1977

Investigation into the Sex Pheromones of Two Species of Flies: Sarcophaga Bullata and Musca Domestica

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SARCOPHAGA BULLATA AND MUSCA DOMESTICA

Fenwick Scholar Thesis
F. Joseph Germino
May, 1977

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Acknowledgements

I would like to thank the Special Studies Committee, Dr. Girard, Dr. McGrath, Dr. Hendry, Dr. Noonan, Joe MacDonald and John Hazlewood for the assistance, support and guidance which they have provided.

Introduction

Prior to 1943, when DDT (DichloroDiphenylTrichloroethane) was first employed against a typhus epidemic in Naples, Italy, man had no really effective way to prevent insects from spreading diseases and devouring crops, although it can not be said that he did not try. During the Middle Ages, severe outbreaks of disease and insect infestations brought the local people streaming into the churches and ecclesiastical courts for heavenly relief. In 1120, the Bishop of Leon excommunicated the caterpillars that were devouring local crops in the hope that they would flee; 368 years later, the high vicar of Autun took a similar step when the local weevil population mushroomed and threatened to wipe out the harvest. Like Martin Luther, the insects ignored the edicts and continued eating.

In an attempt to find more reliable controls, Renaissance man turned to chemicals. Many of these early "insecticides" were probably no more effective than the edicts of excommunication; some, however, showed considerable insight. Seventeenth century French farmers fought lace bugs on their pear trees with infusions of tobacco extract; it is now known that nicotine, found in tobacco leaves, repels many different species of insects.

Despite his ingenuity and toil, man rarely could regulate the populations of his insect pests. Sixty years ago, U.S. entomologist S.A. Forbes wrote:

> The struggle between man and insects began long before the dawn of civilization, has continued without cessation to the present time, and will continue, no doubt, as long as the human race endures. We commonly think of ourselves as the lords and conquerors of nature. But insects had

> thoroughly mastered the world and taken full possession of it before man began the attempt. They had, consequently, all the advantage of possession of the field when the contest began, and they have disputed every step of our invasion of their original domain so persistently and successfully that we can scarcely flatter ourselves that we have gained any very important advantage over them. If they want our crops, they still help themselves to them. If they wish the blood of our domestic animals, they pump it out of the veins of our cattle and our horses at their leisure and under our very eyes. If they choose to take up their abode with us, we cannot wholly keep them out of the houses we live in. We cannot even protect our very persons from their annoying and pestiferous attacks, and since the world began, we have never yet exterminated - we shall probably never exterminate - so much as a single insect species.[1]

Then along came DDT and everything seemed to change. DDT worked incredibly well - it killed a wide variety of insects. A host of other similar, chlorine-containing chemicals was synthesized and tested. Many worked nearly as well as DDT. Almost overnight, man had a whole arsenal of cheap, effective, and reasonably safe (or so scientists then thought) chemicals for his war with the insect world. Many believed that victory was at hand.

No war is ever won that easily. Ominous signs appeared in the early 1950's when researchers discovered traces of DDT - it breaks down very, very slowly in nature - in the tissues of fish, wildlife, and humans. It was later shown that the compound interferes with the shell production of several species of birds. Alarmed scientists tested DDT on laboratory

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[1] "The Bugs Are Coming," Time, July 12, 1976, p.38

animals and found that it could cause cancer. Bad news from the front continued to pour in: farmers noticed that they had to apply ever-increasing amounts of insecticides for the same amount of protection. A study by the U.S. Department of Agriculture revealed that of the 500 species of insects that do significant damage to crops, 267 had built up a resistance to pesticides.[2]

Today, the words of Dr. Forbes appear as true as they did 60 years ago. The use of DDT and many of the other effective insecticides has been banned in this country. The South American fire ant, first arriving in the U.S. as an illegal immigrant in 1918, has spread its domain into 150 million acres in nine Southern states where it causes considerable damage to livestock. Authorities expect it to continue its northward advance. Four species of insects destroyed enough timber in the U.S. in 1975 to build 910,000 houses. The U.S. Department of Agriculture estimates that farmers in the U.S. lose 10% of their crop to insects, despite the heavy use of insecticides. Farmers in poor countries, unable to afford the high cost of pesticides, suffer even more. Kenyan officials estimate that their farmers lose 75% of their crops to insects. The Black Fly, a carrier of river blindness, leaves 700,000 Africans in the Volta River basin sightless each year. The well known Tse-Tse Fly, bearer of sleeping sickness, still freely roams about the African continent. The "killer bee", a hybrid of a European honeybee and an aggressive African fly, and capable of killing both humans and livestock, is reported to be moving northward from Brazil at the rate of 200 miles/year. No one and

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[2] Ibid., p.45

nothing has yet been able to halt the northward advance of this dangerous insect.

These discouraging finds have initiated a mad scramble to find other, safer methods of warfare. Plant geneticists are feverishly working on the development of pest-resistant plants. Biologists and entomologists are searching for predators and parasites of the more common pests. An occassionally efficacious form of birth control entails the release of many millions of sterile male insects. The sterile males can mate with the females but viable offspring are not produced. If there are many more sterile males than fertile males, relatively few fertile males will find an unmated female and the population will decrease drastically (at least theoretically). Pheromonal control also has received considerable attention in the past decade because of its inherent desirable characteristics. Because the chemicals utilized in pheromonal control are identical to the insects' pheromones, it is very unlikely that insects could develop a resistance to them. Conventional insecticides are not specific in their action - they usually indiscriminately kill both harmful and beneficial insects. Pheromones, on the other hand, are very specific in their action: you pick your pheromone for your bug. Finally, the relatively small amounts needed, coupled with the fact that pheromones are natural products and therefore biodegrade into harmless substances, make pheromones environmentally "safe".

A pheromone has been defined as a chemical or mixture of chemicals that is released to the exterior by an organism and that causes one or more specific reactions in a receiving organism of the same species.[3]

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[3]Shorey, H.H., Animal Communication by Pheromones, New York: Academic Press, 1976, p. 3.

If it triggers an almost immediate behavioral, although not necessarily overt, response, it is called a "Releaser" pheromone. The bulk of current research is on releaser pheromones. If, on the other hand, it causes a relatively enduring re-organization of the physiology of the receiving animal by stimulating changes in its developmental or reproductive processes, it is known as a "Primar" pheromone. The "Queen-substance", used by queen ants to produce sterile female workers, is a well-known example of a primar pheromone. Little is known about this class of pheromones.

Researchers have found many different and interesting types of releaser pheromones. Many insect species release aggregation pheromones, which attract both the males and females of a given species, dispersion pheromones, which cause members of the same species to repel each other (useful for maintaining a certain population density), and sex pheromones, which are released by either the male or the female of a species and which attract members of the opposite sex for mating. The more complex insect societies - such as those of the ants and termites - depend upon chemical communication for their survival. If an ant colony is invaded by foreign ants, worker ants release an "alarm" pheromone which alerts the other worker ants in the colony to the danger and causes them to begin defending their colony. Ants are also known to mark trails to food sources with several volatile chemicals; these have been named trail pheromones. There may even be "death" pheromones. Wilson et al[4] found that extracts of dead Pogonomyrmex badius workers, when applied to

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[4] Wilson, E.O., Durlach,N., Roth,L.M., "Chemical Releasers of Necrophoric Behavior in Ants," Psyche (1959), v. 65, p. 108.

filter paper squares, were treated like dead workers and were transported to refuse piles. Several fatty acids - Myristoleic, Palmitoleic, Oleic, and Linoleic Acids - appear to elicit this behavior.

If man could learn the pheromonal language of the insect world, he could disrupt their communication systems and subdue his age-old enemies. A knowledge of the chemicals used in sexual communication would be especially promising since man could then "jam" the signal and prevent mating.

The first recorded experiment involving pheromones was that of Jean Fabre in the nineteenth century.[5] While walking in the forest near his home, he found a cocoon of a rare species of moths and brought it home with him. A female moth eventually emerged and he placed it in a cloth cage near his window. It was not long before he discovered over 60 male moths of that species hovering about the cage. He then transferred his female moth to a tightly closed, transparent glass jar. He reasoned that if the males stayed, the appearance of the female moth attracted the males. If they left, visual stimuli were not responsible for the males' behavior. Fabre was surprised when all the males left. A curious thing happened when the glass jar was emptied of its occupant and allowed to stand open next to the window: the male moths returned. Although this would seem to imply the existence of a chemical attractant, Jean could understand how an imperceptibly small amount of chemicals could attract male moths from so far away. He suggested that the males were attracted

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[5]Beroza,M., Jacobson, M., "Insect Attractants," Scientific American (1964), v. 211, p. 20.

by noises made by the female moth. He "proved" his hypothesis by demonstrating that male moths devoid of both antennae seldom found the female moth. Little did Fabre know that the antennae serve as receptor sites for chemical odors.

Even today very little is known about the mechanisms responsible for odor reception in insects, unfortunately for pheromone researchers. One study, performed on the antennae of the Blowfly Calliphora vicina, found that the odors of decaying meat and of the volatile compounds in several flowers were discerned by different types of receptor cells.[6] Those receptors activated by the odor of decaying meat were named, rather appropriately, meat-odor receptors while the others received the name flower-odor receptors. Six different types of meat odor receptors were isolated and apparently enable the fly to detect the degree to which the meat had decayed. It is interesting to note that one of the meat odor receptors reacted well to compounds with a chain length between five and seven (the maximum response was obtained with six carbon atoms) carbon atoms long and either an alcohol, aldehyde or keto group.

In another series of experiments performed at the Max Planck Institute by Dr. Kafka,[7] receptor cells in several insect species were checked for their reaction spectra, i.e. compounds which could trigger the receptor cells, and about thirty different types were located. In order to study the physicochemical principles involved in odor reception, Dr. Kafka made two simplifying assumptions: the specificity of a cell type

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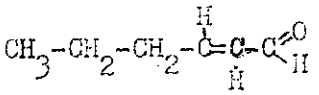
[6]Kaib, M., "Die Fleisch- und Blumenduftrezeptoren auf der Antenne der Schmeißfliege Calliphora vicina," J. Comp. Physiol. (1974) v. 95, p. 105-121.

[7]Kafka, W., "Physicochemical Aspects of Odor Perception in Insects," Annals N.Y. Acad. Sci. (1974) v. 237, p. 115-128.

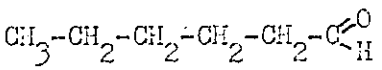
is dependent upon only one type of acceptor site, and the specificty of a cell type is a function of the molecular properties which determine the interactions between the acceptor site and the molecule.

Functional groups (e.g. aldehydes, ketones, amines) or combinations of such groups proved to be an essential element of the specificity: one receptor cell of the insect Locusta migratoria (migratory locust) was very receptive to trans-2-hexenal



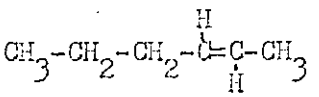
trans-2-hexenal

but unreceptive to hexanal



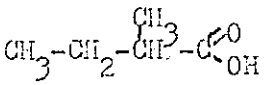
hexanal

and trans-2-hexene.

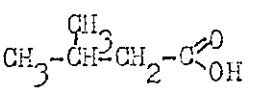


trans-2-hexene

On the other hand, 2-methyl and 3-methyl butanoic acid fired the



2-methyl butanoic acid



3-methyl butanoic acid

receptor cell equally well, implying that side branches are not always critical in odor reception. Other, similar experiments led Dr. Kafka to view the acceptor sites of the receptor cells as "grooves" into which the molecules must fit, either in their most stable state or after an intramolecular rotation, in an acceptable conformation.[8]

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[8] Ibid., p. 119

Since the enantiomeric forms of 4-methyl hexanoic acid (their steric differences are among the smallest known for organic molecules) elicited slightly different cell responses, these "grooves" must be aligned very precisely.

Tests with homologous compounds seemed to rule out bonding between an acceptor site and the entire odor molecule; rather, it appears to be cooperative multiple point interactions. The attachment of the odor molecule (even with alkanes) seems to depend upon two (or more) spatially well-placed binding positions in the acceptor site. If the odor molecule can "fit" into these positions, the receptor cell is stimulated. It must be pointed out that the essential functional groups must be located on one and the same molecule if the receptor cell is to be stimulated: a mixture of trans-2-hexene and hexanal was ineffectual when compared to trans-2-hexenal although the mixture contains the same functionalities (a double bond in the second position and an aldehyde group).

Although this model does not provide a description of odorant discrimination (this undoubtedly depends upon the patterned responses or more than one cell type and on the integrating processes in the central nervous system), it does provide a partial explanation for the high specificity insects exhibit for their own sex pheromones. It has been demonstrated, for example, that one species of boll weevils is attracted to only one (the +) of the two enantiomeric forms of cis-2-isopropenyl-1-methyl cyclobutanethanol.[9] Whether or not the male boll

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[9]Beroza, M., Pest Management with Insect Sex Attractants, Washington, D.C.: American Chemical Society, 1976.

weevils can "smell" the levorotatory form of this compound is not known; however, they certainly are not attracted to it.

The receptor cells are also extremely sensitive, as the early experiments of Jean Fabre attest. In fact, only with the advent of sensitive electronic equipment and chemical tests over the past twenty years has pheromone research been possible. In one fairly typical study in 1973,[10] one of the sex pheromones of the cotton leafworm Spodoptera littoralis was identified with only two micrograms of the substance (cis-9-tans-12-tetradecadien-1-ol acetate), which was obtained from 3000 female leafworms. At that rate, approximately 52 billion females would provide researchers with only one ounce of the compound. Furthermore, these incredibly minute quantities appear capable of attracting the opposite sex from many hundreds of yards (some say several miles) away. Scientists are still not certain how they do it, although it is very unlikely that they are following a chemical gradient.

Scientists are still uncertain about many other aspects of pheromonal communication as well. Are these pheromones synthesized from scratch in the insects' bodies, and if so, how? How are they released? How are they perceived? Do all species rely upon pheromones, or do some employ other means of communication? What are the pheromones of the various insect species and are they related in any way? And, remembering our initial objective, will pheromonal control ever be economically feasible? What are some of the problems associated with pheromonal

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[10] Tamaki, Y., Yushima, T., "Sex Pheromone of the Cotton Leafworm Spodoptera littoralis," J. Insect Physiol., (1974), v. 20, p. 1005-1014.

control of insect pests? Do any higher animals, including man, use chemical communication?

I decided to spend my senior year searching for the sex pheromones of two different species of flies - the housefly (Musca domestica) and the flesh fly (Sarcophaga bullata) - and hopefully provide some answers to the many questions.

Discussion on Previous Work Done with Musca domestica

Actually, the female sex pheromone of the housefly Musca domestica has been reported to be cis-9-tricosene:[11]

$$CH_3(CH_2)_7\overset{H}{C}=\overset{H}{C}(CH_2)_{12}CH_3$$

The published results, however, were not overly convincing. Sex pheromones usually attract at least 65% of the test animals; cis-9-tricosene attracted less than 27% of the male flies tested, or a little better than one in four flies. I could find only one other "sex" pheromone which attracted such a low percentage of the test insects and that was from another fly - Muscidae stomoxyscalcitrans (stable fly).[12] Only 25% of the male stable flies tested were attracted to a solution containing the "sex" pheromone. Not surprisingly, the reported pheromone is a mixture of compounds very similar to tricosene: unsaturated hydrocarbons with 31 and 33 carbon atoms.[13] The similarity of these results (it should be mentioned that a couple of the workers were involved with both projects) might mean that some species of flies really do not utilize chemical communication very extensively for mating ("low-order" sex pheromones). On the other hand, the habitat of both species is often similar: manure and decaying matter. Perhaps long chain alkenes are present in such substances and the flies reacted to them on that basis. The poor responses could be attributed to the fact that these alkenes

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[11] Carlson, D., Mayer, M., Sihacek, D., James, J., Beroza, M., Bierl, B., "The Sex Attractant of the Housefly: Isolation, Identification, and Synthesis," Science, v. 174, pp.76-78.

[12] Muhammed, S., Butler, J., Carlson, D., "The Stable Fly Sex Attractant and Mating Pheromones Found in Female Body Hydrocarbons," J. Chem. Ecol. (1975), v. 1, pp. 387-398.

[13] Uebel,E., Sonnet, P., Bierl, B., Miller, R., "The Sex Pheromones of the Stable Fly: Isolation and Preliminary Identification of Compounds that Induce Mating Strike Behavior," J. CHem. Ecol. (1975), v. 1, pp. 377-385.

are not responsible for the characteristic odor of manure and decaying food but are often associated with these odoriferous substances. Some of the flies were "fooled" into "thinking" that a manure heap or food source was nearby when they "smelled" these unsaturated hydrocarbons. Cis-9-tricosene would then be a weak food attractant and not a sex pheromone.

This hypothesis would be relatively easy to disprove: food attractants should work for both sexes while sex pheromones should work for only one sex. If male and female houseflies responded equally well to cis-9-tricosene, we could conclude that it was not a sex pheromone and try to explain the attraction in some other way, e.g. food attractant, aggregation pheromone, etc.

Field studies with cis-9-tricosene suggest that it is not a sex pheromone. A female sex pheromone should attract predominantly males when tested in the field. Females should not be found near the trapped males unless there was also a male sex pheromone which would attract the females. In that case, the female population would initially be much lower than the male population in the traps but gradually increase as the male pheromone filtered through the air and attracted the females. When the U.S. Department of Agriculture tested cis-9-tricosene in the field, it found equal numbers of male and female houseflies in the traps.[14] Although they found no evidence of a male pheromone in the laboratory, Beroza and Carlson felt that the trapped males were attracting the female flies. They decided to continuously monitor the traps to see whether or not the male/female ratio remained constant throughout the experiment. It did.

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[14]Carlson, D., Beroza, M., "Field Evaluations of (Z)-9-Tricosene, A Sex Pheromone of the Housefly," Envir. Ent., v. 2, p. 555-559.

Do male houseflies emit cis-9-tricosene, or just the females? Mayer and Thaggard[15] found that live virgin female houseflies, live virgin male houseflies, containers contaminated by virgin male houseflies, and containers contaminated by virgin female houseflies attracted live virgin male houseflies equally well and significantly better than an empty container, which served as a control, thus implying than an attractant (possibly tricosene) is released by both male and female flies.

Cis-9-tricosene is also quite different from most other known sex pheromones. The only functionality present in cis-9-tricosene is a double bond while most pheromones identified to date possess at least one keto-, alcohol, acetate, or aldehyde functionality. Several pheromones are depicted in Figure 1. Although this may be merely a coincidence, it would tend to substantiate some aspects of Kafka's model of odor reception. Hydrogen bonding, a fairly strong intermolecular interaction, is possible when a molecule possesses an oxygen atom. Remember that Kafka postulated that the intermolecular interactions between the odor molecule and the acceptor site are partly responsible for odor perception. Keto, alcohol, acetate, and aldehyde functionalities all possess an oxygen atom and could bond fairly well to the acceptor sites in the insects' antennae, thus holding a portion of the molecule in place while the other parts rotated into the proper conformation. Cis-9-tricosene has no oxygen atoms and therefore cannot hydrogen bond to the acceptor site (unless the acceptor site has one of the atoms necessary for hydrogen bonding). Futhermore,

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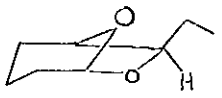
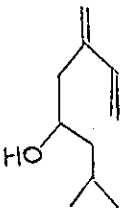
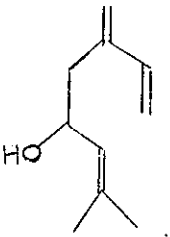
[15]Mayer,M., Thaggard, C., "Investigation of an Olfactory Attractant Specific for Males of the Housefly Musca domestica," J. Insect Physiol., v. 12, p. 891-897.



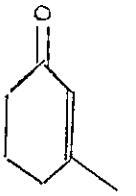
endo-Brevicomin



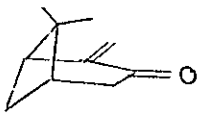
Ipsenol



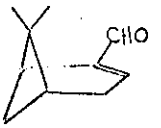
Ipsdienol



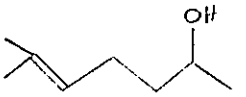
3-Methyl-2-Cyclohexanone



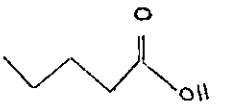
Pinocarvone



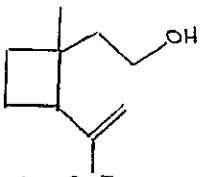
Myrtenal



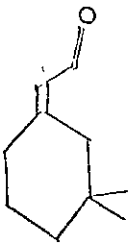
Sulcatol



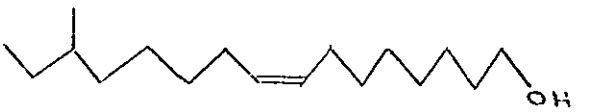
Pentanoic Acid



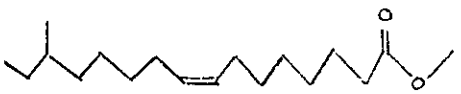
·) cis-2-Isopropenyl
ylcobutanethanol



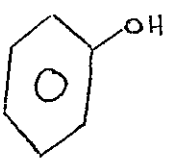
Z-(3,3)-Dimethyl- ',' -
Cyclohexaneacetaldehyde



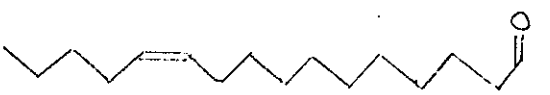
(-) 14-Methyl-cis-8-Hexadecen-1-ol



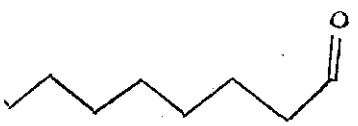
14-Methyl-cis-8-Hexadecenoate



Phenol



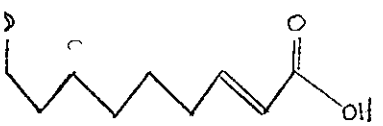
cis-11-Hexadecenal



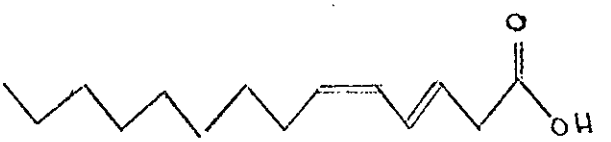
n-Nonanal

Dodecyl Acetate

n-Undecenal



xodec-trans-2-enoic Acid



trans-3-cis-5-Tetradecadienoic Acid

Some Reported Pheromones

Figure 1

the multiple point interaction model suggests that several homologs of cis-9-tricosene might be indistinguishable (to the male houseflies) from the compound itself. In fact, Meresz and Mozogai[16] tested a variety of compounds similar to cis-9-tricosene and found that the spectrum of active materials (unsaturated hydrocarbons from 19 to 25 carbon atoms long, plus, interestingly enough, epoxides of these alkenes) was atypically broad for the classical role of a sex attractant.

