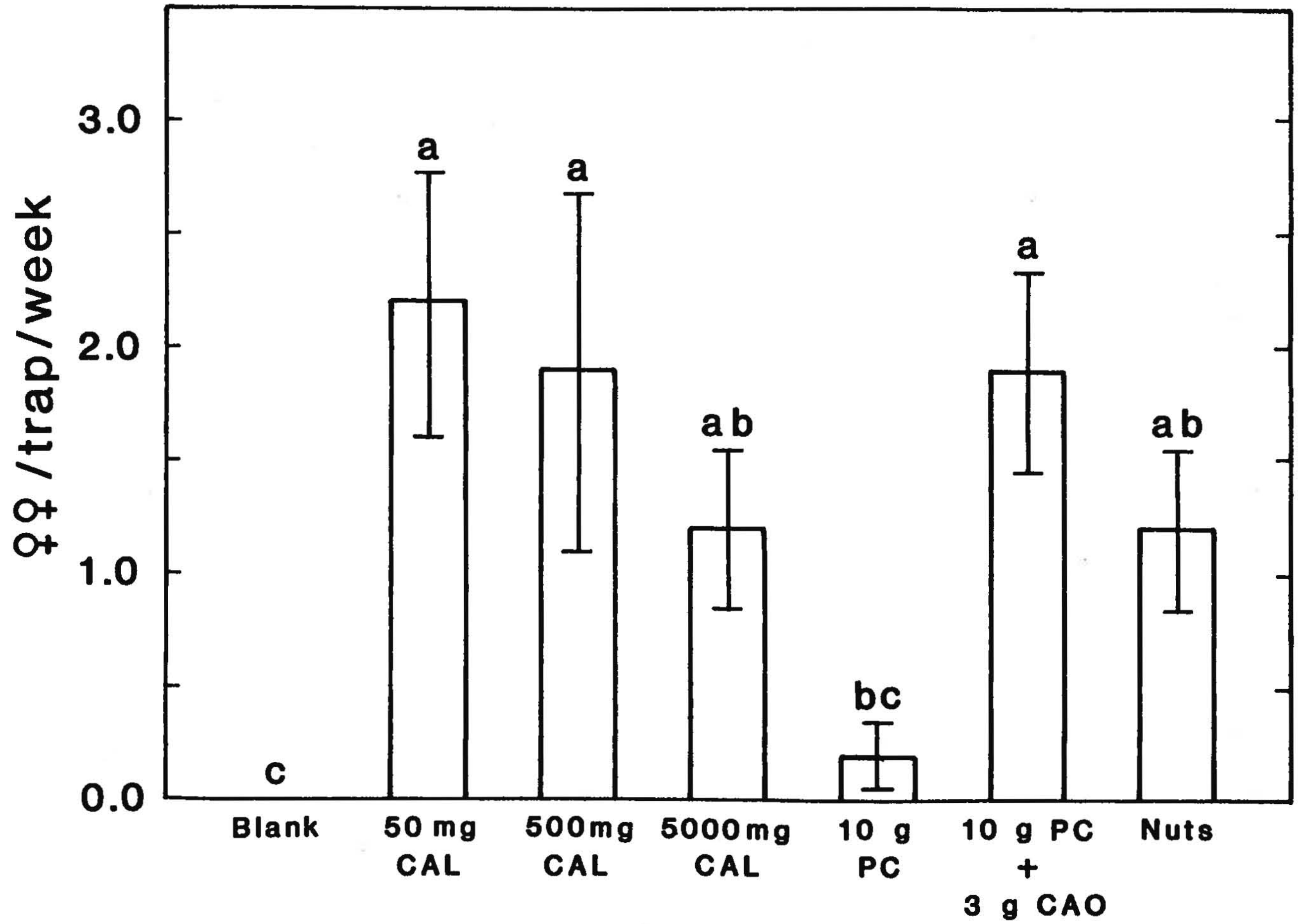


Figure 5. Number of female NOW captured in traps baited with various almond by-products or larvae-infested mummies (n = 9).

arrestment of females flying to traps containing the higher levels of CAO or due to a contamination of vegetation downwind from the trap.

The search for a candidate toxicant for the toxic mummy formulation focused on three pyrethroids: permethrin, cypermethrin, and pydrin. Preliminary studies of topical dosing of NOW adults with these compounds carried out in the laboratory of Dr. Martin Barnes indicated that they possessed similar toxicities that were more than an order of magnitude greater than those of organo-phosphate insecticides presently used to control NOW. Use of the pyrethroids in the field has not been possible due to problems of secondary outbreaks of phytophagous mites, a difficulty that should be circumvented by the toxic mummy formulation. Assessment of the effect of the pyrethroids on attraction to CAO showed that none of the compounds reduced response when present as either 0.1% of 0.5% of the CAO formulation (Figure 6a-c); however, 1% formulations of both permethrin and cypermethrin showed reduced response. Pydrin did not interfere with CAO attraction even at the 1% level. Lack of behavioral disruption by pydrin was confirmed by field trapping (Figure 7).

Finally, the potential for a toxic mummy formulation to attract and kill females was assessed using a non-racemic form of pydrin (M070616), with a toxicity reported to be 3-4X greater than that of pydrin. In the wind tunnel, females were allowed to fly up and contact one of five formulations of M070616 in CAO. Upon leaving the material, females were captured, and mortality was determined after 24 hrs. Significant mortality was brought about by contact with any of the formulations containing toxicant, with almost 90% mortality seen with the 2.5% formulation (Figure 8). It is important to point out that significant sublethal effects are expected in those surviving females, which would render many of them incapable of finding suitable oviposition sites.

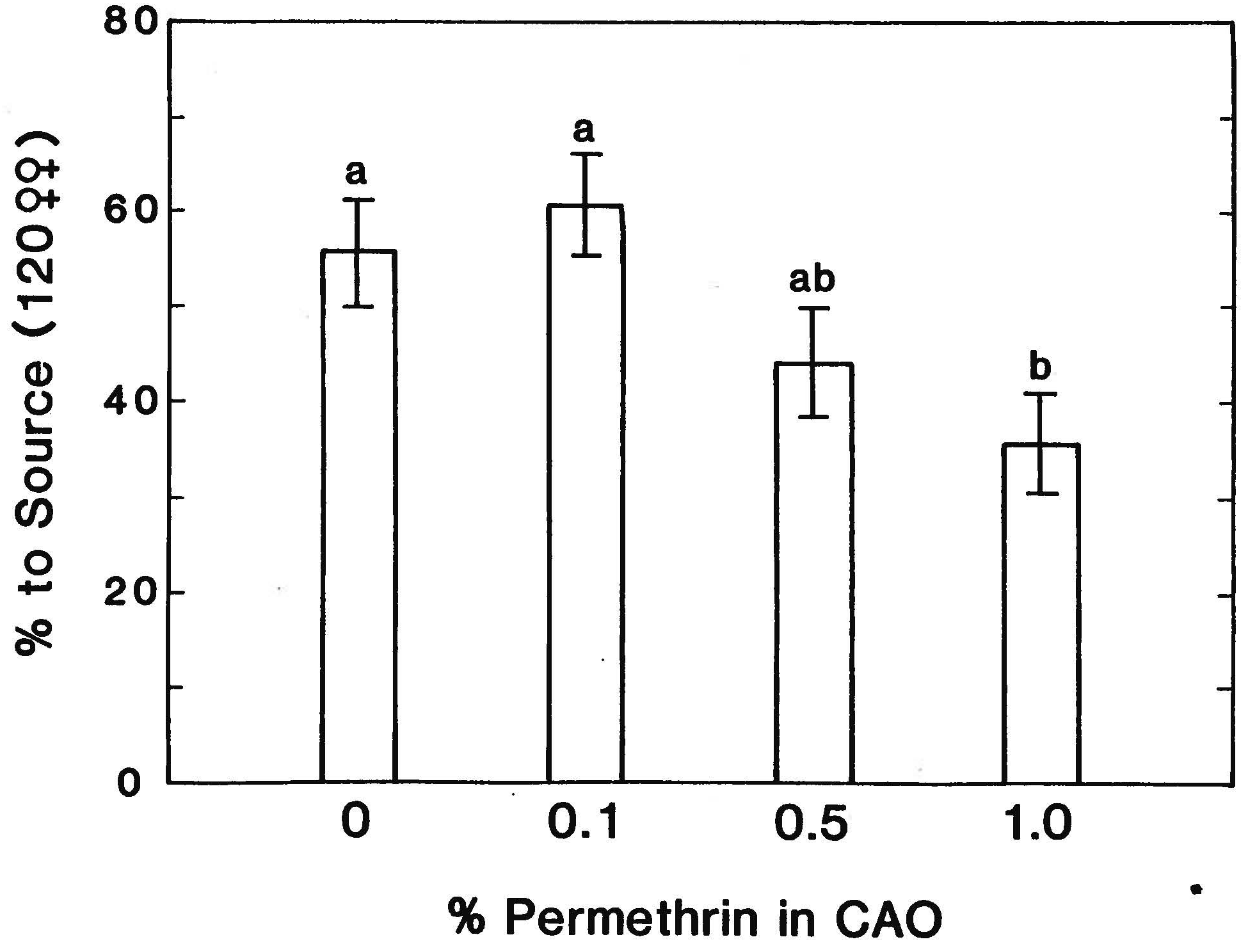


Figure 6a. Response of NOW females to 100 mg CAO containing varying concentrations of permethrin in the wind tunnel.

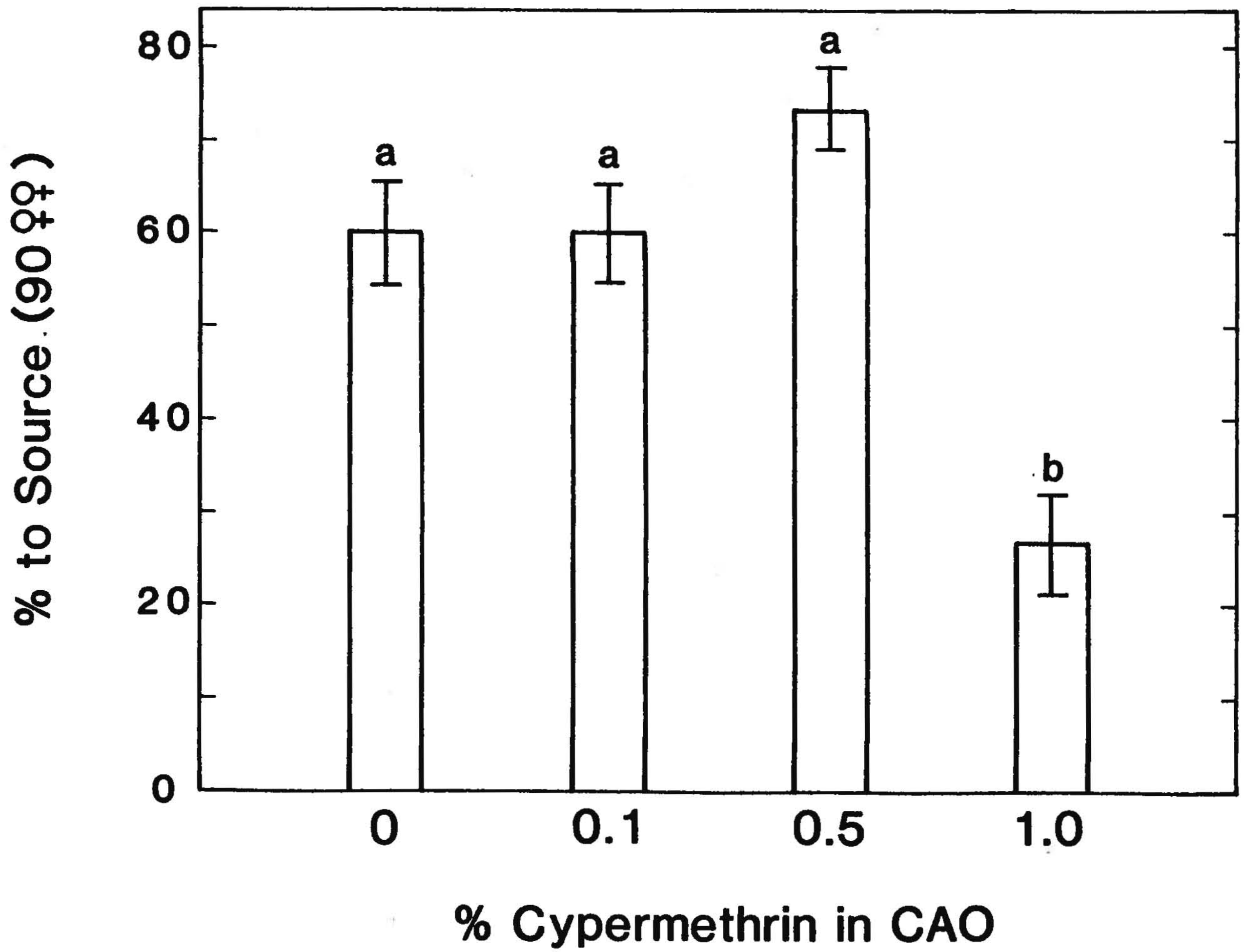


Figure 6b. Response of NOW females to 100 mg CAO containing varying concentrations of cypermethrin in the wind tunnel.