So many things - low specificity, weak attractivity, field tests, lack of an oxygen atom - appear "wrong" with this pheromone. There is yet another problem. Richter[17] found that the mating strikes of male houseflies can be elicited by the visual stimuli of static and moving black objects. A knot, made from a black shoelace and about the size of a female housefly, was attacked an average of 107 times by virgin males in 30 minutes. The optimal amount of cis-9-tricosene, as reported by Carlson et al,[18] when applied to the knot, failed to increase the number of male strikes, indicating that male houseflies may not rely upon chemical stimuli to find their mates. It is conceivable that their food sources attract sufficient numbers of flies to virtually ensure that male and female houseflies are within "sight" of one another when they are ready for mating.

That pretty much exhausts most of the work done with Musca domestica. Studying all the available evidence back in June, 1975

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[16]Meresz, O., Mozogai, C., "Pheromone Effects of Cis-9-Long $C_h$ain Alkenes on the Common Housefly - An Improved Sex Attractant," Can. Ent., v. 104, p. 1963-1965.

[17]Richter, I., "Sex Attraction of the Housefly by Moving Dummies is Not Increased by Pheromones," Naturwissenschaften, July 2, 1975, p. 365.

[18]Carlson, Mayer, Sihacek, James, Beroza, Bierl, op. cit., p. 76-78.

(some of the previously cited research papers were not available then), I concluded that cis-9-tricosene was, at best, a weak aggregation pheromone and not a sex pheromone, and the real sex pheromone, if it existed, remained to be discovered.

Tests with Musca domestica

I thought that the first step would be the development of a sensitive bioassay requiring small amounts of material, the starting point for nearly all pheromone research. It was not for me. The first hurdle proved to be finding a reliable method for distinguishing male houseflies from the females. The pupal coverings were much too dark to separate the sexes before emergence so I had to wait until the first batch of housefly pupae[19] emerged. For most species of flies, it is very easy to identify the male flies: they have a very pronounced "sex comb", or hairy leg. Not so with Musca domestica. Several mating pairs had to be killed, and, with the help of Dr. Madhavan, carefully dissected and checked for internal sex organs. Males were placed on one side of a petri dish, females on the other, and the two groups were compared under the microscope for morphological differences. Fortunately, some were found and I was able to proceed with the development of a sensitive bioassay.

This is not nearly as simple as it sounds: not all pheromones work in the same manner. Some beetles use sex pheromones only for long range guidance - visual guidance takes over once the males get within sight of the females. If the bioassay only tested males within sight of the females, one might conclude incorrectly that these female beetles do not emit pheromones. On the other hand, some species use only short range pheromonal communication and a bioassay which widely separated the insects and the pheromone source also would yield misleading results. Some insects will approach a pheromone source until they get within a certain distance, at which point they stop.

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[19]All housefly pupae were obtained from Carolina Biological Supply Company, Burlington, N.C., 27215

They then emit their own pheromone and await the arrival of the other pheromone source. If the other pheromone source is a piece of filter paper impregnated with the proper chemicals, they could wait a long, long time.

Another seious problem is known as "masking" and is the result of antipheromones and parapheromones. Antipheromones are compounds that interfere with or oppose the normal effect of pheromones and are not produced by the insect. Parapheromones are chemicals not used in intraspecific insect communication but cause responses identical to those cause by true pheromones.[20] Of the two, antipheromones are less desirable because they could lead one to believe that a pheromone is not present when it actually is. And antipheromones need not be uncommon chemicals. According to Riddiford and Williams[21], the action of trans-2-hexenal, needed to trigger the production of the sex attractant by female polyphermus moths, can be masked by numerous volatile agents - including Chanel No. 5. The extreme senstivity of the insects' odor reception apparati compounds the problem: even trace amounts, much too minute for humans to detect, could cause masking.

Pheromone concentration and the time of its application are two critically important factors - and they vary considerably from species to species. If either too little or too much is used, or if it is applied at the wrong time of the day, it is unlikely that the insects will respond, although too high a concentration often repels the insects.

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[20]Mitchell, E., "Disruption of Pheromone Communication Among Coexistent Pest Insects with Multichemical Formulations," Bioscience, v.25, p. 493-49

[21]Riddiford, Williams, "Chemical Signalling between Polyphemus Moths and Between Moths and Host Plants," Science, v. 156, p. 541.

Some species will walk to their attractant; others will not budge unless they have the proper amount of room to carry out an elaborate flight pattern.

Age is often important: sexually immature insects are usually oblivious to sex attractants. Old timers (and there is no a priori way to determine this), too, rarely show any interest in sex pheromones or the opposite sex.

All these factors mentioned above may be important and some of them will undoubtedly influence the design and the conditions of the bioassay.

Learning as much about the natural habits of the species under examination is the best way to avoid some of the pitfalls mentioned above. It also leads to another problem: one of credibility. One source[22] claimed that houseflies become sexually mature 10-14 days after emergence from the pupal stage while one[23] found that 80% of the females reached sexual maturity within two days of emergence (none were mature within 26 hours) and that 88% of the males were sexually mature within 26 hours of emergence (none were mature within 16 hours). A third study[24] reported that male flies less than three days old were not quite sexually mature. I decided to check for myself.

Two plexiglass cages were constructed (H=23.5 cm, W=25 cm, L=40 cm) and arranged with a Y-tube between them, as shown in Figure 2. When two batches of Musca domestica pupae (each batch contained 100-150 pupae) arrived, they were placed in a screen cage (H=25.5 cm, W=29 cm, L=31 cm) and kept in a room heated to 75°-80° F. Within 12 (and usually

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[22] Hewitt, C., Houseflies and How They Spread Diseases, New York: Cambridge University Press, 1912.

[23] Murvosh, C., Fye, R., Labrecque, G., "Studies on the Mating Behavior of the Housefly, Musca domestica," Ohio Journal of Science, v. 64, p.264-27

[24] Merez, Mozogai, op. cit., p.1963-1965.

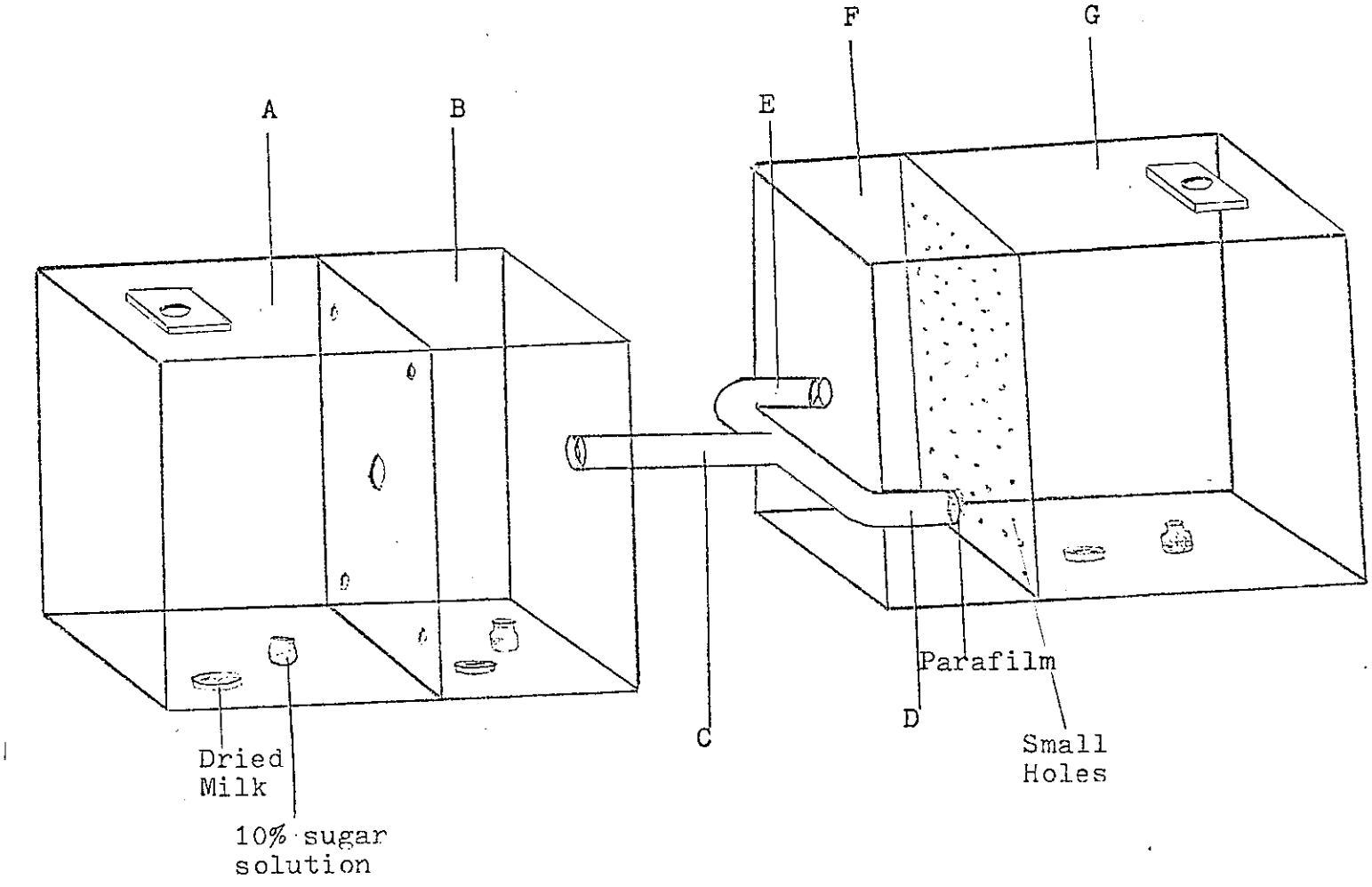

Figure 2.

within four) hours of their emergence, the houseflies were anesthetized with CO_2 and separated according to sex. The males (116) were placed in section A of the setup depicted in Figure 2 while the female flies (114) were placed in G. In sections A,B, and G were placed two vials, one containing dried milk, the other containing a 10% sugar solution. Several Kimwipes were stuffed inside the vial containing the sugar solution to prevent the flies from drowning. Unless otherwise stated, this was the only food fed to all the houseflies used in this and all subsequent tests. To keep the males and females in their places, a plexiglass partition full of very tiny holes was inserted between sections F and G. The day was divided into 12 hours of light and 12 hours of darkness and was maintained throughout the bioassay. The number of male flies in each section was counted periodically and plotted (Figure 3). The plot shows a dramatic increase in the number of male flies in section F during the night hours when the majority of houseflies were a bit less than three days old. Although this did not prove that male houseflies took three days to mature or that they were attracted to the female flies (only 35% of the males made it to F), it did agree with one of the studies and did not contradict a second. In support of this decision, I noticed the first male-to-male strike (a male housefly attacking another male housefly apparently for sexual gratification) in the above experiment when the males were 30 hours old, and increased in frequency until it reached its maximum when the males were nearly three days old. At that times, at least one male-to-male strike could be observed every second. Although numerous experiments agreed that houseflies could mate in the sunlight, I decided, on the basis of this quick test, to start out bioassaying in the dark. It later had to be changed. The plexiglass cages and screen

MALE TO FEMALE HOUSEFLIES

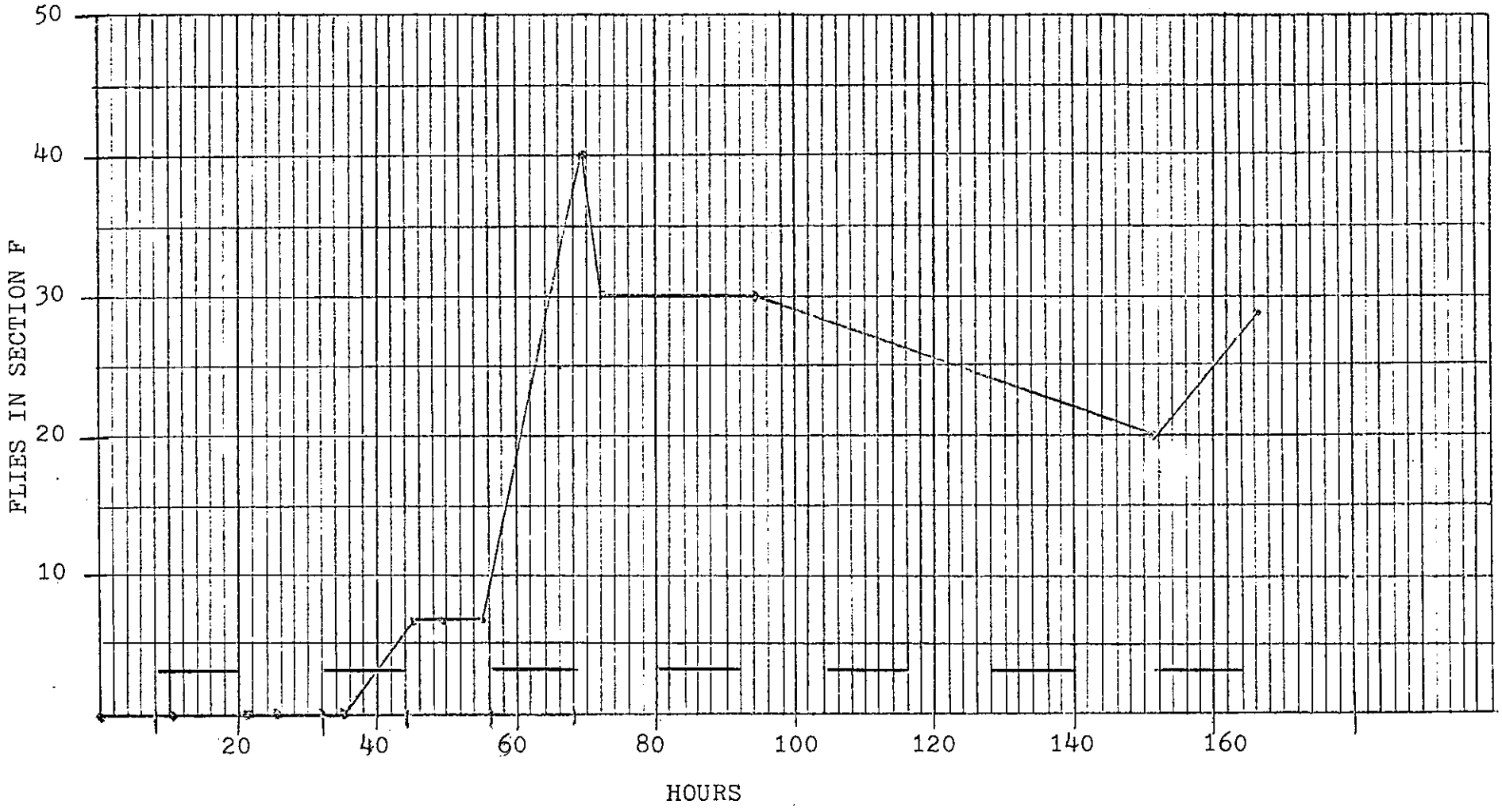

Figure 3

cages were scrubbed in soap, water and chlorox, and allowed to air dry after this and all other subsequent experiments. The Y-tube and food vials were cleaned in soap and water and heated overnight in a special oven.

I next had to decide whether there was a male or female (or both) sex pheromone for Musca domestica. When two more units of pupae arrived, they were handled exactly like the previous batches except that after the insects were separated according to sex, the male flies were placed in screen cages along with some food in a room heated to 75-80, while the female houseflies were put in different cages in a separate room heated to about the same temperature but otherwise were given the same treatment. All the male houseflies which emerged on the same day were put in the same cage. This same procedure was followed for the female flies. I tried to maintain a regimen of 12 hours of darkness and 12 hours of light for all flies. Since all subsequent units of flies were treated in this manner, I shall not repeat this description.

When the flies were three days old, I tried using a test similar to the one successfully employed by John Budris[25] for determining whether the pheromone was a male or female one. The bioassay chamber was very simple and required only two 250ml Erlenmeyer flasks and a Pyrex funnel, which always were washed and baked overnight in the oven before their use. They were arranged as shown in Figure 4a. The funnel served as a one way barrier: flies in the top flask could, with little difficulty, make their way to the flask below while the flies in the bottom flask had an exceedingly difficult time trying to make

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[25] Dr. Girard, personal communique.

FLASK OLFACTOMETERS

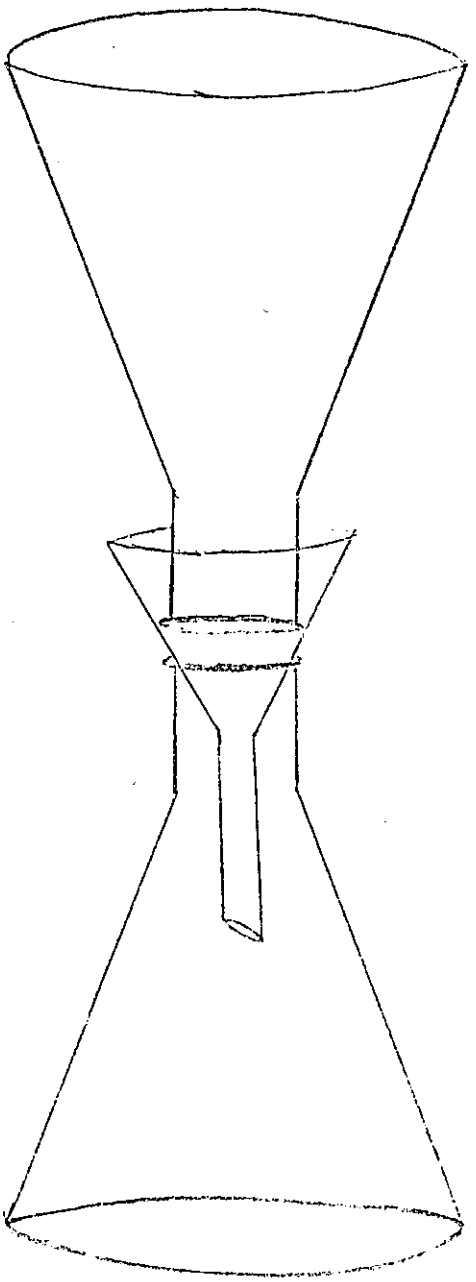

Figure 4a

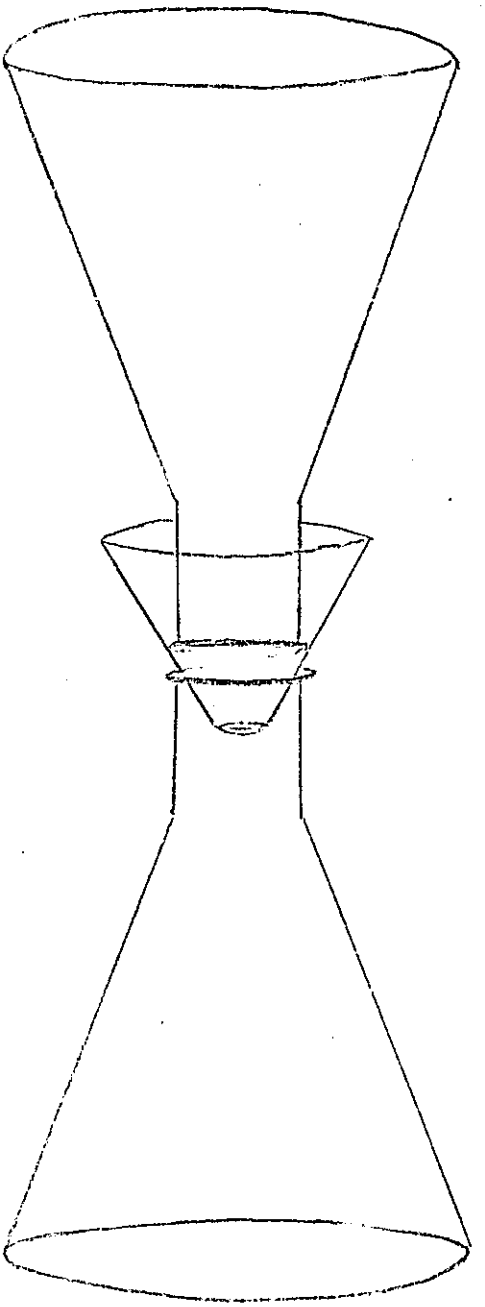

Figure 4b

it to the top flask.

In the first test, five, three-day-old virgin male houseflies were placed in the bottom flask and six, three-day-old virgin females were placed in the top flask. If the females crawled through the funnel, it might indicate the presence of a male sex pheromone. Three of the six female flies journeyed into the bottom flask in 20 minutes. This bioassay was performed in the dark during the "night" cycle. When the bioassay was repeated in the light, four of six three-day-old female houseflies left the top flask to join the males below.

To test for a female sex pheromone, I reversed the positions of the male and female flies: five, three-day-old virgin males were placed in the top flask while five, three-day-old virgin females found themselves in the bottom one. This, too, was performed in the dark, during the "night" cycle. Twenty minutes later, only one male fly had made it to the bottom. When I repeated the bioassay in the light, I noticed that several male flies "tried" to get to the females but had difficulty moving through the long stem of the funnel. So I repeated the experiment during the day under identical conditions except that the stem of the funnel was removed (Figure 4b). This failed to yield a better response, however: only one of five, three-day-old virgin males made it to the bottom flask in twenty minutes.

In a third experiment, 30 abdomens of three-day-old virgin females were ground up in one ml of methylene chloride. Eighty microliters of this extract were placed in the bottom flask while five, three-day-old virgin males were introduced into the top flask depicted in Figure 4b. The lights were left on for the duration of the experiment. Not one male even attempted to descend into the bottom flask within the alloted

20 minutes. When this test was performed again at night, identical results were obtained.

A fourth experiment was tried. Thirty virgin three-day-old female abdomens were crushed in a one ml solution of equal amounts of methanol and benzene. One hundred microliters of this extract was placed in the bottom flask. As in the previous experiment, five, three-day-old virgin male houseflies were put in the top flask. None went down to the bottom flask when the test was performed in the dark. The bottom flask remained devoid of male flies when it was repeated under lights.

The male houseflies did not appear to be interested in either the females or the extracts of female abdomens. The female flies, by comparison, readily crossed into the male flask. This could indicate the presence of a male sex pheromone (which no one else had found before), or it could indicate something else. Only more tests would tell.

On Dr. Girard's advice, I decided to scrap the two flasks and funnel olfactometer. Because it had no "control" response (i.e., no way to measure the natural desire of female flies to descend into the bottom flask), it probably would prove to be a bit unreliable. Different insects in a different location or at a different time would have to be used to measure the control response. This might yield higher variances and uncertainties. Dr. Girard suggested that I try the Y-tube olfactometer which had been employed successfully for the Sarcophaga bullata. The Y-tube olfactometer (see Figure 5) consisted of a Y-shaped tube, two 250ml side arm flasks, Tygon tubing, and two 14/20 adapters. The adapters, which served as one-way barriers, were placed over the

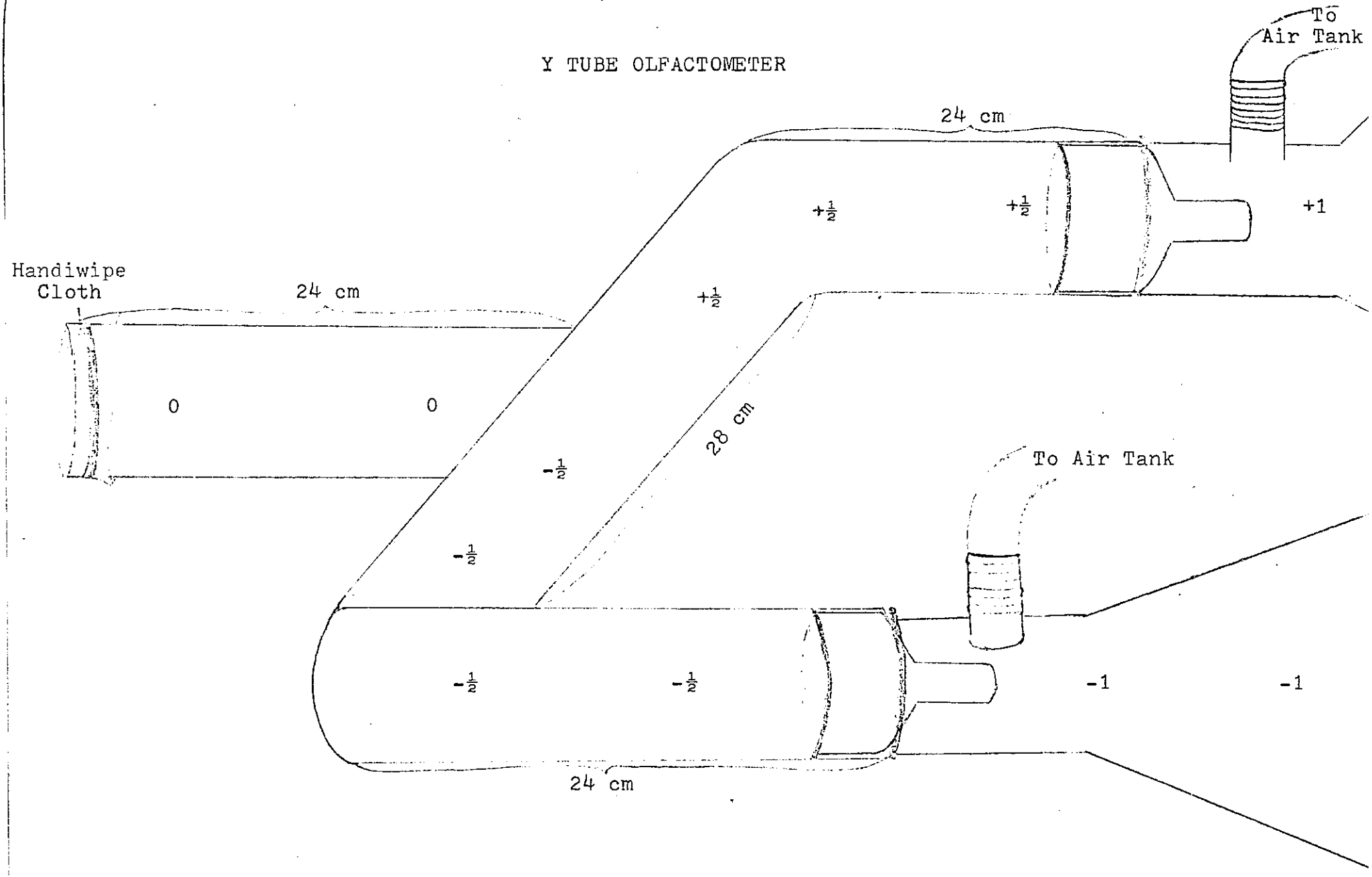
Y TUBE OLFACTOMETER
To Air Tank
24 cm
+½
+½
+1
Handiwipe Cloth
24 cm
+½
0
0
28 cm
-½
To Air Tank
-½
-½
-½
-1
-1
24 cm

Figure 5

side arm flasks, and the flasks were taped to the two arms of the Y-tube. One flask would hold the test substances, the other would serve as the control. Tygon tubing connected the side arms of the flasks to an air tank. An air flow of 150ml/min was maintained for all tests with the Y-tube olfactometer unless otherwise stated.

I started out testing the flies in the dark, turning the lights on whenever I wanted to count the number of flies in each section. This did not work well - the lights would startle them. I then tried performing the bioassays during the day but, to keep it dark, I covered the olfactometer with a black cloth, removing it whenever I wanted to count the number of flies in each section. This did not work well either so I gave up doing the bioassays in the dark. All the tests discussed in the following sections were performed under florescent lights and usually during the "light" cycle of the insect's day.

Four, three-day-old virgin male houseflies were placed in the bottom of one of the two flasks in the Y-tube. The other flask was left empty. Nine, three-day-old virgin female houseflies were admitted into the other end of the Y-tube. The olfactometer was divided into five sections (see Figure 5): a "0" section (the female flies did not move), a $+\frac{1}{2}$ section (the female flies moved to the "male half" of the Y-tube but did not journey into the male flask), a +1 section (the female flies made it to the male flask), a $-\frac{1}{2}$ section (the female flies moved to the "empty half" of the Y-tube but not into the empty flask), and a -1 section (the female flies went into the empty flask). The number of female flies in each section was recorded 5, 10, 15, and 20 minutes after the test began. The results of the first bioassay were:

	Males				Empty	
	+1	+½	0	-½	-1	Total
5 min	1	2	2	4	0	0
10 min	1	4	3	1	0	+2.5
15 min	1	5	0	2	1	+1.5
20 min	1	2	3	2	1	0

The final tally showed that 6 of 9 (67%) female flies left the 0 section, a response very similar to that obtained with the two flasks and a funnel olfactometer. At the end of the bioassay, three female flies were in the male half of the olfactometer but an equal number were in the control half, thus discrediting the male sex pheromone hypothesis. Several replications of this test yielded very similar results.

I then tried the reverse situation: six, three-day-old virgin males in the 0 end of the Y-tube with five, three-day-old virgin females in one of the flasks. The other flask was left empty, and the sections were labelled as they were in the previous set of bioassays except that the female flask and half were +1 and +½, respectively. The first bioassay worked very well:

	Females				Empty	
	+1	+½	0	-½	-1	Total
5 min	2	3	0	1	0	+3.0
10 min	2	3	0	1	0	+3.0
15 min	2	2	1	1	0	+2.5
20 min	2	3	1	0	0	+3.5

but might be misleading: at several times between the five minute intervals nearly all the flies in the +½ section (the males in the +1 section could not get out of the flask) went into the -½ section. In fact, several replications of this failed to confirm this bioassay's results. The males did not appear interested in the female houseflies.

In another experiment, eight, three-day-old virgin male abdomens were crushed on a piece of filter paper and placed in one of the flasks of the Y-tube. Eight, three-day-old virgin female abdomens were crushed on another piece of filter paper and placed in the other flask. Since the pieces of filter paper could not move, the 14/20 adapters were not necessary and therefore not used in this experiment. Sixteen, three-day-old virgin male houseflies were placed in the 0 section of the Y-tube. The first bioassay showed that the male flies seemed to prefer the male abdomens to those of the females (after 20 minutes, six males were in the crushed male abdomen flask (+1), two males were in the crushed male abdomen half of the Y-tube ($+\frac{1}{2}$), four males remained in the 0 section, only one male was in the crushed female abdomen half of the Y-tube ($-\frac{1}{2}$), and three males went to the crushed female abdomen flask (-1)). Subsequent bioassays, however, demonstrated no real preference.

This constant lack of reproducibility was, I felt, due to the Y-tube olfactometer: "good" results were not really as convincing as the numbers indicated - very often the flies would spend a lot of time on one side of the Y-tube and, just before it was time to count the numbers in each section, crawl into the beginning of the other half. They did not appear to get "settled down" within the 20 minute time limit. Tests run longer than 20 minutes usually resulted in several flies crawling through the side arm and into the Tygon tubing.

I tried two more tests with the Y-tube olfactometer before scrapping it. In one of the tests, fifteen, three-day-old virgin female abdomens were crushed on a piece of filter paper and placed in one of the flasks (+1) while a clean piece of filter paper was

put in the other flask (-1). No adapters were used and eight, three-day-old virgin males were used in the bioassay. The results were:

	Abdomens				Empty	
	1	½	0	-½	-1	Total
5 min	1	1	1	5	0	-1.0
10 min	1	0	1	6	0	-2.0
15 min	1	0	1	6	1	-3.0
20 min	1	0	2	4	1	-2.0

Having no luck with live females or crushed female abdomens, I decided to try to use the reported sex pheromone. However, one report found that cis-9-heneicosene attracted twice as many three-day-old virgin male flies as did cis-9-tricosene[26] so I purchased 100 mg of cis-9-heneicosene.[27] I did not check the purity of the sample which I had received. Ten micrograms of cis-9-heneicosene elicited the best response from the male houseflies.[28]

Such small quantities are difficult to handle so it was necessary to dissolve the heneicosene in some solvent. The solvent had to evaporate very quickly so that there would be only a small fraction of the solvent left and a large portion of the heneicosene when the bioassay was started. The solvent also had to have only a minor effect on the flies: chemicals like ether would probably kill the test flies as they approached them. Methylene chloride was chosen as the solvent because it has a high vapor pressure (B.P.=40-41° C) and did not appear to adversely affect male and female houseflies when used in moderate amounts.

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[26]Meresz, Mozogai, op. cit., p. 1963-1965.

[27]Purchased from Suppelco, Inc., Suppelco Park, Bellefonte, Pa.

[28]Meresz, Mozogai, op. cit., p. 1963-1965.

The 100 mg of cis-9-heneicosene were dissolved in 100 ml of methylene chloride (1µg/1µl of solution) and stirred for an hour. Henceforth, this solution will be referred to as "heneicosene". Whenever it was not being used for a test, the heneicosene was kept in the refrigerator.

The Y-tube olfactometer was set up as before and 10 µg of cis-9-heneicosene (10 µl from the 100 ml solution) were shot onto a piece of filter paper, fanned for about 20 seconds (to get rid of most of the methylene chloride), and then put into one of the two flasks(+1). Ten µl of methylene chloride, shot onto a piece of filter paper and fanned for about 20 seconds, were placed in the other flask (-1). Eleven, thre-day-old virgin males were used in this bioassay. They were not overly impressed with the heneicosene:

	Heneicosene			Methylene Chloride		
	+1	+½	0	-½	-1	Total
5 min	1	0	8	2	0	0
10 min	1	3	4	3	0	+1.0
15 min	1	2	4	4	0	0
20 min	1	1	4	5	0	-1.0

The Y-tube olfactometer indicated that there are no sex pheromones for Musca domestica; however, this olfactometer may not be the best way to test for the pheromones. Perhaps the sex pheromone is present but only effect for very short distances, much shorter than the distances involved with the Y-tube.

To test this possibility, I used another type of olfactometer (see Figure 6). This cubic olfactometer was constructed from plexiglass and had a middle compartment which was divided into two sections - one for the test material (+1), the other for a control (-1). Should the

CUBIC OLFACTOMETER

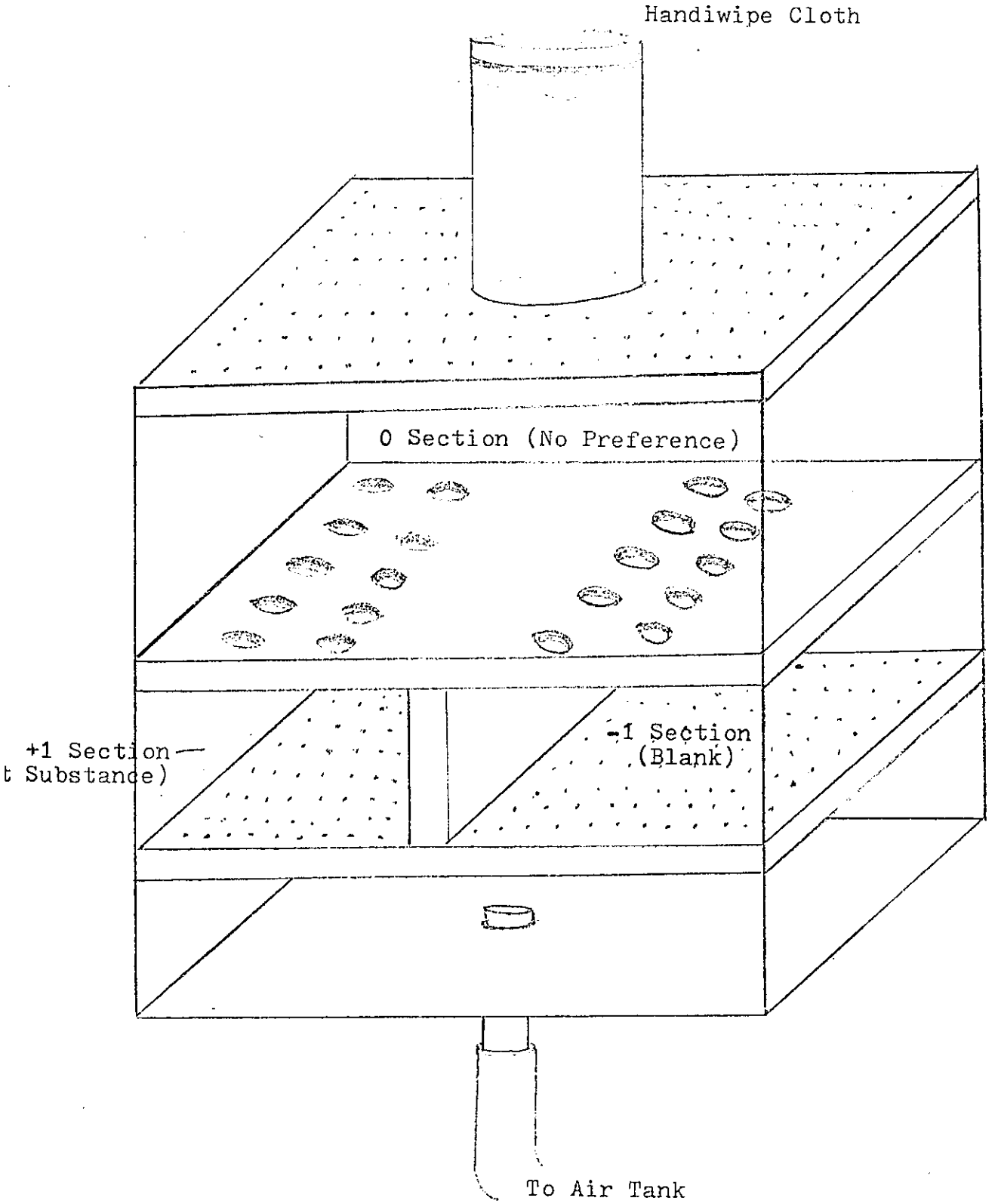


Figure 6

majority of test flies congregate in one side, we can assume that something either attracted them to that side or repelled them from the other side. A hole (diameter=4cm) was drilled into the middle of the top of this cubic olfactometer (measuring 15cm on a side). Three inch long cylindrical glass tubes (diamter=3.5cm), which were used to introduce the test flies into this olfactometer, easily fit into this hole. In the middle of the bottom plate was another hole (diameter= 1cm) which was fitted with a glass tube and Tygon tubing and connected to an air tank. An air flow of 150 ml/min was used in each of the experiments with this olfactometer.

Ten μg of the heneicosene were placed on one side of the olfactometer's test section while ten μl of methylene chloride were shot into the other section. After 30 seconds had elapsed (to allow the vapors of methylene chloride to escape), the glass tube containing ten, three-day-old virgin male houseflies was fitted into the hole in the top plate. The flies seemed more interested in investigating the contents of their new cage than the samples placed before them. They quickly and indiscriminately moved from compartment to compartment. No male-to-male strikes were witnessed. After half an hour had elapsed, three males were in the compartment with the heneicosene, three chose the methylene chloride, and four remained in the top chamber, which was taken to mean "no preference". Things were not going well.

Since live females could not be used as a test substance (there was no way to keep them in the test section), I killed five, three-day-old virgin females and placed their abdomens in one of the test chambers. The other test chamber was left empty. Twelve, three-day-old virgin male houseflies were used in this test. At the end of the bioassay (30

minutes), five males were in the top section (no preference), four were in the control section, and three were with the female abdomens. This olfactometer was not yielding spectacular results either and so was scrapped.

Houseflies might require more than a half hour to become acclimated to the test chamber and to find the sex pheromone; this could explain the frequently observed shifting from the pheromone (?) source to the control and back. So a third type of olfactometer was set up (see Figure 7) which would all the bioassay to run for an hour, a day, a week, or whatever was necessary.

For every bioassay (unless otherwise stated), the olfactometer was divided into seven sections (A through G - see Figure 7). The newly emerged test flies were placed in section A while the test material was put in section G. Food (dried milk and a 10% sugar solution) was available only in sections A and B to avoid any possiblity of flies being attracted to the food in another section and not the test material in section G. The flies experienced 12 hours of darkness and 12 hours of light each day. Additional test meaterial was added every 12 hours, usually at the onset of "day" and "night". The number of flies in each section was recorded periodically.