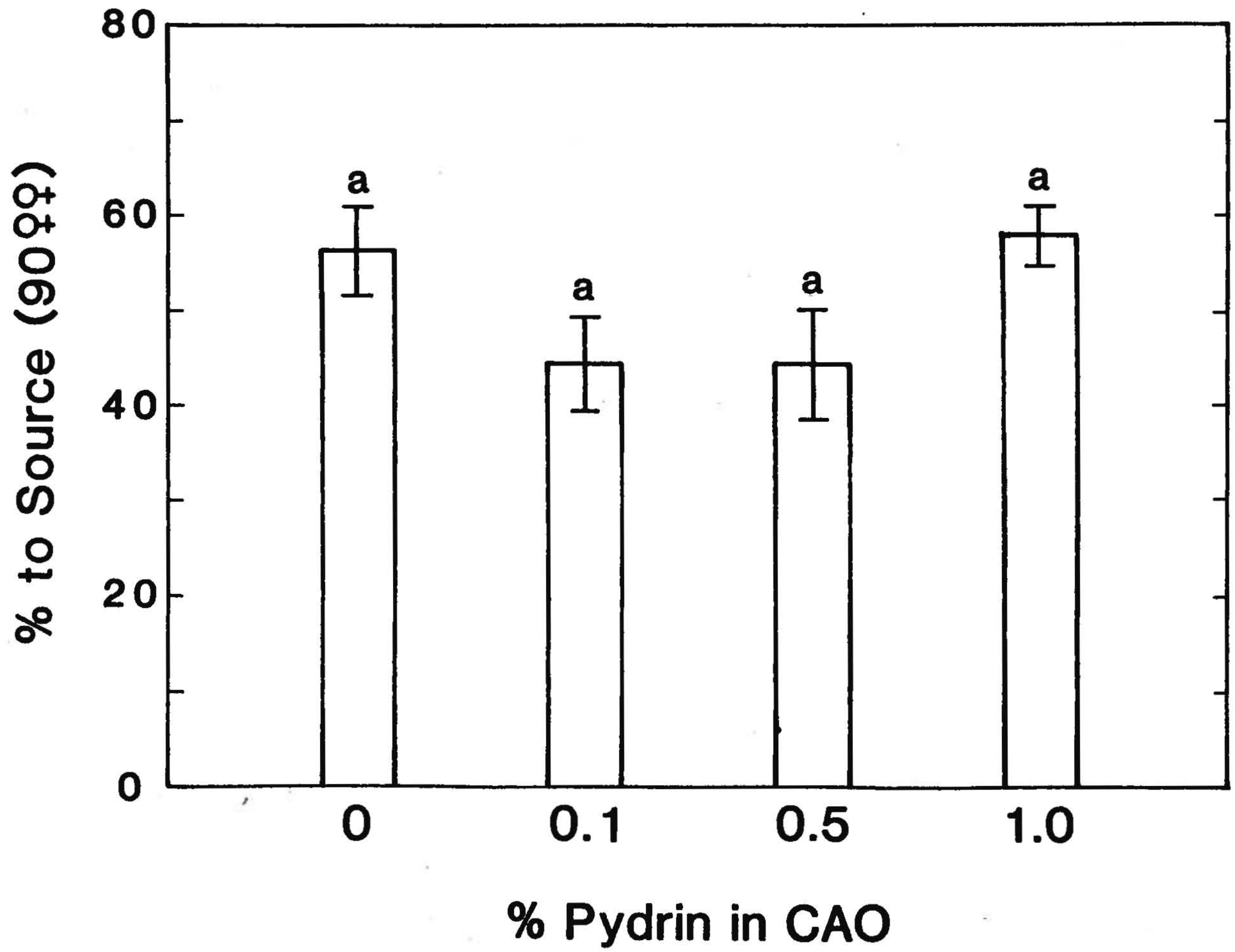


Figure 6c. Response of NOW females to 100 mg CAO containing varying concentrations of pydrin in the wind tunnel.