In the first bioassay with this new olfactometer, a crude extract of one kilogram of male and female houseflies ground up in methylene chloride was the test material. Fifty newly emerged male flies (all but 16 emerged within seven hours of one another) were placed in A. I did not "start" the bioassay until the majority of flies were a day old (five were nearly two-days-old while 11 were only about three-hours-old) when 150 μl of the crude extract were injected into a vial in section

DOUBLE CAGE OLFACTOMETER

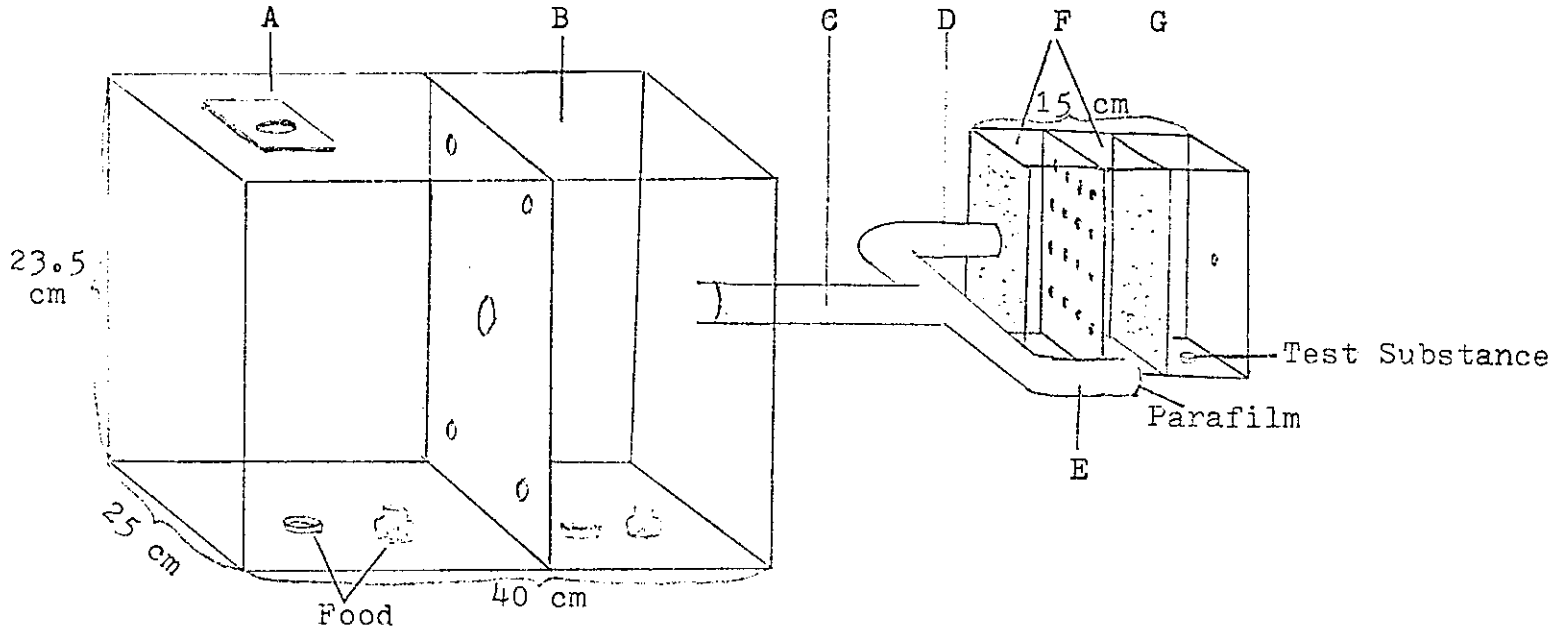


Figure 7

G (one male fly had already made it into section F before the test had started). The male houseflies slowly moved from section A into B. Two hours after the test started, the number of flies outside section A exceeded the number in section A. Furthermore, although the majority of flies appeared lethargic, the six flies in F were very active. The flies continued to leave A and to become more active. About one day after the bioassay had started (the majority of male flies were two-days-old), the first male-to-male strikes were observed in section F. By the next day (the males were three-days-old), the males literally were going after one another, although the number of flies in section F had not yet reached its maximum. For the next 24 hours, the population in F steadily declined to a low of eight flies, or 16% of the total population. Seventy eight and a half hours after the bioassay had begun (when most of the males were a little more than four-days-old), the number of flies in F reached its maximum: 23 flies (46% of the total population). Three flies were also in section E at the time. The F population then declined steadily for the next three days (it dropped to six flies), at which time the bioassay was discontinued. The variation of the population in F with time is plotted in Figure 8. This was very encouraging: the male flies left their food in sections A and B for something in section F, presumably the smell of the crude extract.

How would the female flies respond to the crude extract? I decided to find out using a similar set-up. As before, food was available only in sections A and B while the crude extract was put in section G. One hundred ten newly emerged females were used for this bioassay. The majority of flies (76) was less than four-hours-old when the test

MALE HOUSEFLIES TO CRUDE EXTRACT

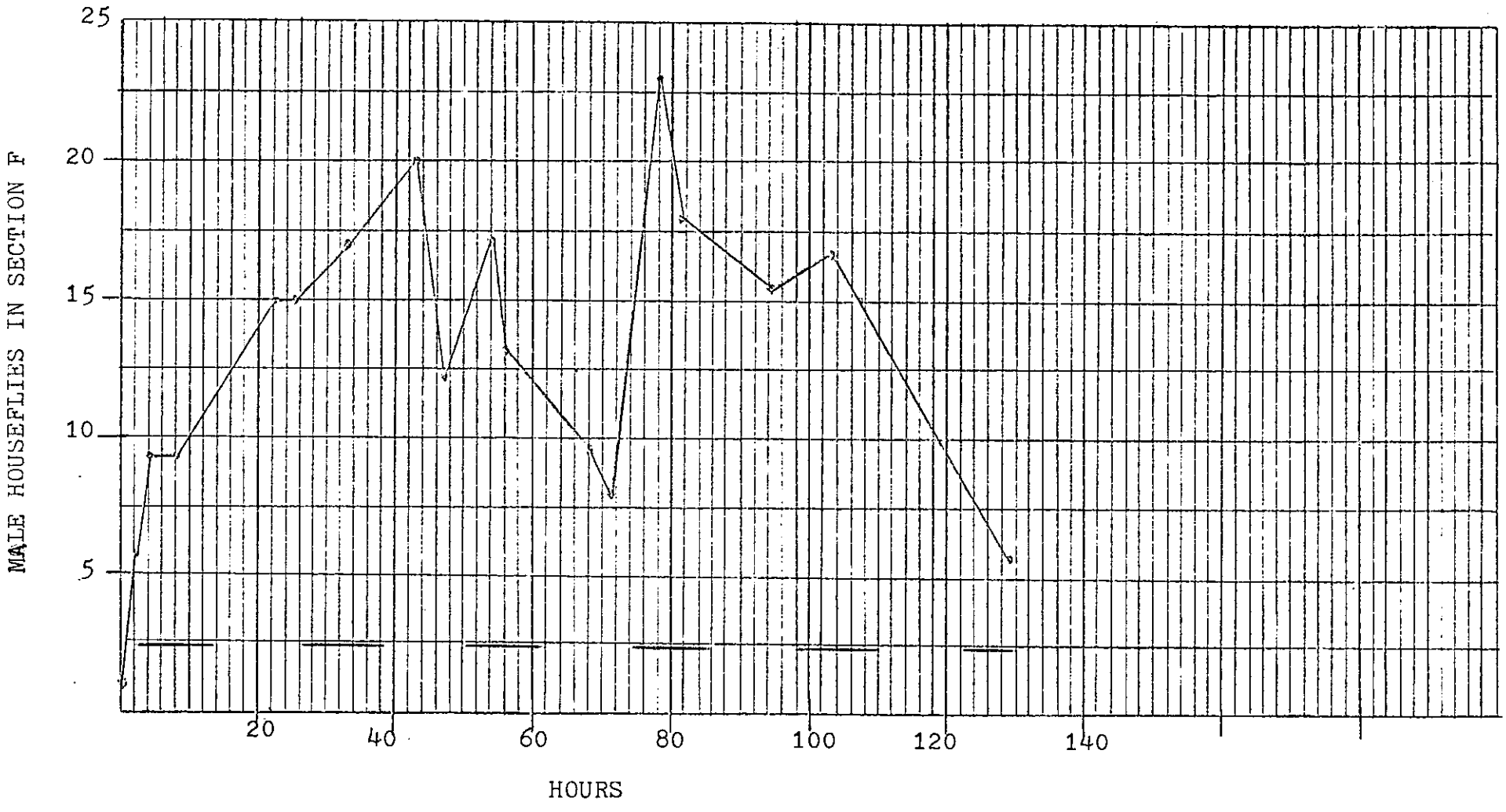


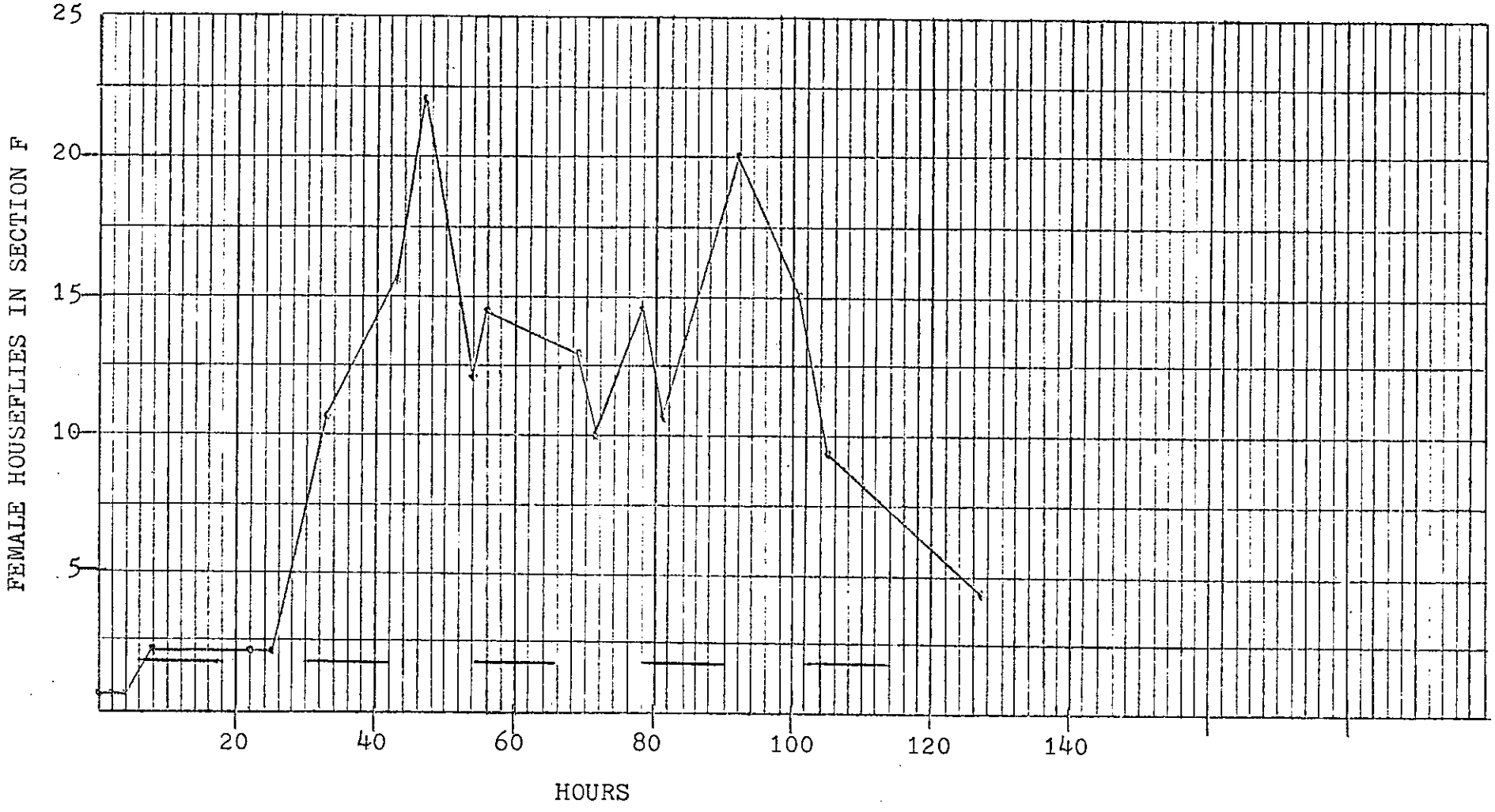
Figure 8

——Night

started (the first injection of 150 µl of the crude extract) although three females were two-days-old and 31 were only a-day-old. One female housefly made it into F before the test had started. The females did not respond as well as the males: it took the females nearly two days to reach "the majority of flies not in A" stage, as opposed to four hours for the male flies. Interestingly enough, section F achieved its maximum population (22 females or 20% of the total) a couple of hours later when most of the female flies were barely two-days-old. As wtih the males, the female population in F declined after reaching its maximum until it hit 11 (10%) flies, then rebounded to 20 flies, and then declined again (see Figure 9, the plot of the variation in the population of F with time). This latter decline might be attributable to the escape of 56 female flies. Sometime after the population reached 20 females in section F, one of the seals (between B and C) fell off, leaving a hole big enough for the flies to escape. More females preferred the escape route to the crude extract. Unlike in the previous experiment with the males, a substantial number of female flies were found in the Y-tube (sections C, D, and E).

Although the female houseflies did not respond well to the crude extract, they did exhibit very unusual behavior two days after the bioassay had started: many female flies (about 15 to 20) clustered about the body of one apparently dead female fly. It even looked as though they were fighting over the body and continued it for over two hours. But not every dead female fly aroused the interest of living female houseflies: a couple of days later, five dead flies were noticed in the cage but only two of them had attracted any females (and there were many around the two dead females). Perhaps the age of the corpse

FEMALE HOUSEFLIES TO CRUDE EXTRACT



——Night

Figure 9

had something to do with the strange behavior. I killed one, day-old virgin female housefly and dropped it into section A. Although it was approached by several female flies within one minute of its entrance into A, it virtually was ignored for the next 30 hours (the bioassay was then terminated).

In the third bioassay with this "double cage" olfactometer, the test substance was the heneicosene and the test insects were male houseflies. Seventy-nine newly emerged male houseflies (all had emerged on the same day) were placed in section A. One hundred μg of cis-9-heneicosene were injected into section G immediately after the flies were put in the olfactometer. The first male reached F 2½ hours later. The population of F steadily grew (see Figure 10) until it reached 49 (62% of the total) 41 hours after the bioassay had begun (the males were also 41-hours-old). It then declined to about 30 flies (38% of the total). There were also four male flies in section E when F was at its maximum. The flies were remakably inactive, however. Very few male-to-male strikes were observed when F was at its maximum or even afterwards. In fact, the males in F looked as though they were resting. Only the flies in A and B occassionally attacked each other. The population in F never surpassed 49 and the bioassay was terminated when the flies were a week old.

I then tried to see how attractive heneicosene would be to the female houseflies. Fifty-four newly emerged female houseflies were placed in section A while 100 μg of cis-9-heneicosene were placed in G. These females moved considerably faster than the females used in the crude extract bioassay. Within two hours, the majority of females had left section A and three had made it to F (5% of the total). One

MALE HOUSEFLIES TO CIS-9-HENEICOSENE

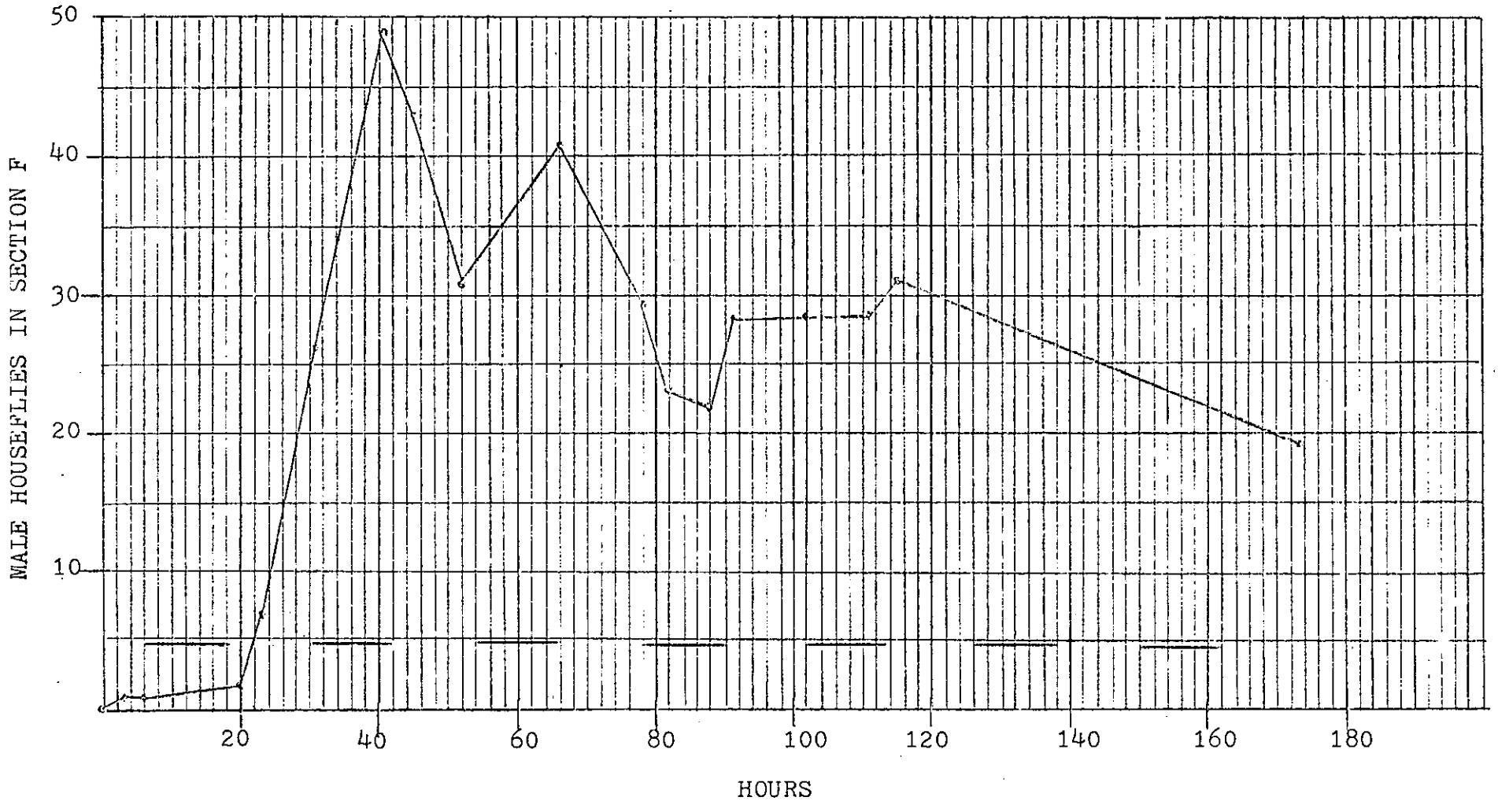
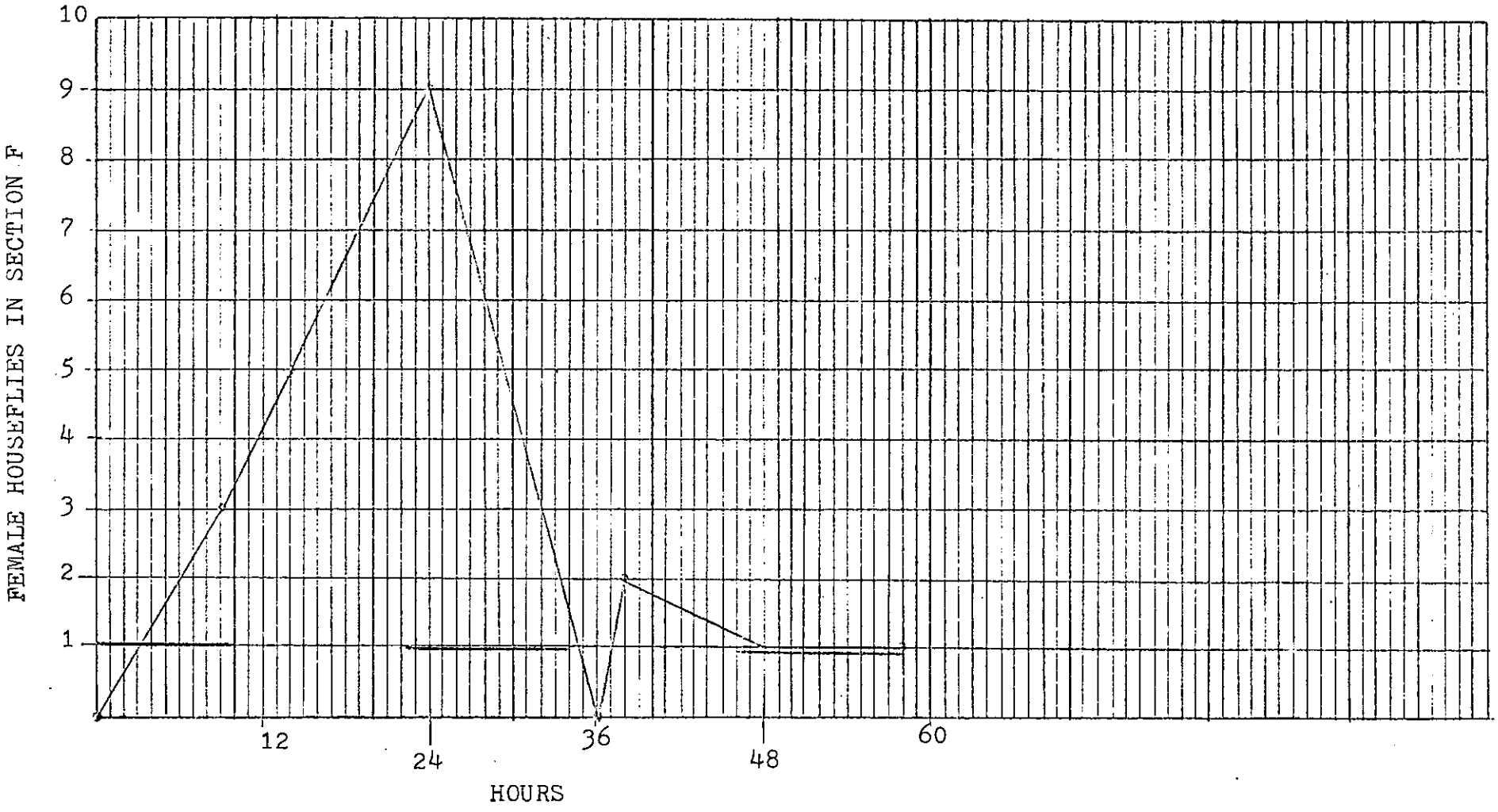


Figure 10

day after the bioassay had started (the females were 24-hours old), section F attained its maximum population: 9 females (about 17% of the total). Thereafter, the population in F quickly decreased (see Figure 11) while that in A gradually increased until it again contained the majority of the females. There was also a significant number of females in the Y-tube (sections C, D, and E) for the duration of the bioassay. The females apparently were not interested in heneicosene.

How well could the male houseflies find their way through the test apparatus? I decided to find out. Since all of the small plexiglass cages (sections F and G) were tied up into other experiments, I used screen cages at the two ends of the Y-tube (see Figure 12). This olfactometer was divided into five sections: one screen cage was "A", the other screen cage was "B", the Y-tube was section "C", the front end of the large plexiglass cage was labelled D, and the back end of the large plexiglass cage was E. Food was placed only in section E to encourage the flies to move. Fifty-six, one-day-old virgin male houseflies were placed in A; seven, one-day-old virgin male houseflies were also put in B. The insects were placed in the screen cages instead of the plexiglass cage to ensure that their response was not caused by a build-up of odors inside the cage which might repel the males. One male made it to the food nine hours after the bioassay had started. Twenty four hours after the bioassay had begun, eleven flies were in B (a gain of four), ten were in E, two were in D, and 39 remained in A. By the end of the second day (when the males were three-days-old), 16 male flies were in E, five were in D (33% of the total were in sections D and E), eleven were in B, and 30 were in A. Several male-to-male strikes were noticed in the plexiglass cage while only one was witnessed in A even though A had

FEMALE HOUSEFLIES TO CIS-9-HENEICOSENE



———— Night

Figure 11

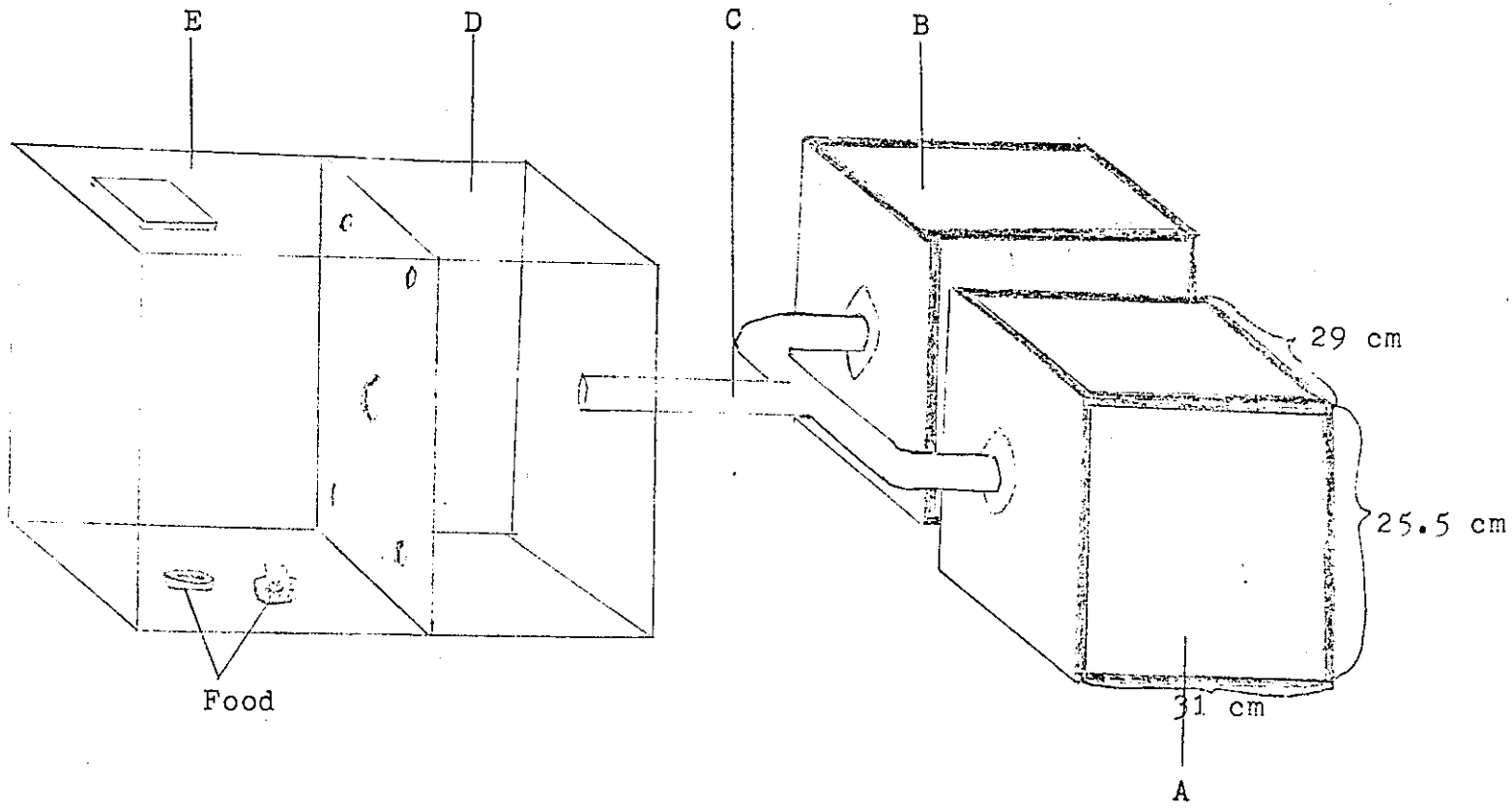


Figure 12

many more flies than did sections D and E. Unfortunately, this bioassay had to be stopped prematurely and ended only two days after it had started.

Another bioassay with heneicosene and the male houseflies was in order. However, I changed the olfactometer a bit (see Figure 13). A flask containing a methylene chloride blank (just pure methylene chloride) replaced the parafilm covering on one end of the Y-tube. Also, to eliminate the possibility that male flies left sections A and B because they were repelled by their own odor or by the crowded conditions, I installed a screen cage above the hole in the top plate of the large plexiglass cage. The sections were labelled in the following manner: the screen cage was A, the section of the large plexiglass cage directly below the screen cage was B, the other section of the large plexiglass cage was C, the arm of the Y-tube leading to the flask containing the methylene chloride blank was E, the other arm of the Y-tube was D, the flask was F, and the small plexiglass cage was G. Food was available in sections B and C.

Twenty-three, one-day-old virgin male fles were placed in section A, 100 μg of cis-9-heneicosene were put in G, and 100 μl of methylene chloride were put in F. Whenever heneicosene was added to G, an equal amount of methylene chloride was added to F. Other than this, these male flies were treated exactly like the males in the previous heneicosene bioassay.

It took nearly fifteen hours for the first flies to leave the screen cage and to descend into B and C. Two days after the test had started (the flies were three-days-old), one fly had made it into G, one fly had escaped from the cage, and two had died. Twenty four hours

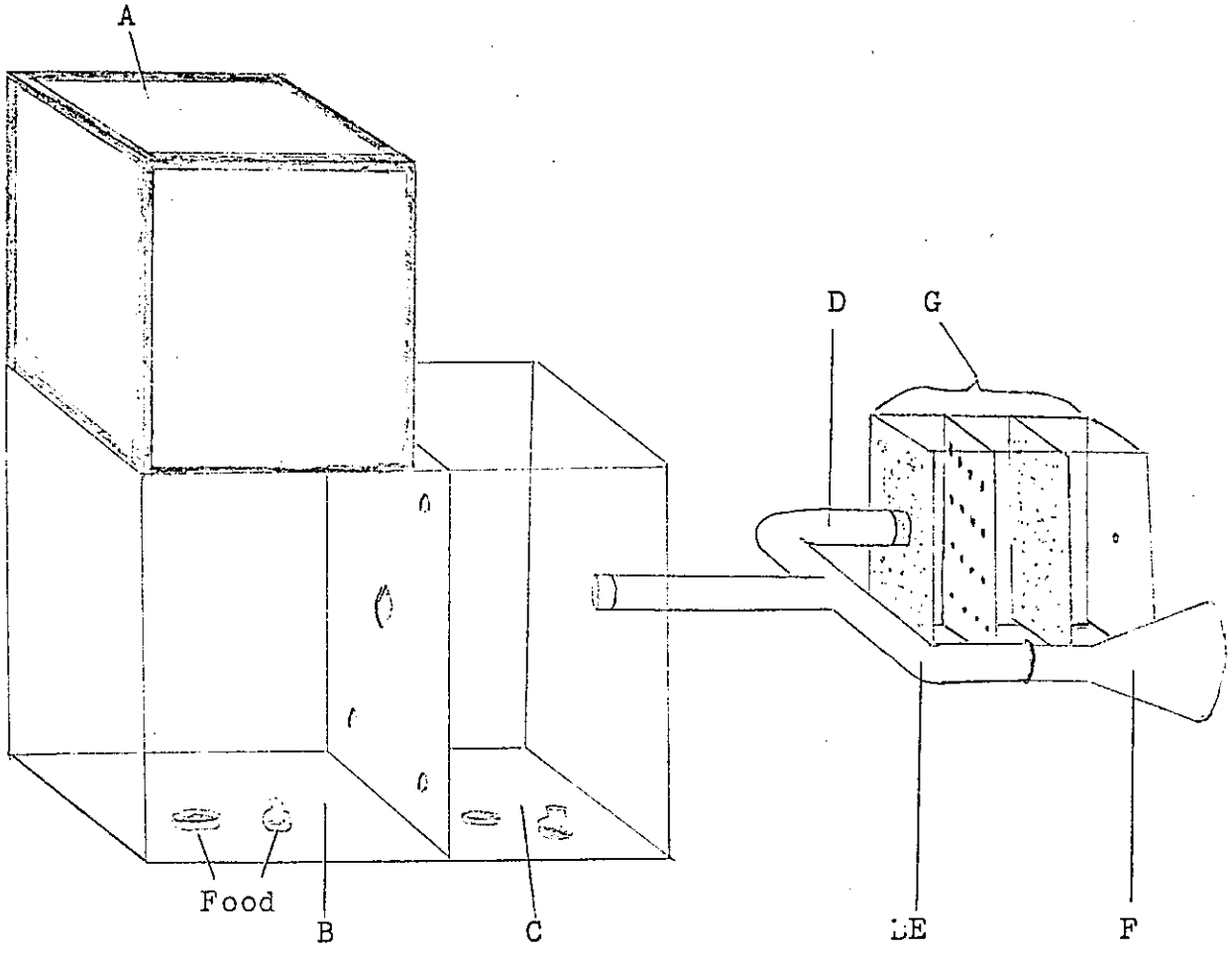


Figure 13

later (the flies were four-days-old), five flies were dead, three males were still in the screen cage, eight were in B, three were in C, two were in D, one fly was in E, and five were in G (a 30% response discounting the five dead males and the one which had escaped). The population of G declined over the next 24 hours and then resurged to six male flies (33%). By the next day (when the flies were a little over five-days-old), all but three of the flies were dead so the bioassay was discontinued.

I repeated the bioassay above with 60 newly emerged male housflies. It took the first fly only one hour to get to B. The first male fly was in section G fifteen hours after the bioassay had begun. The population in G remained low (a maximum of one fly at any one time) until the bioassay was 2½ days old when it reached 6 flies (10%). It then declined for a while but rebounded to its maximum (eight flies, or 13% of the total) when the males were three-days-old. The test was continued for another two days, after which time it had become evident that these males were not attracted to the heneicosene.

I then tried 60 newly emerged females in this olfactometer with 100 μg of heneicosene in section G and 100 μl of methylene chloride in section F. The female houseflies quickly left the screen cage: within an over, over 33% of the female flies had left. Less than one day after the bioassay had started, only five females remained in the screen cage. But the females were not attracted to the heneicosene: only one female fly (less than 2%) went to section G during the entire week-long test.

One last bioassay with another batch of female houseflies indicated that they might not be willing to travel through the test apparatus. Seventy-one newly emerged female flies were placed in the screen cage.

No food was placed in sections B and C and the barrier between these two sections was removed (see Figure 13). Dried milk was placed in the small plexiglass cage (G), and some cooked hamburger meat (from Kimball) and the 10% sugar solution went into the flask (F). Only one female fly went to either food section for the duration of the bioassay (five days). In fact, 30 remained in the screen cage until their death. Most of the flies were dead by the end of the bioassay. Perhaps the Kimball food repelled them.

Discussion of Results and Conclusions

Is there a sex pheromone for Musca domestica and, if so, is it cis-9-tricosene (or heneicosene)? Unfortunately, my tests do not provide a definitive answer because there appear to be large variations from bioassay to bioassay which might indicate the absence of a pheromone or might be the result of uncontrollable factors (amount of time the pupae were exposed to the cold when they were in transit, etc.). However, 35% of the male houseflies left their cage and food for the female flies (section F), a value not too unlike that found by some other researchers. Although no control was attempted (time did not permit it), it seems unlikely that the males would leave their food for no reason. If it were a "diffusion" process - flies leaving areas of high population density for areas of low population density - we would expect a fairly gradual increase in the F population and a significant number of flies dispersed about the Y-tube (C, D, and E). In fact, the reverse happened: the population in F increased quickly and few male flies were found in the Y-tube.

On the other hand, the female flies' responses to the crude extract might be the result of such a diffusion process: a much less dramatic increase in the F population as well as a significant number of female flies in the Y-tube were noticed. If we assume that a 17%-20% response (the females' response to the crude extract and the heneicosene) is the control response of both the male and female houseflies, there does appear to be some sort of male sex attractant, although not a very good one.

It is doubtful that the males' poor response could be attributable to some masking agent: all researchers who have worked with Musca

domestica have received equally poor results. It is difficult to believe that masking agents would be present in all these different laboratories. It is much more reasonable to believe that there was something wrong either with the diet of the houseflies, or with the design of the olfactometer, or with the insects themselves (a low-order sex pheromone). These possibilities will be discussed later.

The crude extract of the houseflies also attracted a large number of male houseflies (46%), although the maximum response came when the flies were four-days-old - over a day later than in the first bioassay with the female flies in G. However, when the test substance was heneicosene, the maximum response of the males came when they were less than two days old. Recalling the large variations reported for the attainment of sexual maturity by male houseflies, the small variations which I have found might not be too unreasonable: perhaps even slight temperature differences can cause relatively large differences in developmental rates.

The results of the three male-to-heneicosene bioassays are a bit puzzling: the first bioassay had a 62% positive response, the second had a 33% positive response, the third had a dismal 13% positive response (the second bioassay, because it was based on 17 insects, may not be a reliable figure). Although this may have resulted from a loss of the heneicosene with time, it seems more likely to be the result of a simple change in the olfactometer: the use of a screen cage above the plexiglass cage.

The screen cage was used because there seemed to be little air flow inside the olfactometer and the odors inside the large plexiglass cage became quite strong after several days, and I did not want the flies to head for section F because they were repelled by smells in

sections A and B. A screen cage would allow greater air flow and keep the population density low.

At first glance, it would appear that the male flies were not attracted to the heneicosene but repelled by their own smells because the use of the screen cage sent positive responses plummeting. But then again, the 62% positive response in the first heneicosene assay seems much too high to be the result of some sort of repellent. Why did the crude extract bioassay, using the same olfactometer, only give a 46% positive response? And, unless it was a male dispersion pheromone, should not the females be repelled in a somewhat similar manner? Could something else be involved?

Male-to-male strikes were observed when the males were kept in screen cages and in the plexiglass cage. However, the frequency of male-to-male strikes appeared much greater in the plexiglass cages than in the screen cages and were usually observed a couple of days after the male flies were placed in the plexiglass cage. Male flies in screen cages usually took longer to exhibit this behavior. This may mean that certain chemicals emitted by the male can stimulate the male housefly although it must reach a certain concentration level before it really turns the males on. Screen cages, because they allow more air circulation, do not allow a large build-up of the chemicals and therefore the number of male-to-male strikes is reduced. Recalling the habitat of many houseflies - decaying food and manure - one is tempted to suggest that this "stimulant" chemical might be a compound present in decaying food or fecal matter. It is known that several compounds likely to be present in decaying food - ammonia,

butyric acid, ethanol, valerianic acid, and isovaleraldehyde - are very effective housefly attractants.[29]

The presence of this "stimulant" may be necessary in sufficient amounts before the male houseflies will respond to heneicosene (or tricosene). This synergistic relationship could explain the poor results obtained with the Y-tube olfactometer and the olfactometer with the screen cage above the plexiglass cage. The screen cage and the short duration of the tests in the Y-tube (20 min) may have kept the concentration of this compound at too low a level, thus preventing the males from responding "well".

This may not be as far-fetched as it sounds: the absence of food, or an appropriate place to deposit eggs, might discourage male flies from mating. It has been demonstrated that the synthetic sex pheromone of the boll weevil attracts many more male weevils when the odor of cotton is also present.[30] When cis-9-tricosene was tested in the field,[31] it attracted 12.4 times as many flies as the control with electric grid traps, 7 times as many with sugar bait traps, 2.8 times as many with flypaper strips, and 3.4 times as many with sticky panels. There is a large variation in these ratios which may be the result of of the different capacities and killing efficiencies of these different types of traps. But it is interesting to note that the field evaluations were carried out in a barn containing a lot of manure and that those traps with the highest ratios were those placed on the ground - presumably

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[29]Sharma, R., Saxena, K., "Orientation and Developmental Inhibition in the Housefly by Certain Terpenoids," J. Med. Ent., v. 11, p. 617-621.

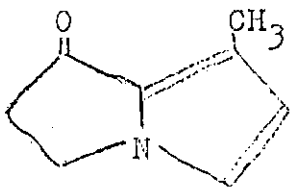
[30]Hardee, D., Wilson, N., Mitchell, E., Huddleston, P., "Factors Affecting the Activity of Grandlure, the Pheromone of the Boll Weevil in Laboratory Bioassays," J. of Ec. Ent., v. 64, p. 1454-1456.

[31]Carlson, Beroza, op. cit., p.555-559.

closer to the manure. The lowest ratio came from the trap placed farthest from the floor (3 m).

I once tried testing this hypothesis with isovaleric acid, a very foul-smelling compound. I had no idea how much (if any) would be the proper amount so I arbitrarily chose ten μl. Unfortunately, isovaleric acid is so strong that even ten μl of it is enough to fill an entire room (and other parts of the Chemistry department) with its foul odor. I decided to scrap the test rather than to continue experimenting to find the proper dosage.

It is unlikely that natural populations of male and female houseflies subsist solely upon dried milk and sugar water. Perhaps these other foods contain the necessary precursors of the pheromone, and flies raised without these necessary precursors exhibit only low-order sex recognition. This, too, could explain the poor responses from the flies (but not the male-to-male strikes). In one study with male Danaus butterflies,[32] it was found that the mating success of males emitting little or no 2,3 dihydro-7-methyl-1H-pyrrolizine-1-one



2,3 dihydro-7-methyl-1H-pyrrolizine-1-one

was low compared to that of normal males. Further investigations revealed that natural populations of male D. chrysippus butterflies obtained this pyrrolizidinone from a certain plant in a slightly

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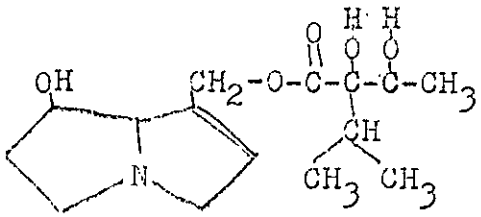
[32]Schneider, D., Boppre, M., Schneider, H., Thompson, W., Boriack, C., Petty, R., Meinwald, J., "A Pheromone Precursor and Its Uptake in Male Danaus Butterflies," J. Comp. Physiol., v. 97, p. 245-256.

different form and then modified it to the correct form. Males not



lycopsamine

fed the leaves of this plant, or this pyrrolizidine alkaloid (lycopsamine were found to be deficient in 2,3 dihydro-7-methyl-1H-pyrrolizine-1-one, implying that the males must obtain the precursor of their sex pheromone from their food sources and cannot synthesize it from simple chemicals. Perhaps the male or female houseflies lacked the necessary food source.

A third explanation for the poor responses could be the design of the olfactometers: perhaps it hindered in some way the flies' normal responses. As mentioned earlier, field tests of cis-9-tricosene attracted male and female houseflies equally well while my laboratory bioassays (and those of other people) were unable to show that female houseflies were attracted to the crude extract or heneicosene. The inability of the female flies to make it through the olfactometer to their only source of food would be evidence against the olfactometer employed. Perhaps the male and female responses would have been better if the olfactometer were better.

In conclusion, the female sex pheromone of Musca domestica does not appear to be a strong one and may require the presence of some other chemical, perhaps in fecal matter, to attract the males. There does not appear to be a male sex pheromone.

Previous Work Done with Sarcophaga bullata

I also was involved with the attempt to isolate the sex pheromone of the flesh fly Sarcophaga bullata, although considerable progress already had been achieved and will be summarized in this section.

Using the Y-tube olfactometer, John Budris[33] found that two-day-old virgin female flesh flies were attracted to virgin male flesh flies and the abdominal tips of virgin male flesh flies. Virgin male flesh flies did not appear to be attracted to either virgin female flesh flies or to the abdominal tips of virgin female flesh flies. Evidently a male sex pheromone was responsible for bringing the sexes together. He then checked a crude extract of ground-up flesh flies for activity; this, too, proved attractive to the female flesh flies. With the presence of the pheromone in the crude extract established, the long task of finding the sex pheromone among the thousands of different compounds present in the crude extract could then begin.

The active crude extract, which was placed on top of a 120 cm x 6 cm Sephadex LH-20 column, was eluted from the column with a 50:50 methanol-benzene solution, and was separated into seven fractions (labelled 2G1 - 2G7). Each fraction had to be tested for activity. Only fractions 2G6, which worked very well, and 2G7, which worked poorly, attracted the females. The number of possible compounds had been narrowed down somewhat.