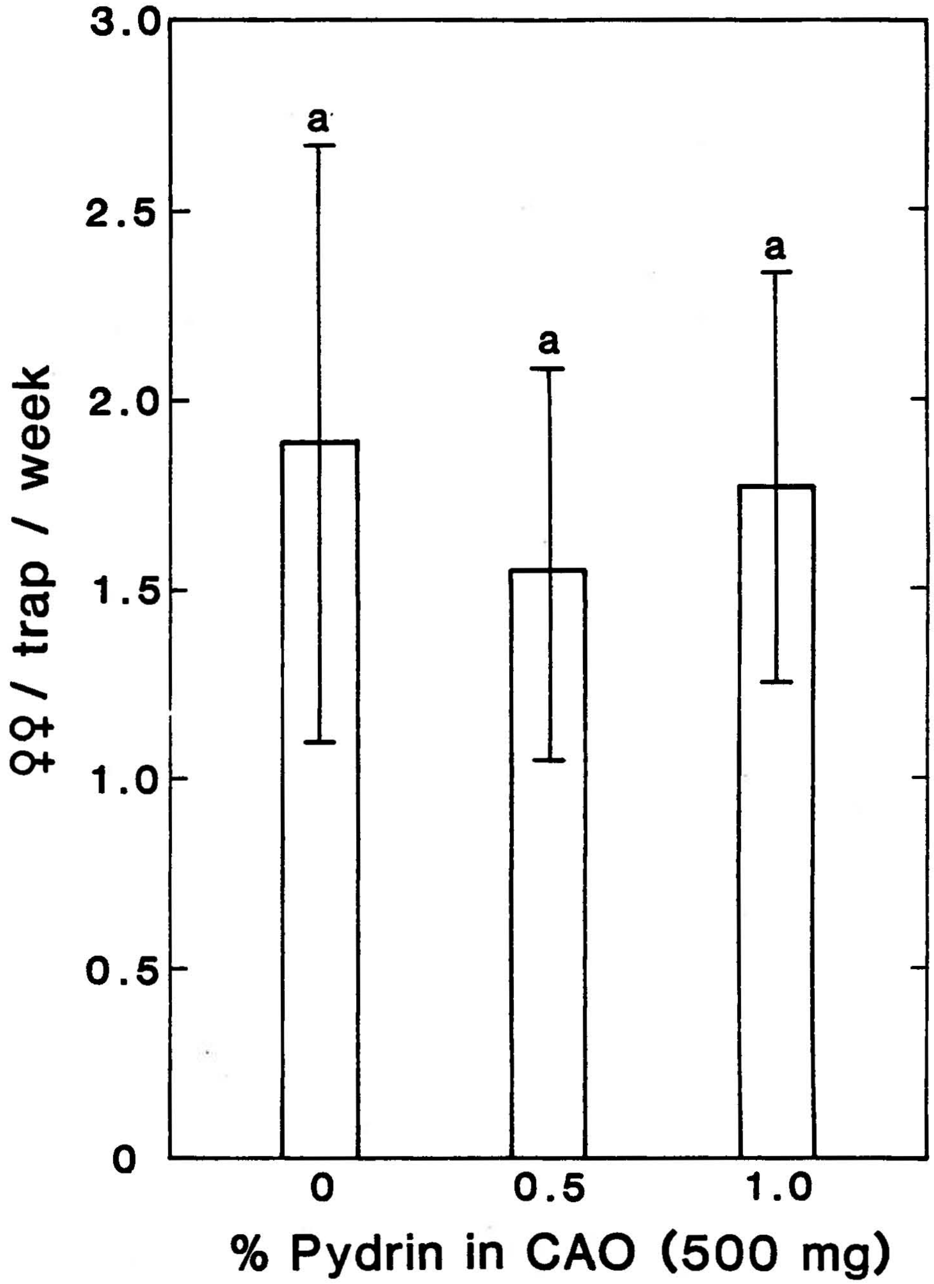


Figure 7. Number of NOW females in traps baited with 500 mg CAO and three concentrations of pydrin (n = 9).

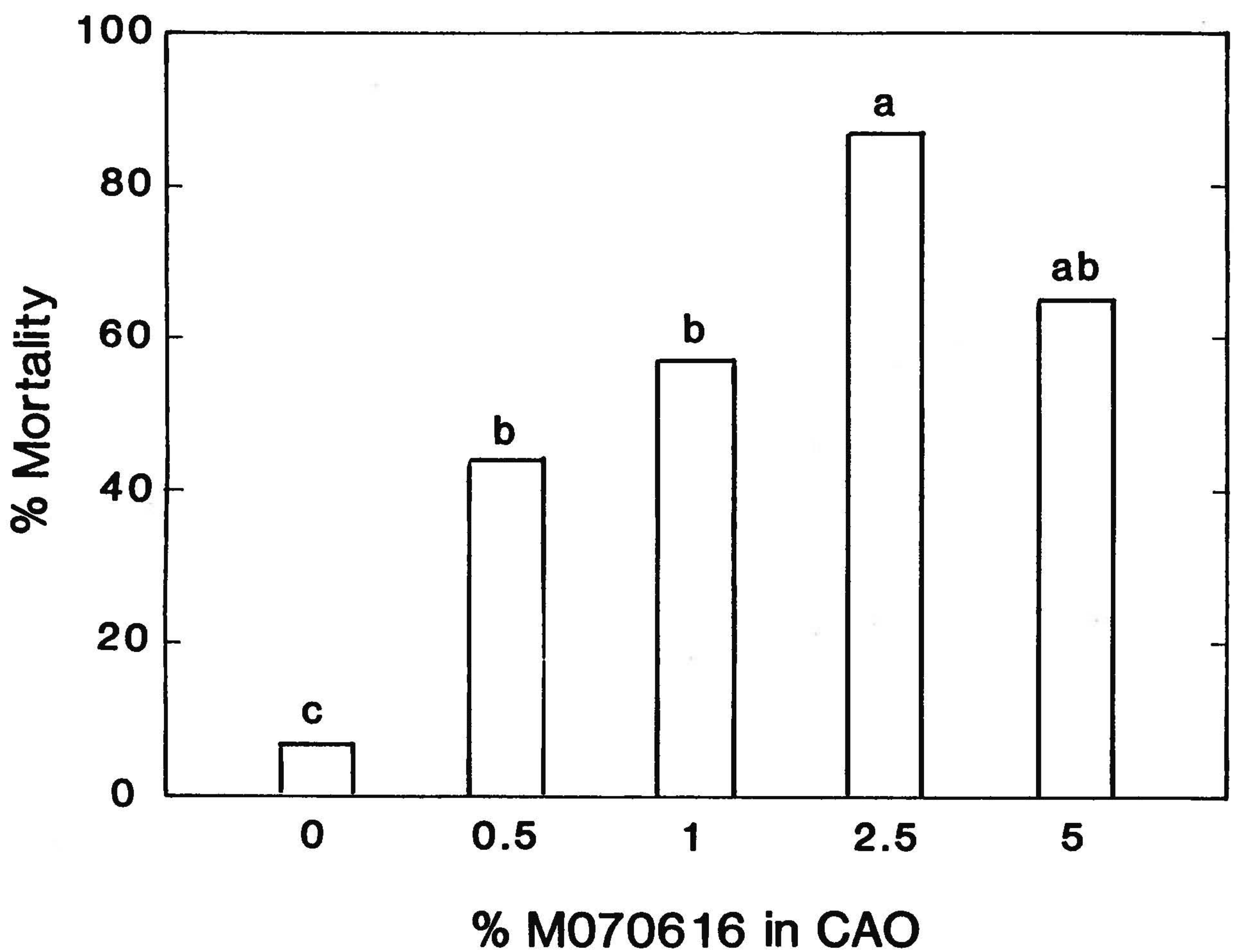


Figure 8. Percent mortality after 24 hrs of NOW females after flying up to and landing on formulations of non-racemic pydrin (M070616) in CAO (n = 40).

The reduced level of mortality with the 5% formulation was probably due to the fact that these females spent considerably less time on the substrate than with formulations containing less toxicant.

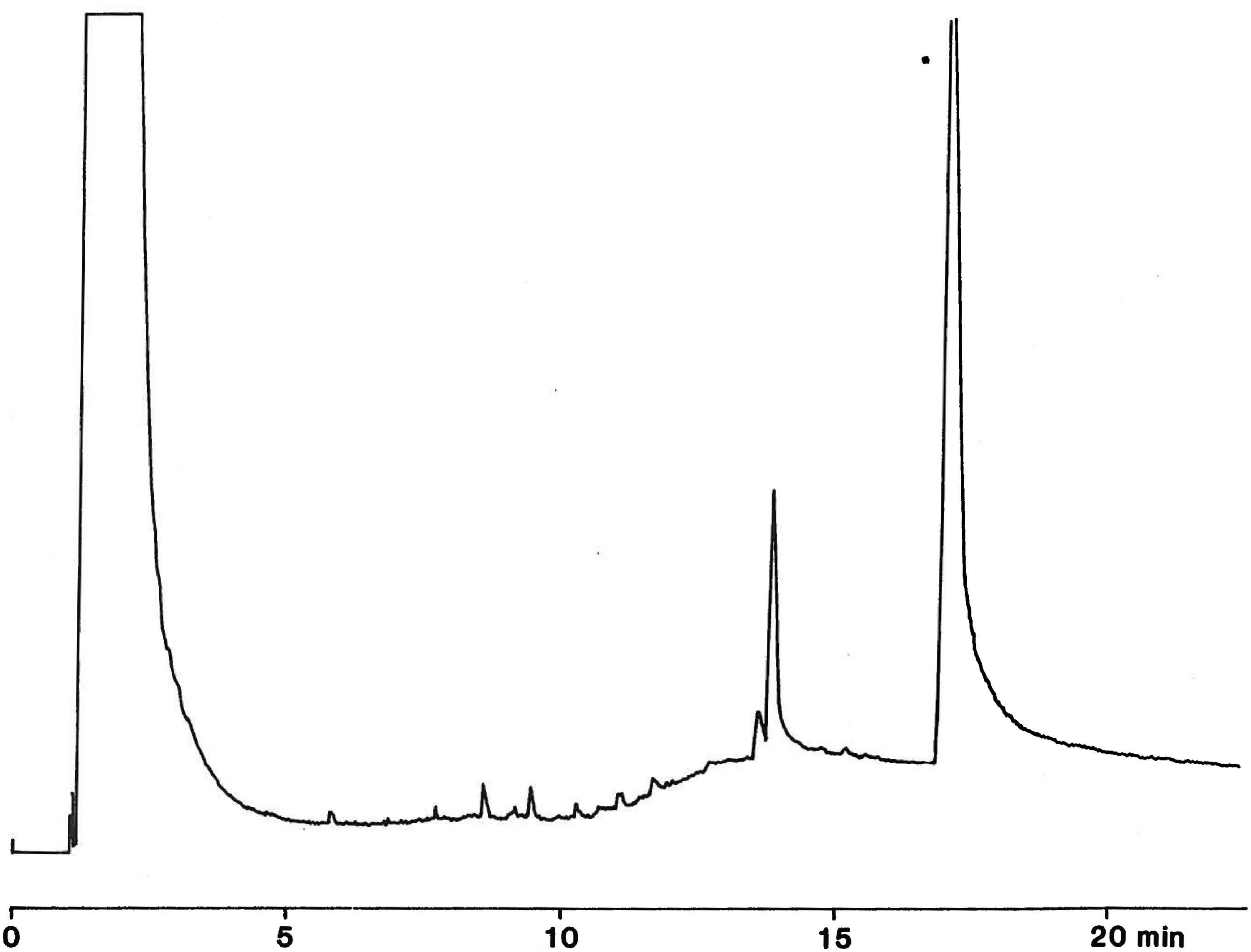
Thus, we have developed an attracticide formulation that is competitive with natural oviposition sites and that produces high levels of mortality in gravid female NOW, with significant levels of sublethal effects in the surviving females expected. Future efforts with focus on developing the characteristics of field durability to this formulation.

ID of behaviorally active constituents

In an effort to chemically characterize the behaviorally active constituents of CAO, 100 mg of CAO was fractionated by high performance liquid chromatography (HPLC) using a normal phase column and a solvent program of 100% methylene chloride to 100% methanol in 10 min. Fractions were bioassayed in the wind tunnel and behavioral activity was isolated to two adjacent 1-ml fractions. After combining the fractions, analysis was made by gas chromatography (GC, Figure 9) and then Fourier-transformed infrared spectroscopy (FT-IR, Figure 10). The FT-IR analysis was particularly informative, indicating the predominance of long-chain fatty acids, as evidenced by the broad band between 2400 and 3600, and by the sharp band at 1711. This assessment was confirmed by treatment of the active fraction with diazomethane and re-analysis on capillary GC (Figure 11). The dramatic sharpening of the peaks (cf. Figure 9) is due to the derivatization of the fatty acid moiety to the methyl ester by the diazomethane; the resulting chromatogram indicates the presence of four primary constituents.

The four-component mixture was next submitted to mass spectrometry (GC/MS) for identification. Fragmentation patterns suggested the following determinations for the peaks in Figure 11: peak A-methyl ester of saturated sixteen-carbon

Figure 9. Capillary gas chromatogram of active HPLC fraction of CAO (column = DB-1).