When a sample of 2G6 was subjected to TLC, it was separated into two fractions, neither of which attracted the female flesh flies. When the two fractions were recombined, it still failed to attract the female flesh flies - indicating that the "active" component(s) of 2G6 probably

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[33]Budris, John, M. S. Thesis, College of the Holy Cross, Worcester, Mass.

had evaporated, decomposed or oxidized. This was confirmed with another sample of 2G6: when it was left out in the air at room temperature for several hours, activity was lost. Apparently the sex pheromone was either fairly unstable, easily oxidized or highly volatile.

Gas chromotography is quite useful for separating the chemicals in a multicomponent solution, providing the collecting apparatus is arranged properly, so it was natural to turn to this technique once TLC had failed. A Varian (Model #2240-10) gas chromatograph was used in these preliminary separations with dry ice (sublimes at -78° C) around the collecting tube to facilitate the condensation of the gaseous effluents.

The temperature program dial on the G.C. was set to 8°/min, the initial temperature set at 50° C, and the final temperature set at 250° C. This 200° temperature range was divided into four fractions: 50°-100°, 100°-150°, 150°-200°, 200°-250° C. Each fraction was collected in a special collecting tube and sealed for future tests. When the four fractions were bioassayed, only the first fraction - from 50° to 100° C. - evidenced any activity.

For the next set of collections, the temperature program rate was set at 2°/min to expand the 50°-100° region. Collections were made between 50°-70°, 70°-90°, 90°-100°, and 100°-250° C. and bioassayed as before. Only the second cut - from 70°-90° - showed any activity this time. A third set of collections from the G.C. narrowed the range to 80°-90° C. while the fourth set of collections showed the active compound(s) to come off the G.C. between 80°-86° C. John Budris reported the presence of a rather broad double peak in this region and, because he was

unable to resolve it any further, stopped here.

Mike Gottschalk[34] performed a number of chemical tests on the active fraction and noted any changes in the activity of the fraction to the female flesh flies. Any loss of activity after a chemical test was attributed to the rearrangements or changes in the structure of the sex pheromone caused by that chemical test, thus revealing some of the structural characteristics of the pheromone. After Gottschalk finished all his tests, he concluded that the sex pheromone in fraction 2G6 was a polar, volatile, unsaturated carboxylic acid.

When Robin Vita[35] joined the search for the pheromone, she needed more of the active fraction for more bioassays. Five kilograms of flesh flies gave 2.8 grams of the sixth fraction coming off the 120 cm x 6 cm Sephadex LH20 column. This fraction was labelled 183-6. It was bioassayed and found to attract the female flesh flies.

She tried to "clean-up" the 183-6 fraction with High Pressure Liquid Chromatography (HPLC). The active compound(s) came off the μ-Porasil column with 100% hexane.

Robin Vita then returned to the G.C. with her crude 183-6 fraction and her "cleaned-up" 183-6 fraction. Activity was found in all fractions collected between 40°-110° C. but not in those collected from 110°-195° C. The trace of the crude 183-6 is shown in Figure 14. Notice the large solvent peak (methylene chloride) and the two prominent peaks in the 50°-100° C. region. A number of small, volatile compounds must have been coming off the G.C. column with the methylene chloride

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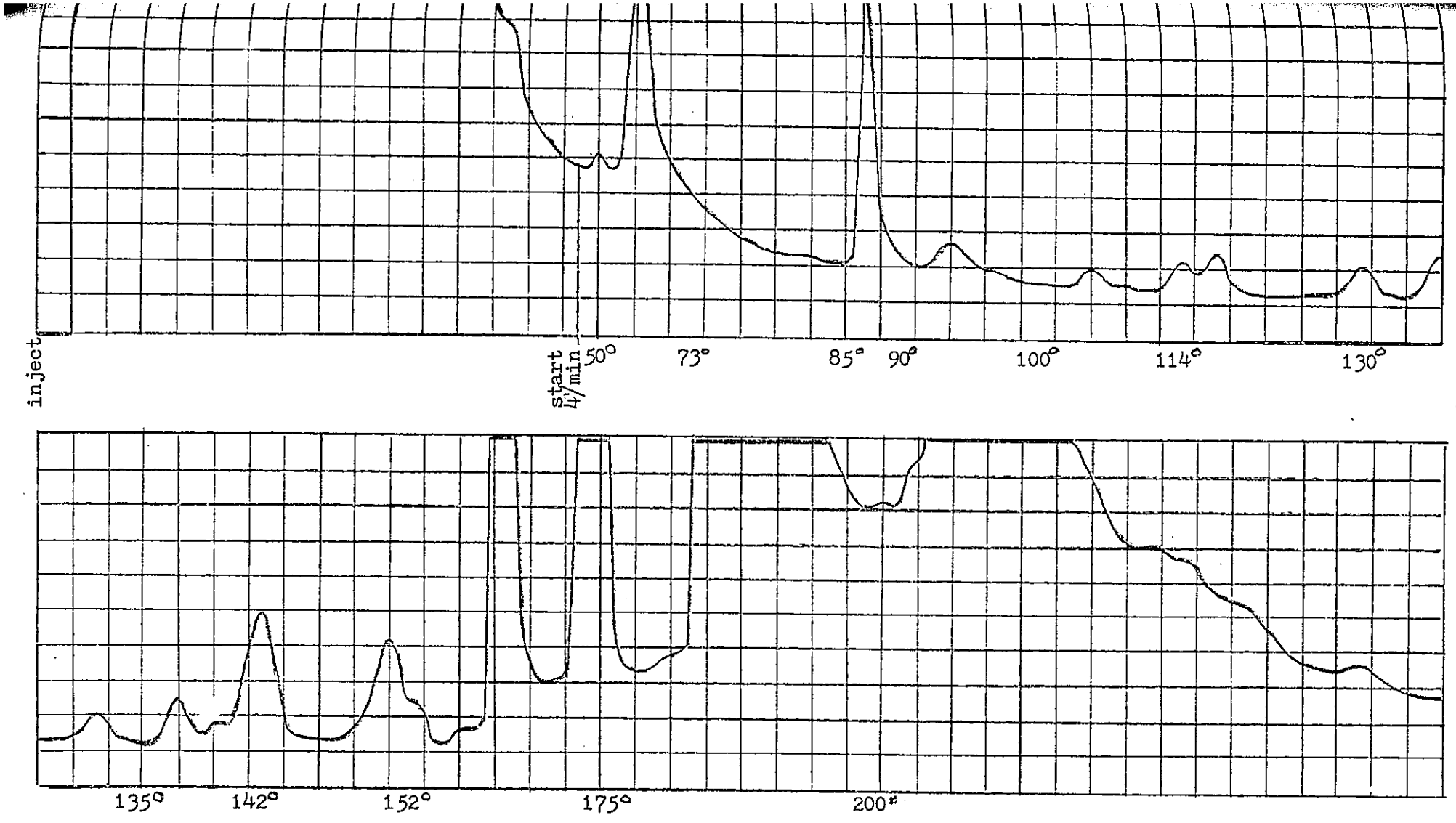
[34]Gottschalk, M., Honors Thesis, College of the Holy Cross, Worcester, Massachusetts.

[35]Vita, R., Honors Thesis, College of the Holy Cross, Worcester, Mass.



Column: SV-17
Chart speed: ½ in/min
Sensitivity: 1 x 10^{-11}
Chart: Cool

Active Extract 183-6 (6/29/76)

Figure 14

to yield such a broad peak - an injection of pure methylene chloride would not give such a broad peak.

John Budris' work had indicated that the pheromone came off the OV-17 column between 80°-86° C. In Figure 14, this would correspond to the tiny peak immediately preceding the large peak at 85°-90°. It was doubtful that this small peak could be isolated and collected in significant quantities from Holy Cross' G.C. So Dr. Girard journeyed to Pennsylvania State University with the active 183-6 fraction and injected it into their G.C.-Mass Spectrometer (Finnegan 3200F-6103). Unfortunately, the number of compounds coming off the G.C. between 80° and 86° was too great to determine the structure of the pheromone. However, several peaks indicative of an aromatic ring and molecular weight about 120 amu were noticed.

With this in mind, Robin Vita bioassayed a number of different compounds including several short chain fatty acids, toluene, ethyl benzene, and other benzene-containing compounds. The female flesh flies were not attracted to any of the compounds, although it must be noted that the concentrations may not have been correct. These tests were completed by the end of the summer of 1976, just before my arrival on campus.

Isolation of the Sex Pheromone of Sarcophaga bullata

Little more could be done at Holy Cross: the resolving power, sensitivity and collecting apparatus were just not good enough to collect the active 80°-86° region in significant quantities. To get any further with this project, a better G.C. had to be used. Dr. Girard asked me if I would be willing to travel to Pennsylvania State University and collect some fractions of 183-6 off their Finnegan (Model #9500) G.C. Thus began my involvement with the project.

I travelled to Pennsylvania State University with about 15 ml of the "crude" (unpurified) 183-6 fraction (solvent: methylene chloride), 15 ml of the "purified" (cleaned on the HPLC) 183-6 fraction (solvent: methylene chloride), and 15 ml of a ripe liver extract for another project. All were sealed in glass ampoules prior to my departure from Holy Cross. Upon arrival at Penn State, I opened the sealed ampoules and, using a nitrogen tank, reduced the 15 ml samples to about 2 ml to concentrate the active peak. These concentrated samples were stored in a freezer until their use. Two analytical, six-foot-long, U-shaped glass columns were cleaned and then heated to 350°C. for several hours. Upon cooling, one column was packed with 6% OV-17 material while the other was packed with DiEthyleneGlucoSuccinate (DEGS) material, which is better than the OV-17 material for separating compounds with double bonds. Both columns were "activated" that night.

The next morning I worked with the unpurified 183-6 fraction. With the chart speed set at $\frac{1}{2}$ in/min, the sensitivity of the G.C. at 1×10^{-10}, the temperature program rate at 4°/min, the intial

temperature at 40°C., and the carrier gas (Helium) flow rate at 40 ml/min, I injected five μl of the unpurified 183-6 sample into the G.C. fitted with the OV-17 column and, at the same time, started a special digital stopwatch. The time on the stopwatch, because it was more accurate and reliable than the temperature showing on the G.C., was recorded for each major peak appearing on the G.C. (these were later converted to approximate temperatures). The first trace which I obtained in shown in Figure 15: note the large peak between 52° and 64°, the small peak between 64° and 70°, the large double peak around 70°, and the lack of any large peak between 84° and 90°. The column was baked at 250° for 20 minutes (to rid it of any large, polar compounds which may have been retained) and then cooled to 40°. The entire process of injecting a sample of the unpurified 183-6 into the G.C., recording its trace, and baking the column was repeated two more times to find out how reproducible the results of the first trace were. Aside from minor variations in retention times and the appearance (or disappearance) of several peaks, all three traces were remarkably similar.

After all three traces were compared and all variations in retention times were averaged, I was ready to begin collecting fractions off the G.C. But where should I begin collecting and where should I end - there was no significant peak between 80° and 86° C.? I did not know if this different G.C. and column could shift significantly the retention time of the active peak so I decided to cut the trace into six fractions (labelled A1, B1, C1, D1, E1, F1). See Figure 16. The first cut, between 48° and 57°, captured the first large peak coming off the G.C. The region between 57° and 66°, which at times

Flow Rate: 40 ml/min

183-6 Fraction in Methylene Chloride

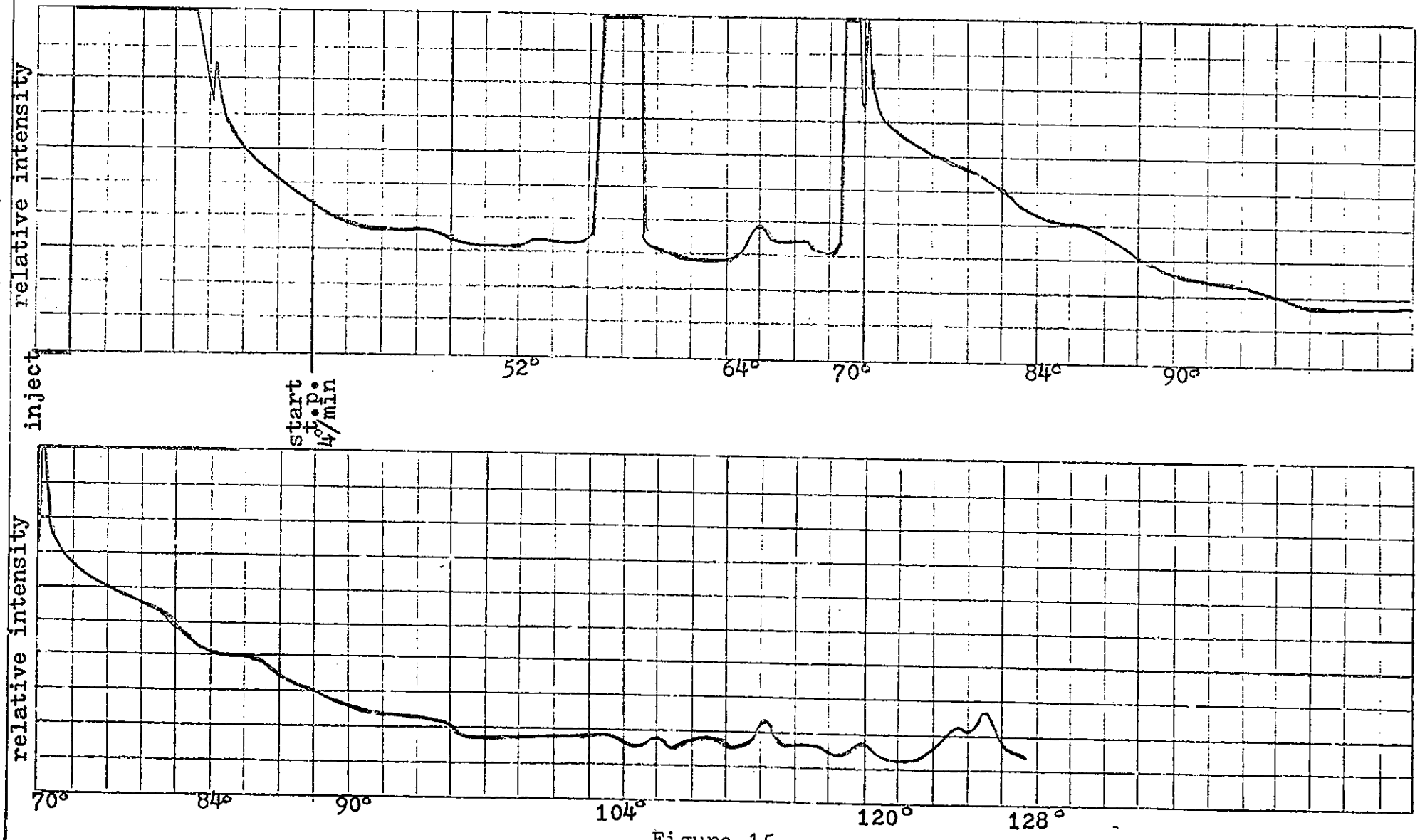


Figure 15

Column: OV-17

183-6 Fraction in Methylene Chloride

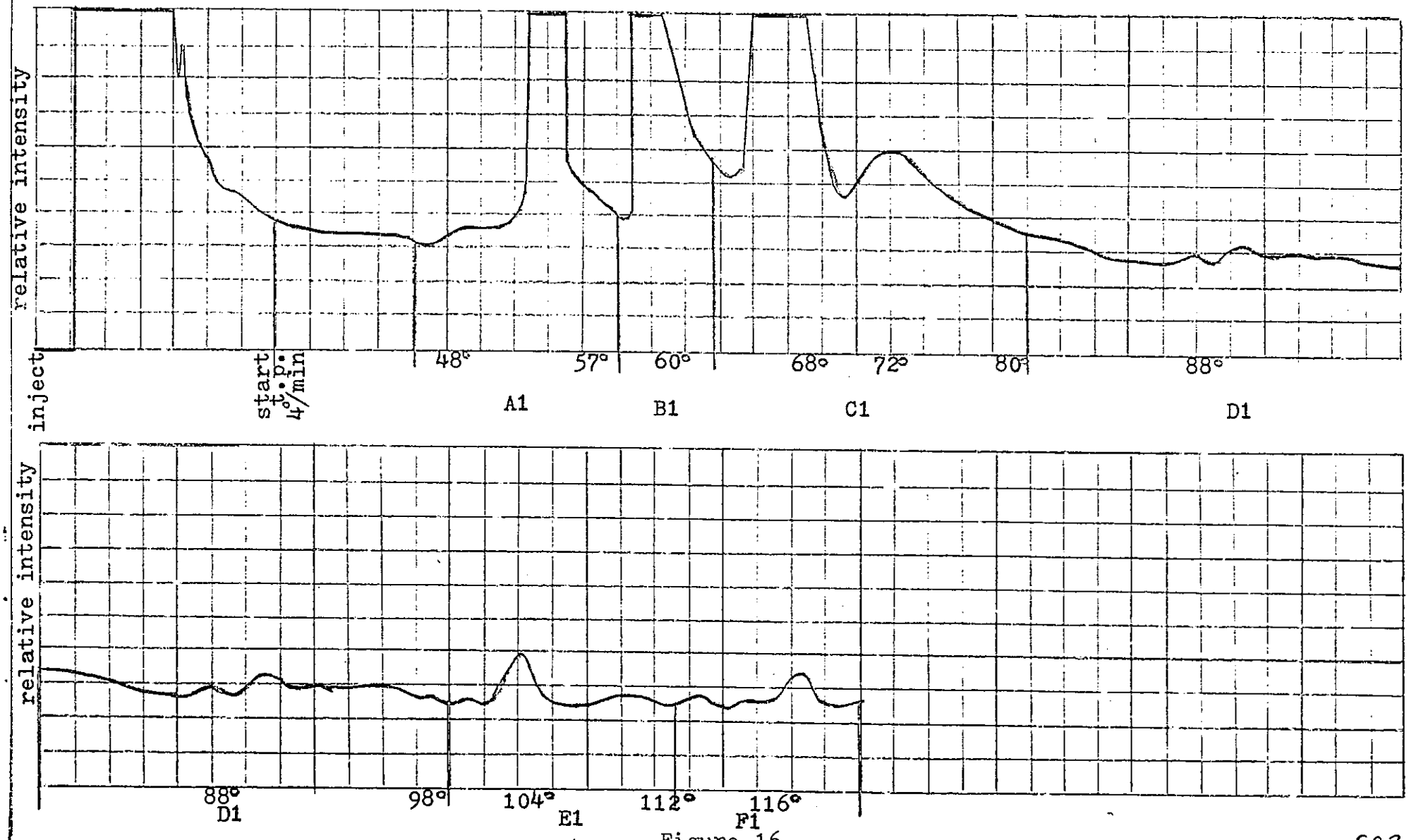


Figure 16

GC3

contained a large peak but at other times had only a small peak (compare Figures 15 and 16), was caught in cut B1. Cut C1 (between 66° and 80°) contained two large peaks - one of which was fairly broad and possibly the "active" peak found by John Budris. Cut D1 was collected over a wide region (80°-98°) because there were few prominent peaks in this region. The fifth (E1: 98°-112°) and sixth (F1: 112°-120°) cuts were not expected to contain the active compound(s) but were collected just in case they did.

The collections were performed in a slightly different manner at Pennsylvania State University. Instead of the dry ice cold trap, an aluminum coil was immersed in liquid nitrogen (B.P.=-195.8°C.). One end was connected to a nitrogen tank, the other end was placed a few inches from the collector port. At the start of every collection period, the nitrogen tank was turned on and a six-inch-long capillary tube was inserted into the collector and the free end of the aluminum coil was directed at the capillary tube. A very cold stream of nitrogen issued out from the end of the aluminum coil and rapidly cooled the collector tube. At the end of the collection period, the capillary tube was quickly removed and another one took its place. The "filled" capillary tube (even though it appeared empty) then was sealed, labelled and stored in a freezer. Four sets of collections (labelled 1A1,....,1F1, 2A1,...,2F1, 3A1,...,3F1, 4A1,...4F1) were made for the unpurified 183-6 on the OV-17 column.