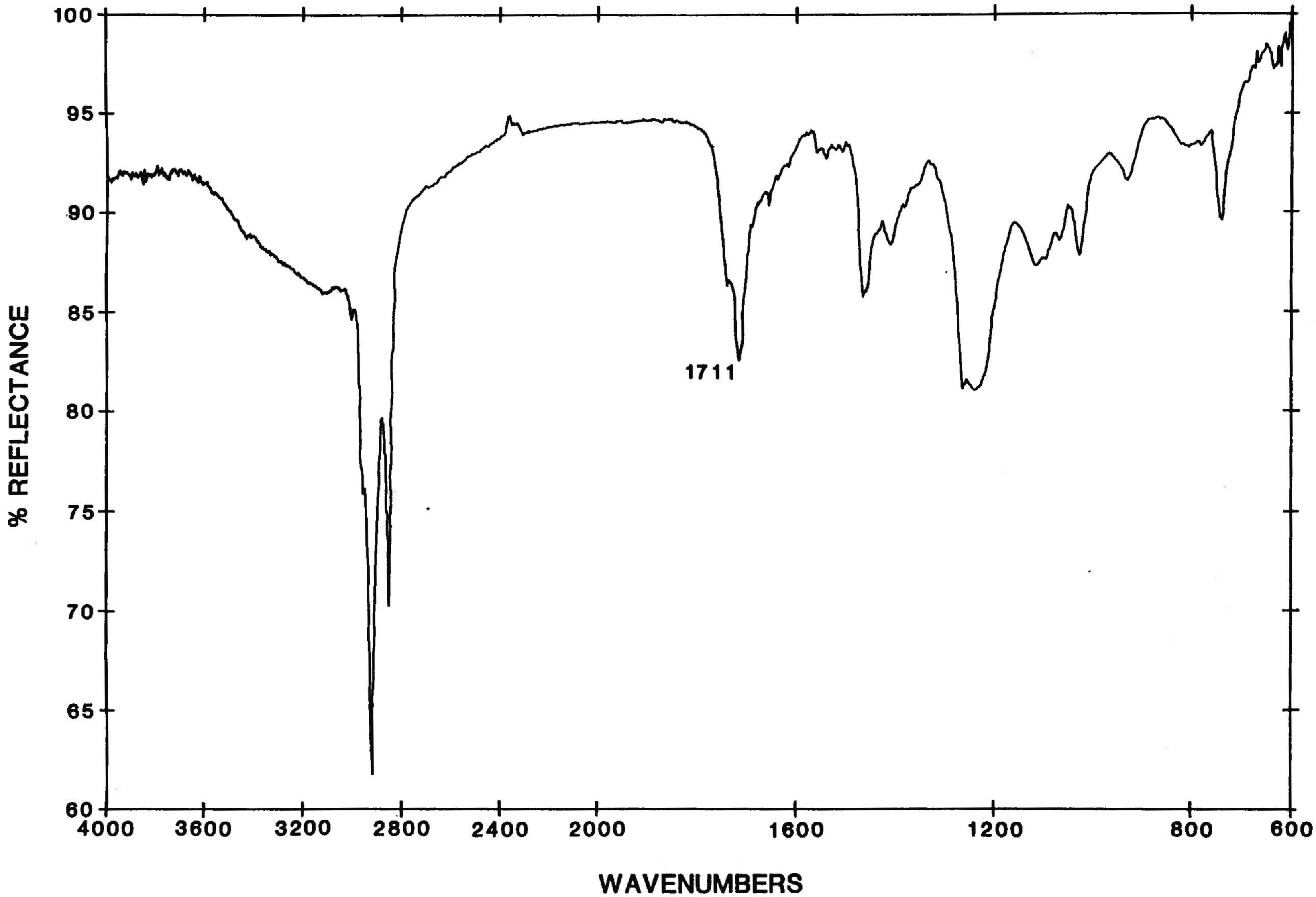
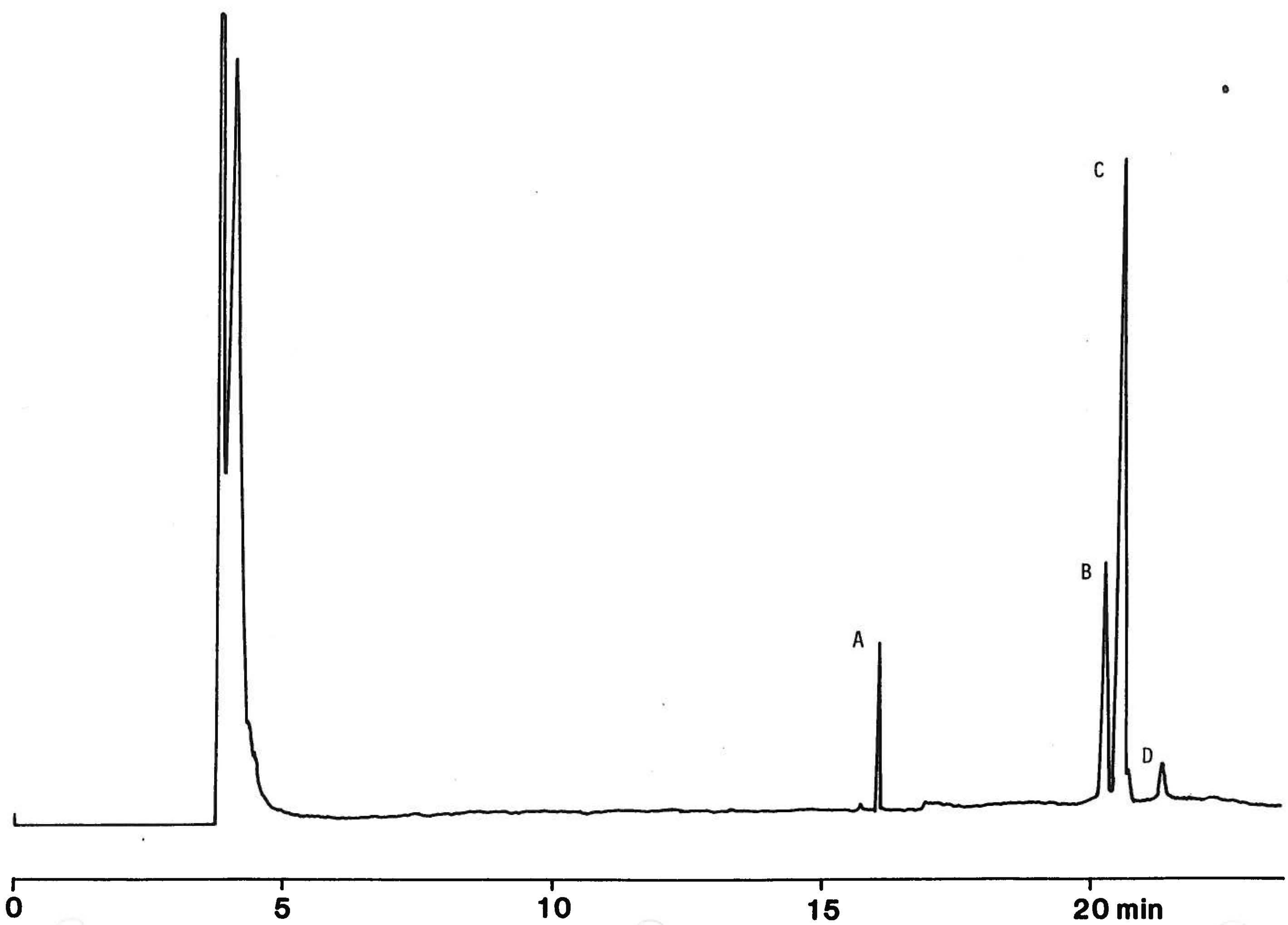


Figure 10. FT-IR analysis of behaviorally active HPLC fraction.

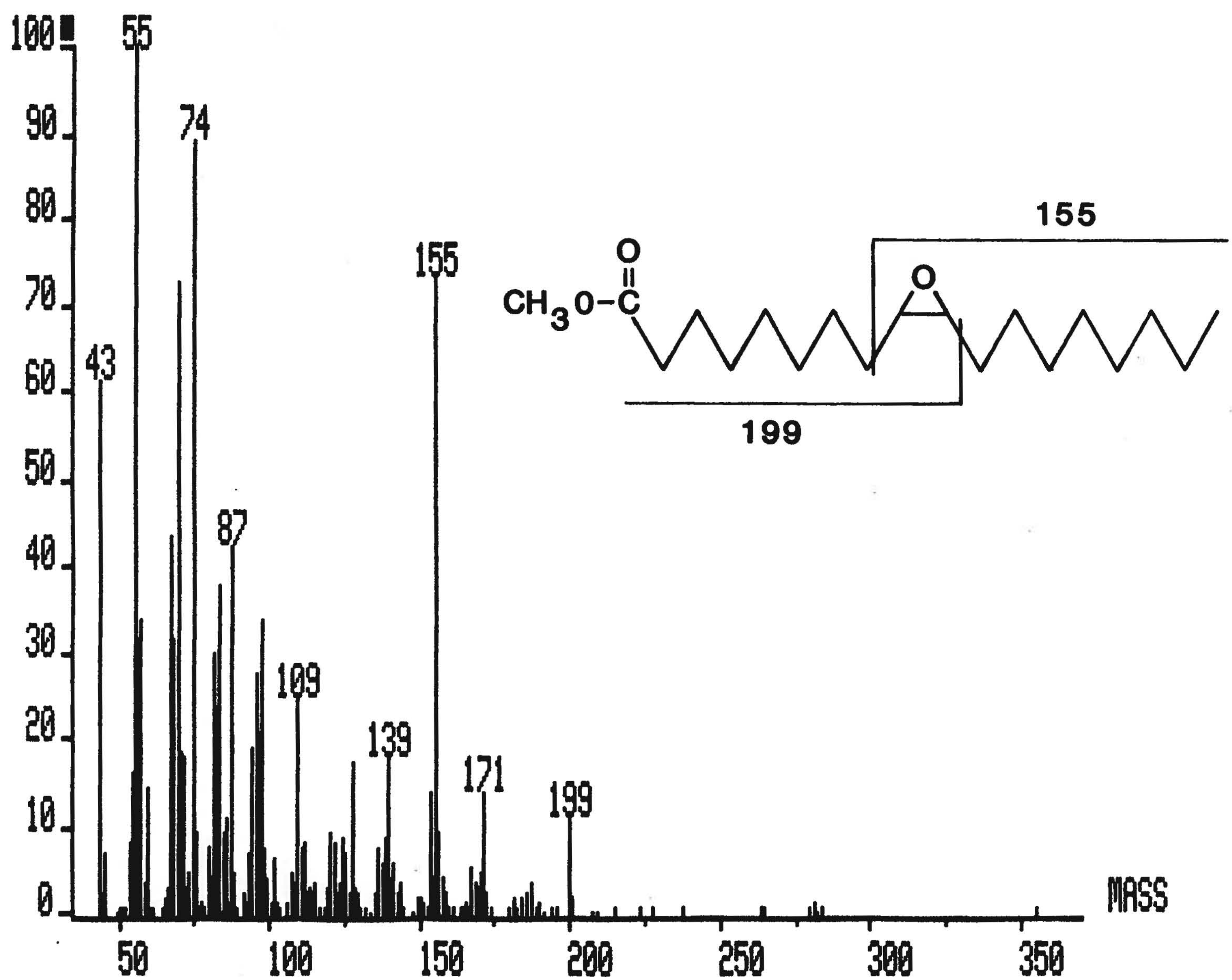
Figure 11. Capillary gas chromatogram of active HPLC fraction after derivatization by diazomethane (column = DB-1).

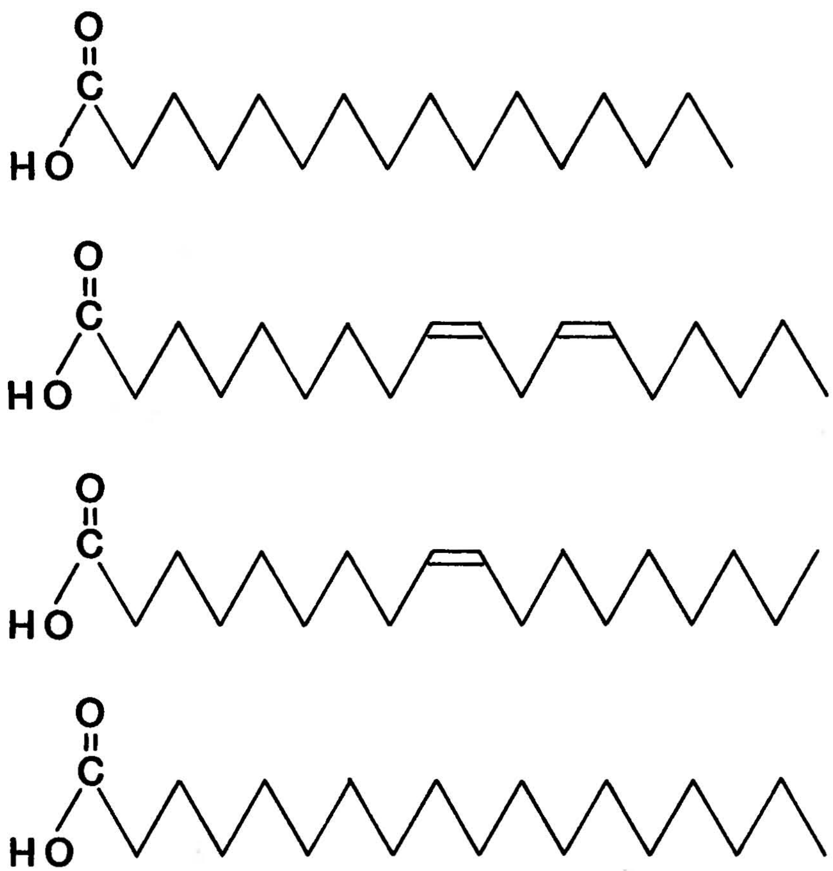


fatty acid (Palmitic acid), peak B-methyl ester of doubly unsaturated eighteen-carbon fatty acid, peak C-methyl ester of mono-unsaturated eighteen-carbon fatty acid, peak D-methyl ester of saturated eighteen-carbon fatty acid (stearic acid). Double-bond positions for compounds B and C were determined by formation of epoxide derivatives, followed by re-submission to GC/MS. Examination of fragmentation patterns indicated sites of unsaturation to be at the 9-10 and 12-13 position for compound B and 9-10 position for compound C (Figure 12). Comparison of capillary GC retention times with synthetic standards indicated all double bonds to be in the cis configuration. Thus, the final characterization of the four-component mixture is given in Figure 13.