After the column was baked for an hour at 250°, I was able to begin work with the purified 183-6 sample. Five microliters of the purified 183-6 were injected into the G.C. and its trace recorded (see Figure 17). As before, two more injections were made and any variations in retention times were averaged to give a middle value. Since none of the settings on the G.C. were changed, we can compare

Column OV-17
Sensitivity: 1×10^{-10}

183-6 Fraction " Hexane

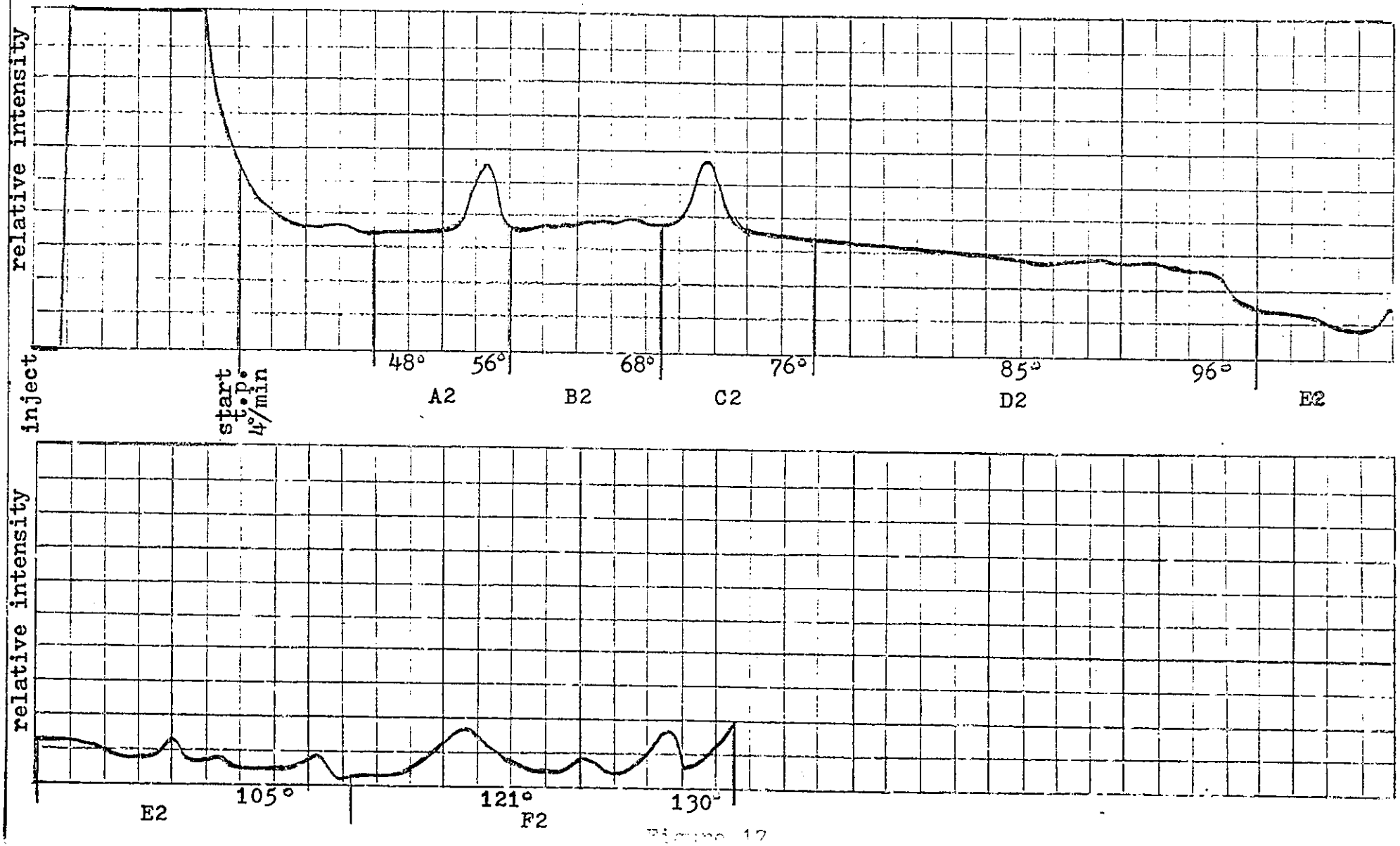


Figure 12

the traces of the purified and unpurified 183-6 injections. There are some obvious similarities and differences between the two traces (see Figures 16 and 17). Perhaps the most striking difference in the two traces is the size of the peaks coming off the G.C. before 98°: the early peaks of the purified 183-6 fraction are much, much smaller than those of the unpurified 183-6 fraction. Apparently a lot of the smaller, more volatile compounds present in 183-6 were lost during the purification process either through evaporation or retention upon the μ-Porasil column.

Although the size of the peaks may have differed, the locations of the peaks remained nearly the same in the traces of the purified and unpurified 183-6 fractions. This was hardly surprising: the only difference in the two 183-6 fractions was that one was subjected to HPLC to clean it up. Aside from the loss of "dirty" compounds, the purified 183-6 fraction should contain exactly the same compounds as the unpurified 183-6 fraction. Because the locations of the peaks were so similar, I was able to take nearly the same cuts with the purified 183-6 as I did with the unpurified 183-6: A2 (48°-56°), B2 (56°-68°), C2 (68°-76°), D2 (76°-96°), E2 (96°-107°), and F2 (107°-130°). Three sets of collections were made and labelled 1A2,...,1F2, 2A2,..., 2F2, 3A2,...3F2. They were stored in a freezer until my departure.

The six-foot-long, U-shaped DEGS column replaced the OV-17 column in the G.C. All the settings on the G.C. were kept the same except that the temperature program dial was changed to 6°/min. The collecting apparatus was set up as it was in the previous set of collections. Since the DEGS column "bled" more than the OV-17 column, the bake temperature was held to 200° C. The bake time

between injections was increased to 40 minutes.

The first five μl injection of the unpurified 183-6 fraction yielded a trace quite different from those obtained with the OV-17 column (see Figure 18). The first large peak (aside from the solvent peak) did not appear until 94°. The three early peaks present on the traces from the OV-17 column probably came off with the methylene chloride - notice how wide the solvent peak is. However, the DEGS column appears to have done a better job separating the bigger, less volatile compounds - notice the number of small peaks after about 106°.

Since there were few major peaks in the DEGS traces, I decided to collect only four fractions instead of six: W1 (66°-106°), X1 (106°-144°), Y1 (144°-166°), and Z1 (166°-196°). Three sets of collections were made and labelled 1W1,..., 1Z1, 2W1,..., 2Z1, 3W1,..., 3Z1 (Figure 19). After these collections were finished, the column had to be baked overnight.

The next morning I returned to try the purified 183-6 fraction on the DEGS column. I injected a five μl sample into the G.C. This new trace (Figure 20) was surprisingly different from the previous two (Figures 18 and 19). Upon closer examination, however, it appeared that the peaks were the same but that their retention times were a bit less. For example, the region in the trace between 130° and 168° in Figure 20 appears quite similar to the region in Figure 19 lying between 170° and 200°. This shift may be attributable to the loss of some of the DEGS material through bleeding and to the slightly smaller length of the column in the morning (about six inches shorter) after it broke.

In any event, I decided to switch the collecting regions to try

Fraction 183-6 in Methylene Chloride on DEGS Column

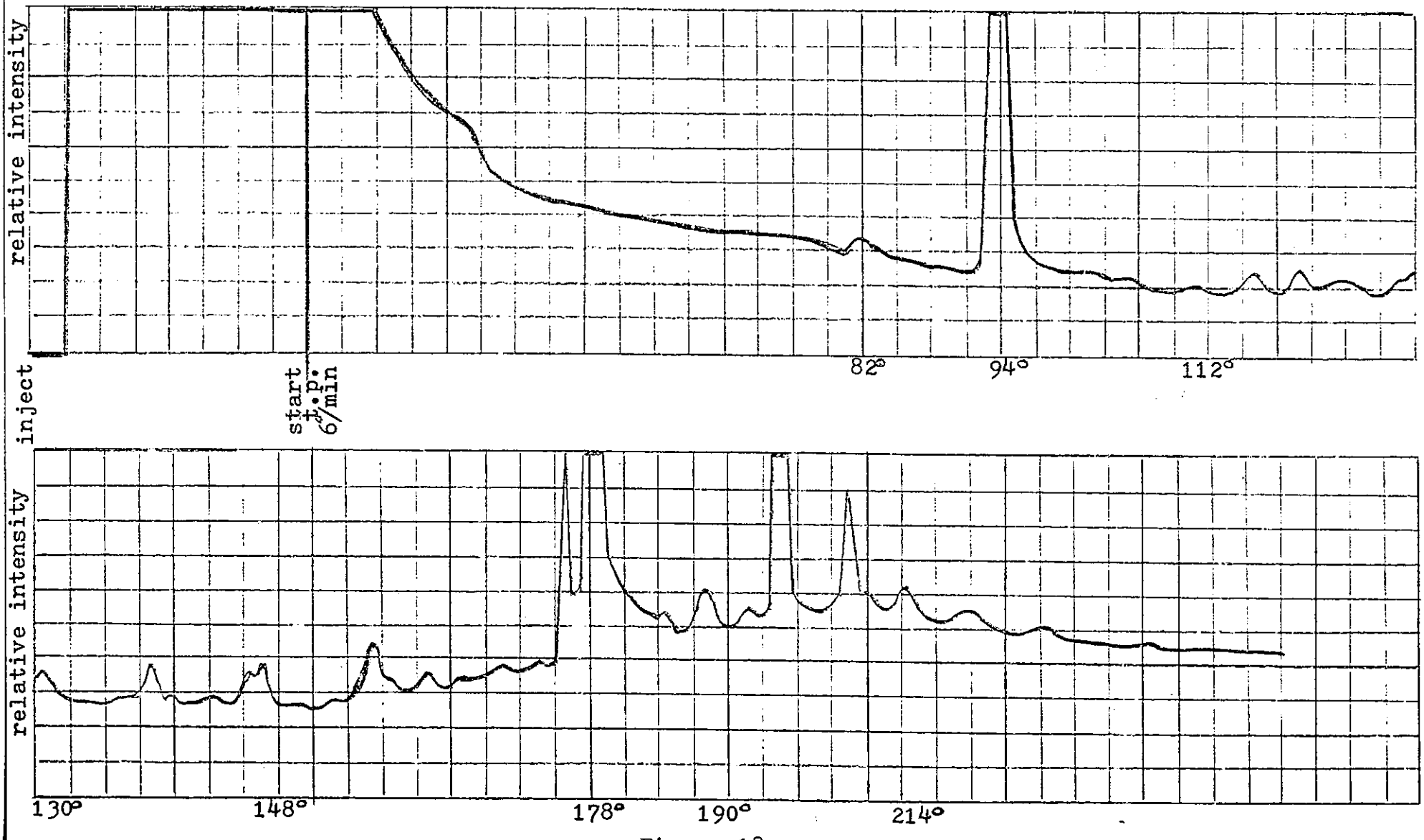


Figure 18

GC-6

183-6 Fraction in Methylene Chloride on DEGS Column

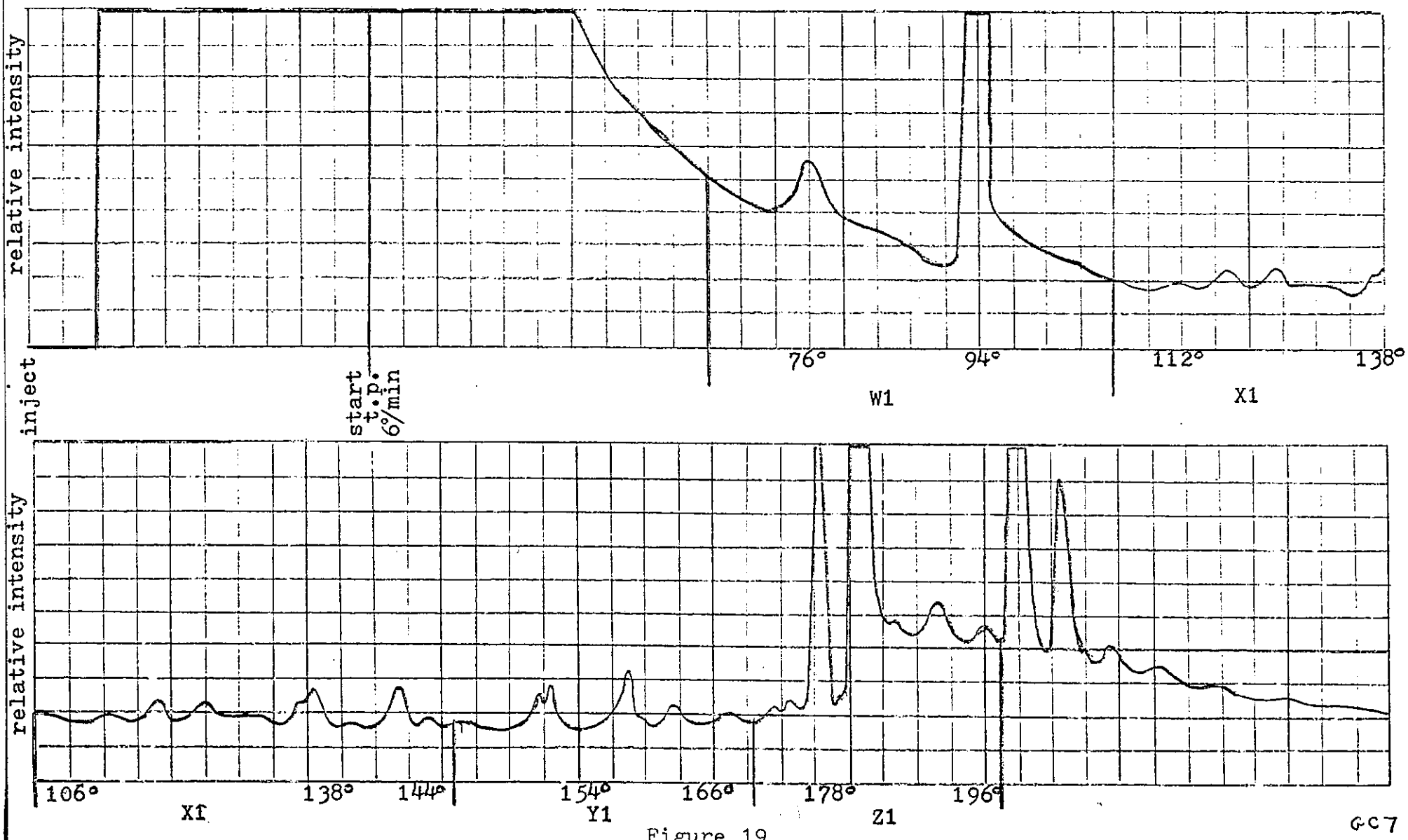


Figure 19

183-6 Fraction .. Hexane on DEGS Column

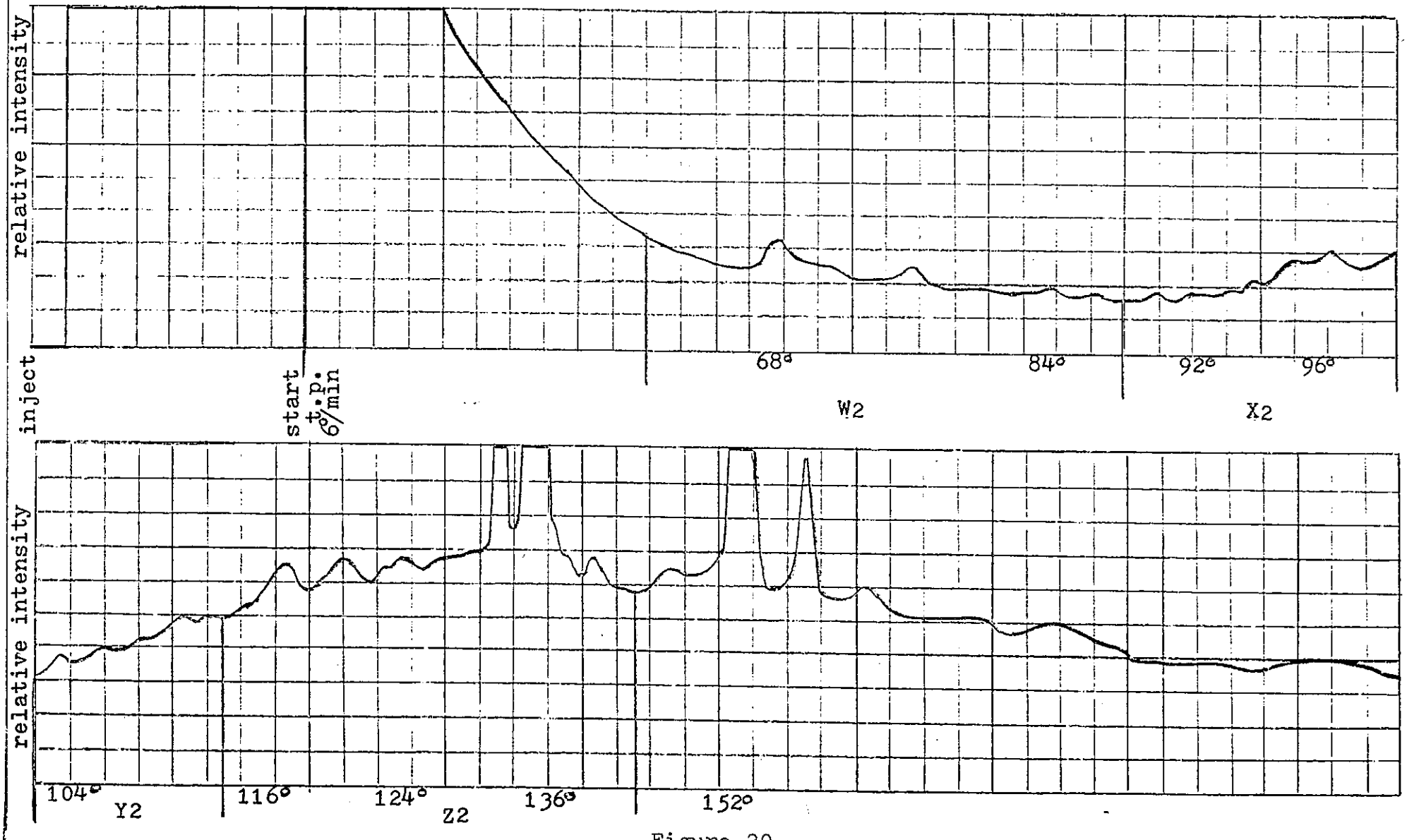


Figure 20

to offset the change in retention times. The following cuts were made: W2 (60°-88°), X2 (88°-104°), Y2 (104°-116°), and Z2 (116°-144°). Three sets of collections were made and labelled: 1W2,..., 1Z2, 2W2,..., 2Z2, 3W2,..., 3Z2.

I returned to Holy Cross with the capillary tubes and promptly placed them in the refrigerator. As soon as the female flesh flies turned three-days-old, bioassays were begun on each of the fractions.

The first bioassay[36] used the 1A1 capillary tube (the unpurified 183-6 sample collected between 48° and 57° from the OV-17 column). The two ends were broken off and the tube was washed with methylene chloride (100 µl). This was injected into the +1 flask depicted in Figure 5. An equivalent amount of pure methylene chloride was injected into the other flask and the air flow was set at about 125 ml/min. Fifteen female flies were introduced into the 0 end of the Y-tube. The number of flies in each section of the Y-tube was recorded five, fifteen and thirty minutes from the start of the test. The very surprising results of the first bioassay were:

	1A1				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	0	4	0	6	-1.0
15 min	7	1	4	0	3	+4.5
30 min	8	1	3	0	3	+5.5

There seemed to be definite activity in this fraction.

Cut 1B1 was assayed next in a similar fashion and gave the following:

---

[36]This and all subsequent bioassays were done by Robin Vita.

	1B1 +1	+½	0	-½	Blank -1	Total
5 min	3	1	8	0	3	+.5
15 min	5	0	6	0	4	+1.0
30 min	6	1	5	0	3	+3.5

This appeared to be attractive to the female flesh flies although the slow response and the large number of zeroes clouded the reliability of this bioassay.

The third cut in this set of collections, 1C1 (62°-80°), was tested in the third bioassay. This cut was not well received by the female flies:

	1C1 +1	+½	0	-½	Blank -1	Total
5 min	5	0	5	0	5	0
15 min	6	0	4	0	5	+1.0
30 min	4	0	5	0	6	-1.0

The fourth cut, 1D1 (80°-98°), was bioassayed and seemed to slightly repel the female flesh flies:

	1D1 +1	+½	0	-½	Blank -1	Total
5 min	4	1	2	0	8	-3.5
15 min	4	1	2	0	8	-3.5
30 min	4	1	3	0	7	-2.5

The fifth cut, 1E1 (98°-112°), probably did not contain the sex pheromone:

	1E1 +1	+½	0	-½	Blank -1	Total
5 min	5	1	4	1	4	+1.0
15 min	4	1	4	2	4	- .5
30 min	3	1	5	2	4	-1.5

The last cut, 1F1 (112°-120°), may have contained a trace of some attractant:

	1F1				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	2	4	0	4	+2.0
15 min	5	3	4	1	2	+4.0
30 min	5	2	4	2	2	+3.0

Of the six cuts, the first one - collected between 48° and 57° - most likely contained the major component of the sex pheromone. The slight activity of 1B1 might be another attractive compound or it might be due to some "overlap" from cut 1A1. Both cuts 1C1 and 1D1, one of which should have corresponded to the active region found by Budris, appeared inactive (or repulsive). The large number of zeroes in the 1F1 bioassay might imply that the activity is not "real". Cuts 2A1 and 3A1 were used to test the reproducibility of the results from 1A1. The first bioassay, with 2A1, was not very encouraging - its results seemed to indicate that no attractant was present:

	2A1				Blank	
	+1	+½	0	-½	-1	Total
5 min	0	0	15	0	0	0
15 min	1	2	10	2	0	+1.0
30 min	2	2	6	2	3	-.5

It is quite possible that no pheromone was collected in 2A1 - either because none came off the G.C. at the proper time or because the collecting apparatus failed to condense it inside the capillary tube as it came off the column. The flesh flies may not have been good either: there were a large number of zeroes.

Cut 3A1 worked much better:

	3A1				Blank	
	+1	+½	0	-½	-1	Total
5 min	6	2	2	0	5	+1.0
15 min	9	1	2	0	3	+6.5

The bioassay using 2B1 failed to confirm the results obtained with 1B1:

	2B1				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	1	5	1	3	+2.0
15 min	4	2	4	2	3	+1.0

The third cut of the series (2C1), however, did show some activity: after fifteen minutes, there were seven females in the +1 section, two others on the +½ side, four remained in the 0 section, and two were in the -1 section (Total = +6.0). So 3C1 was opened and bioassayed. The female flies were strongly repelled by the substance at first but gradually approached it:

	3C1				Blank	
	+1	+½	0	-½	-1	Total
5 min	1	1	5	0	8	-6.5
15 min	2	0	4	0	9	-7.0
25 min	4	1	4	0	6	-1.5

It is possible that there was too much of the attractive compound present in 3C1 and actually repelled the females. The drop in the Total might have been the result of a decrease in the concentration of the active component through evaporation. Had the test run longer, it might have shown a net positive response.