A final set of bioassays was carried out to assess the role of fatty acids in the orientation of females to CAO. Females were present one of four treatments in the wind tunnel: 1) blank, 2) 100 mg CAO, 3) 100 mg CAO treated with diazomethane to destroy free fatty acids, or 4) 1 mg synthetic fatty acid four-component mixture (free fatty acids represent approximately 3% of CAO weight. Looking at three levels of female upwind-flight response (Figure 14), when CAO is treated with diazomethane to remove fatty acids (diazo-CAO), attraction to within 10 cm of the odor source is reduced to a level not significantly greater than the blank. In contrast, the synthetic fatty acid mixture was found to be equally as effective as CAO at this level of attraction; however, response at subsequent steps (touching source and landing on source) were significantly lower for the synthetic fatty acids compared to the natural CAO. This inability to elicit short-range behaviors may be due to either qualitative or quantitative problems, i.e. either a necessary (and as yet unidentified) constituent is missing from the synthetic mixture or the release rates used

Figure 12. Mass spectrum of epoxide derivative of oleic acid methyl ester.





	RATIO
Palmitic	11
Linoleic	28
Oleic	100
Stearic	2

Figure 13. Chemical determination and relative ratio of four-component mixture of Figure 11.

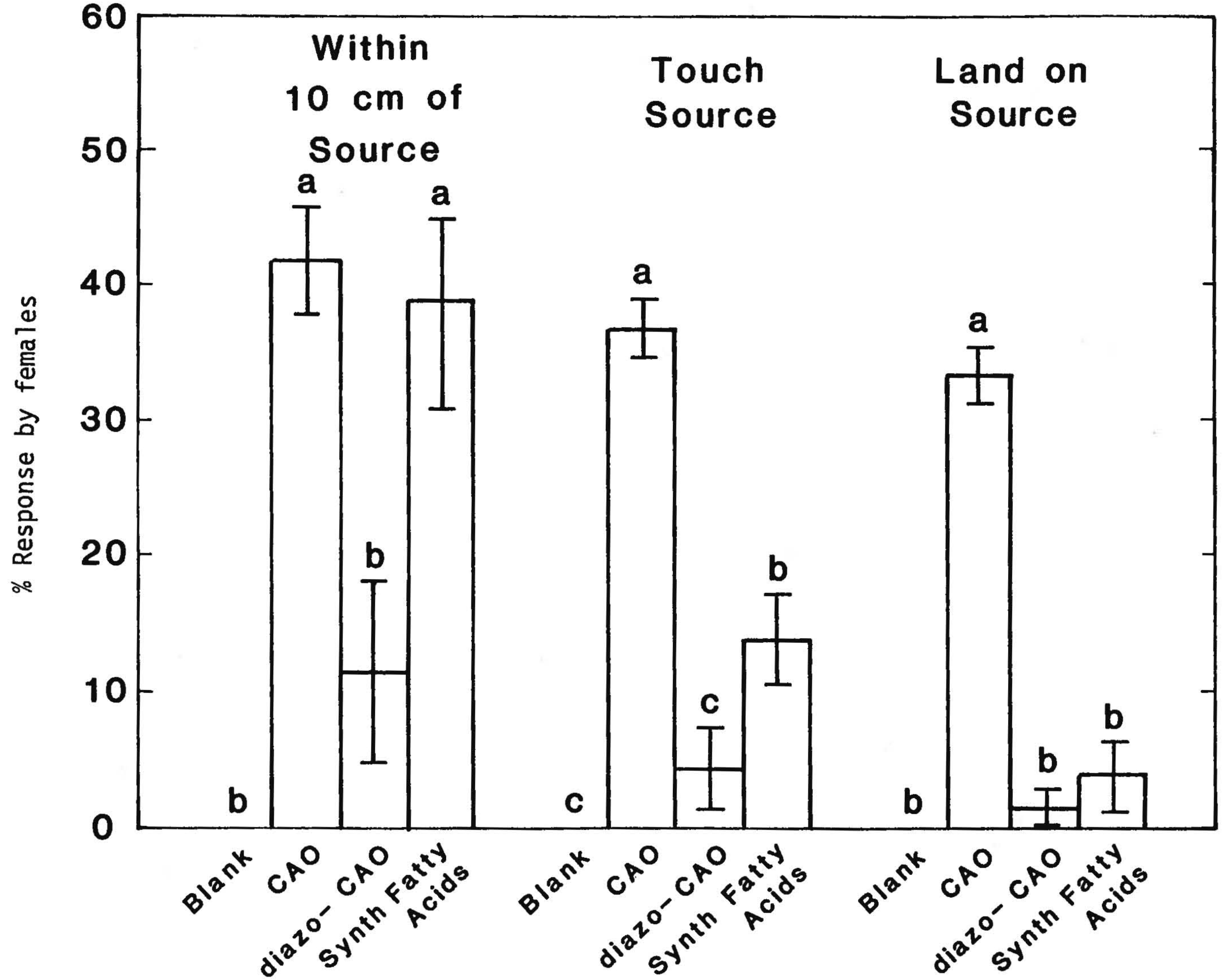


Figure 14. Role of fatty acids in attraction to CAO analyzed at three points in the behavioral sequence (60 females).

for the synthetic mixture are too high, causing premature in-flight arrestment. Nevertheless, these results do indicate that one or more of the four fatty acids is necessary for attraction to CAO and is responsible for at least the long-range components of the orientation sequence.

In conclusion, during the past year, we have: 1) developed a laboratory prototype for the toxic mummy strategy, elicidating the necessary parameters for such a bait, and 2) chemically characterized the primary constituents of CAO eliciting long-range attraction in NOW females. Additional work is necessary (and planned) to allow use of the toxic mummy in the field and to complete the chemical determination of host-plant attraction in NOW females.