Since there were no more A1 or C1 tubes left for testing (4A1 and 4C1 were saved for analysis with the G.C.-Mass Spectrometer). Robin tried bioassaying the next best thing: the A2 and C2 cuts from the purified 183-6 since they were collected at nearly the same time as A1 and C1. The first cut bioassayed, 1A2, attracted slightly more

female flies than did the methylene chloride blank:

	1A2				Blank	
	+1	+½	0	-½	-1	Total
5 min	4	0	0	4	7	-3.0
15 min	2	3	0	5	5	-4.0
40 min	8	0	2	0	5	+3.0

The bioassay of 2A2 was run only 15 minutes and gave:

	2A2				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	1	6	0	3	+2.5
15 min	4	2	6	0	3	+2.0

Finally, the last bioassay with the purified 183-6 cuts was done with 1C2. The fifteen minute bioassay did not provide evidence of an attractant in this cut:

	1C2				Blank	
	+1	+½	0	-½	-1	Total
5 min	3	2	1	3	6	-3.5
15 min	3	0	4	3	5	-3.5

Only one test was done using a cut from the DEGS column. Cut 1W1 was bioassayed with fifteen female flies. The results of the fifteen minute bioassay were:

	1W1				Blank	
	+1	+½	0	-½	-1	Total
5 min	3	1	6	1	4	-1.0
15 min	6	1	4	1	3	+3.0

The primary component of the sex pheromone, if it was collected, probably was collected in the first fraction (48°-57°) coming off the G.C. with the OV-17 column. Activity had not been found in this region when J. Budris had tried it a couple of years earlier so we

were quite surprised. The "C" cuts, which might contain the active peak which John Budris had found, might contain a secondary or minor component of the sex pheromone since the one bioassay with 2Cl attracted a good number of the female flesh flies.

Since the A cuts contained only one large peak, it was thought that the use of the G.C.-Mass Spectrometer at Pennsylvania State University would aid in the identification of the active compound in A. So I returned to Pennsylvania State University with capillary tubes 4Al and 4Cl and about 10 ml of the unpurified 183-6 extract.

When I arrived at Pennsylvania State University, the OV-17 column which I had used during my previous stay there was found and baked overnight at 250°C. The next morning it was installed inside the G.C.-Mass Spectrometer.

There is one major problem associated with G.C.-Mass Spectrometry work: to operate the Mass Spectrometer, the pressure in the M.S. ionizer must be very low (below 10^{-4} Torr). The solvent peak, because it is so large and broad, usually will send the pressure way above the 10^{-4} Torr limit so it must be diverted away from the Mass Spectrometer and G.C. recorder until it drops to an acceptable level. This means that any compounds coming off under or very close to the solvent peak will probably be lost.

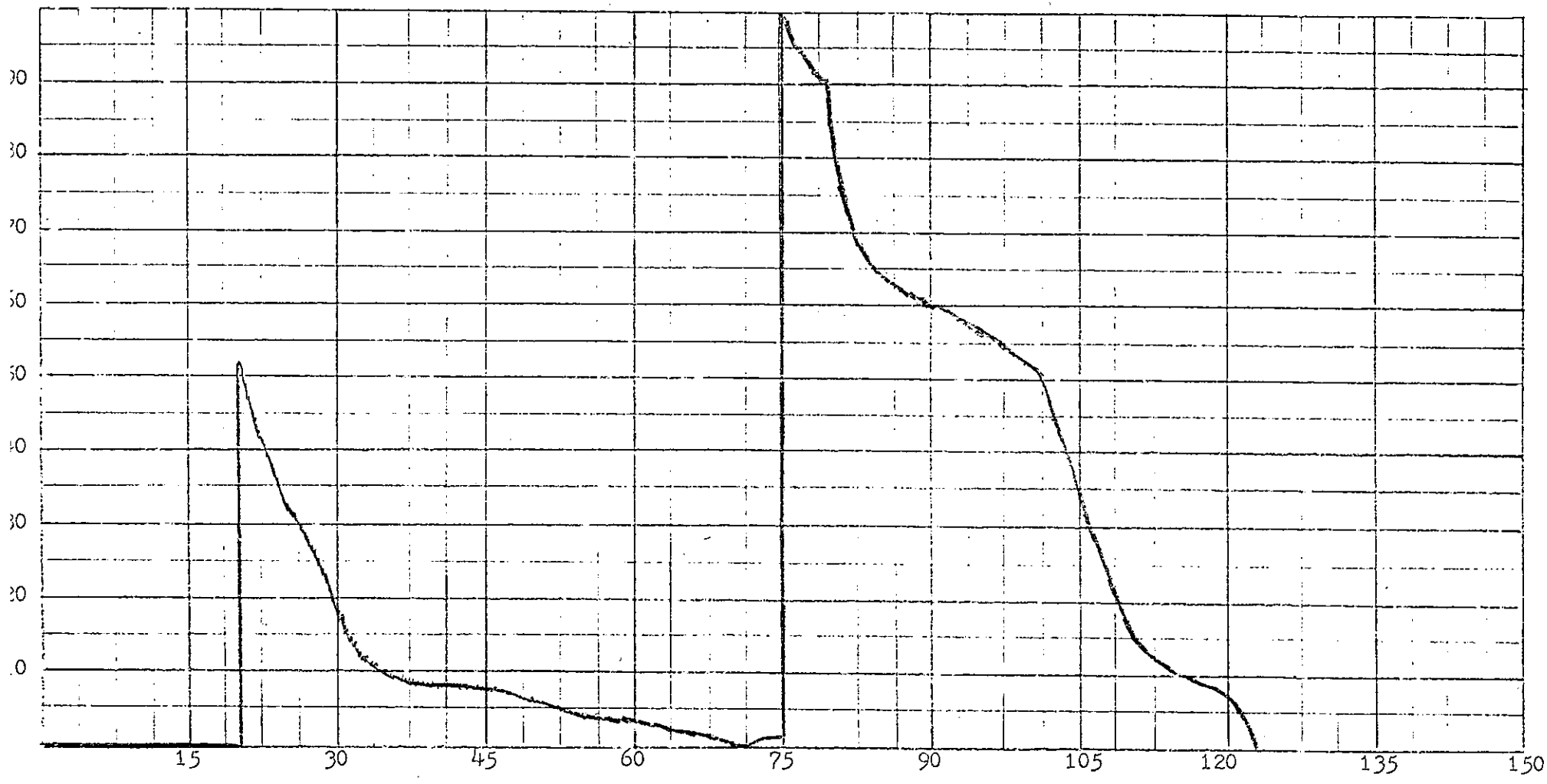
Once the G.C.-M.S. was ready, I broke open the 4Al capillary tube and washed it with 10 µl of methylene chloride. This then was injected into the G.C.-M.S. and the methylene chloride peak was diverted until it was at an acceptable level. However, no peak was observed coming off the G.C. between 48°-57° although the sensitivity was set at 1×10^{-11} and the temperature program rate was 4°/min. In

fact, no peaks appeared at all. The 4C1 cut did not yield any peaks either when it was injected into the G.C.-M.S..

Fortunately, I had about 10 ml of the unpurified 183-6 extract. Using dry nitrogen gas, I reduced this to about 1 ml. Five μl of this concentrate was injected into the G.C.-M.S. The methylene chloride had to be diverted but a large peak did appear between 40°-62° (see Figure 21). The region between 20 and 60 in Figure 21 is the tail end of the methylene chloride peak while the large peak between 75 and 120 (the tail end of the peak is exaggerated greatly) is, in all probability, the peak captured in the A1 cuts. The numbers in Figure 21 do not refer to temperature - they refer to Mass Spectra number. For example, to get the Mass Spectra of the tip of the peak, we could instruct the computer part of the G.C.-M.S. to print out Mass Spectra of #76 or #77 since the peak reaches its maximum near there. If we wanted to eliminate the "background" compounds, we could have the computer subtract the portion immediately preceding the peak under consideration from the peak itself and then print out the result.

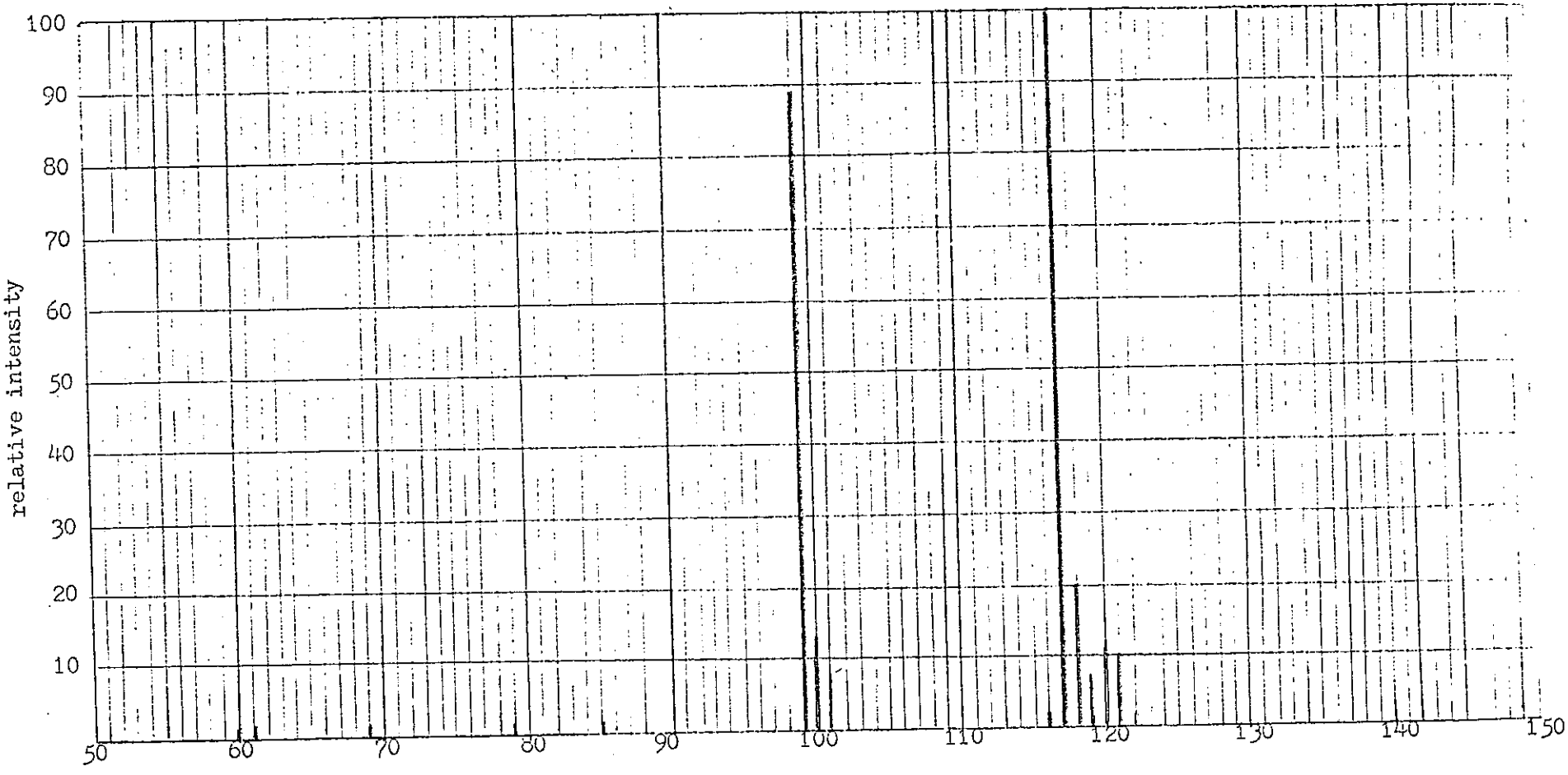
This was done with the one large peak depicted in Figure 21. Unfortunately, the peak was probably not pure, and the Mass Spectra suggested that two or more substances were coming off the OV-17 column at nearly the same time (see Figures 22, 23, 24). All three Spectra were very similar: large peaks at m/e 99 and 117, and smaller, but significant peaks at m/e 100,101, 118, 119, 120, and 121. In Spectra #88 (Figure 23), there were also two small peaks at m/e 155 and 163. Since Chemical Ionization Mass Spectrometry was used, all peaks are 1 amu heavier than they would appear if Electron Impact Mass Spectrometry had been employed.

Second Penn State Trip



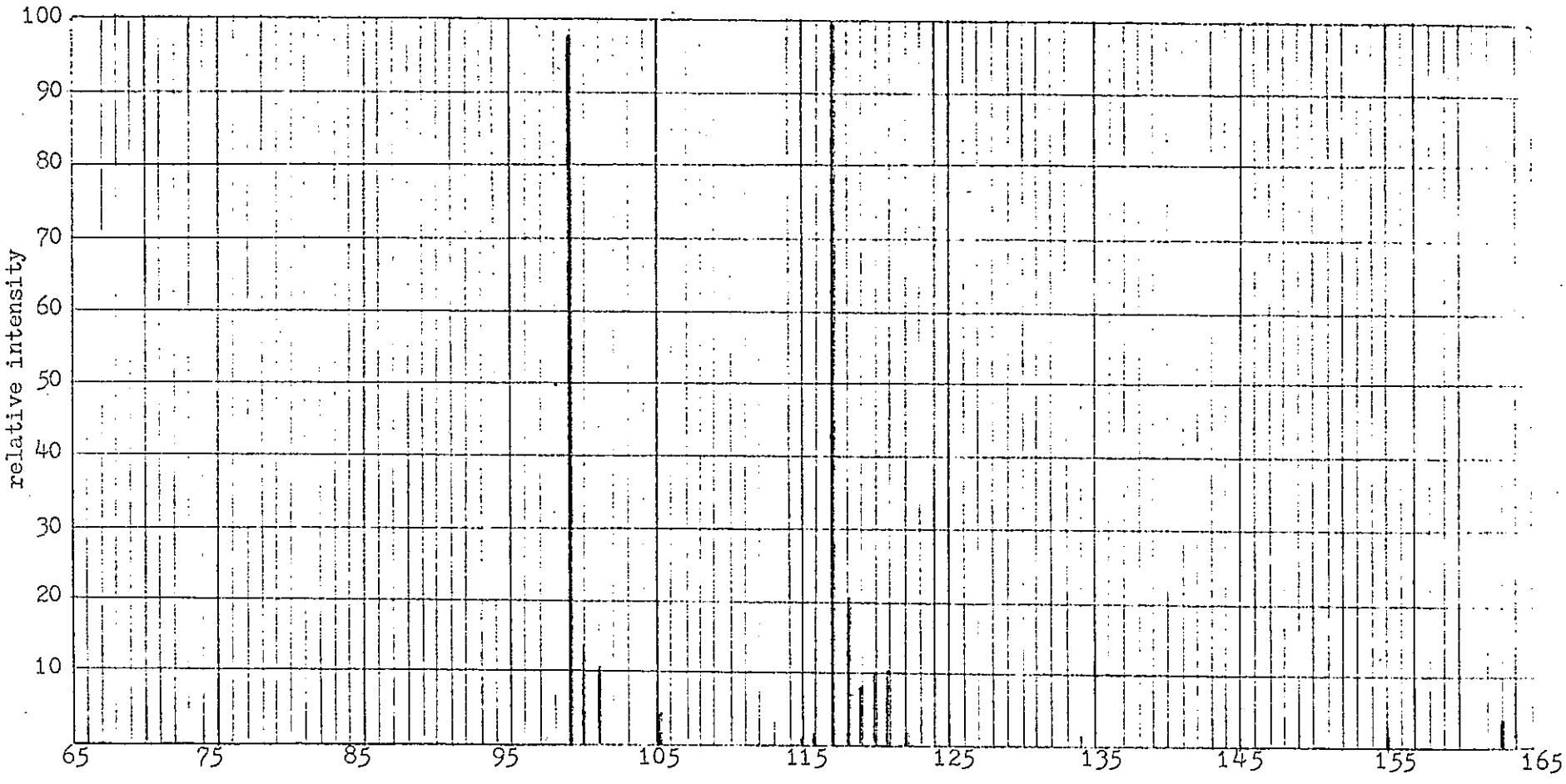
Fraction "A" on GC-MS

Figure 21



Spec #87 (CI)

Figure 22



Spec #88 (CI)

Figure 23

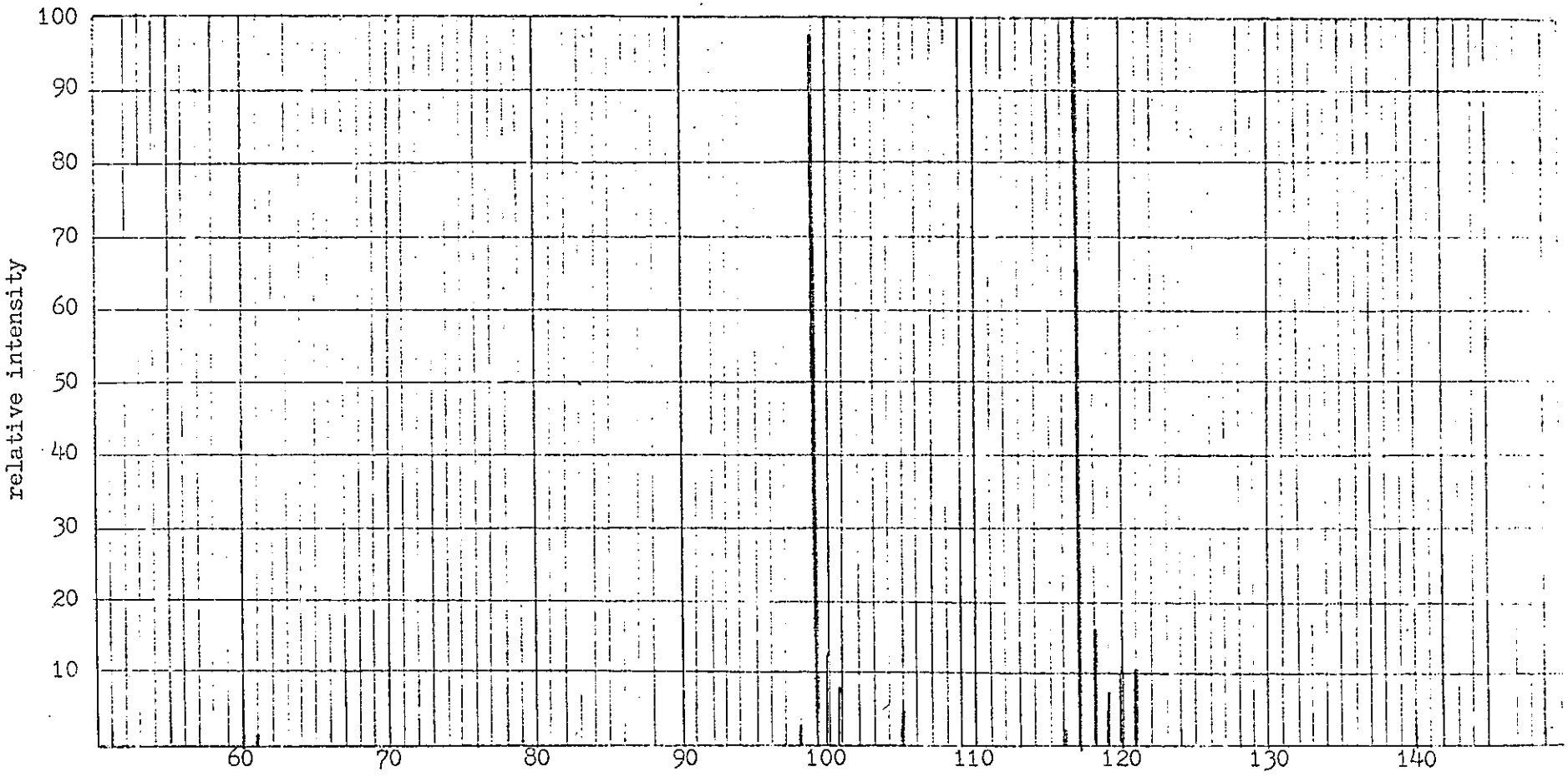

100
90
80
70
60
50
40
30
20
10
relative intensity
60
70
80
90
100
110
120
130
140
mass/charge
Spec #89 (CI)

Figure 24

Since CI M.S. usually yields a noticeable parent ion peak, it seemed likely that the molecular weight of the active compound (or one of the active compounds) fell between 98 amu and 120 amu but, unless the compounds under the peak could be separated and EI M.S. performed, little else could be learned about the structure of the active compound.

When I arrived back at Holy Cross, I went through all the Mass Spectral data which Dr. Girard had recorded during his summer visit to Pennsylvania State University. However, since it was thought at the time that the pheromone came off the OV-17 column much later, the Mass Spectra for the early peaks were not recorded and few similarities between the two sets of data could be found.

Earlier work had established that the active compound was very volatile. Assuming that the pheromone molecule possessed an oxygen atom - nearly all pheromones do - and had a molecular weight of 98, the weight of the molecule without its oxygen atom would be 82 amu, or the equivalent of C_6H_{10}. Cyclohexanone and any unsaturated straight chain aldehyde or ketone with six carbon atoms would have a molecular weight of 98 amu. All would be fairly volatile.

Cyclohexanone is a very common chemical in the laboratory and for that reasons was singled out for a bioassay. When I mixed a drop of cyclohexanone and a ml of methylene chloride together and injected seven microliters of this into the G.C., I obtained the trace shown in Figure 25. Cyclohexanone seemed to have remained in the column a little too long (it comes off between 56° and 60°) to have been the sex pheromone. It was bioassayed anyway. Four hundred fifty nanograms (10^{-9}g) of cyclohexanone dissolved in 100 μl were used in the bioassay

Cyclohexanone in Methylene Chloride

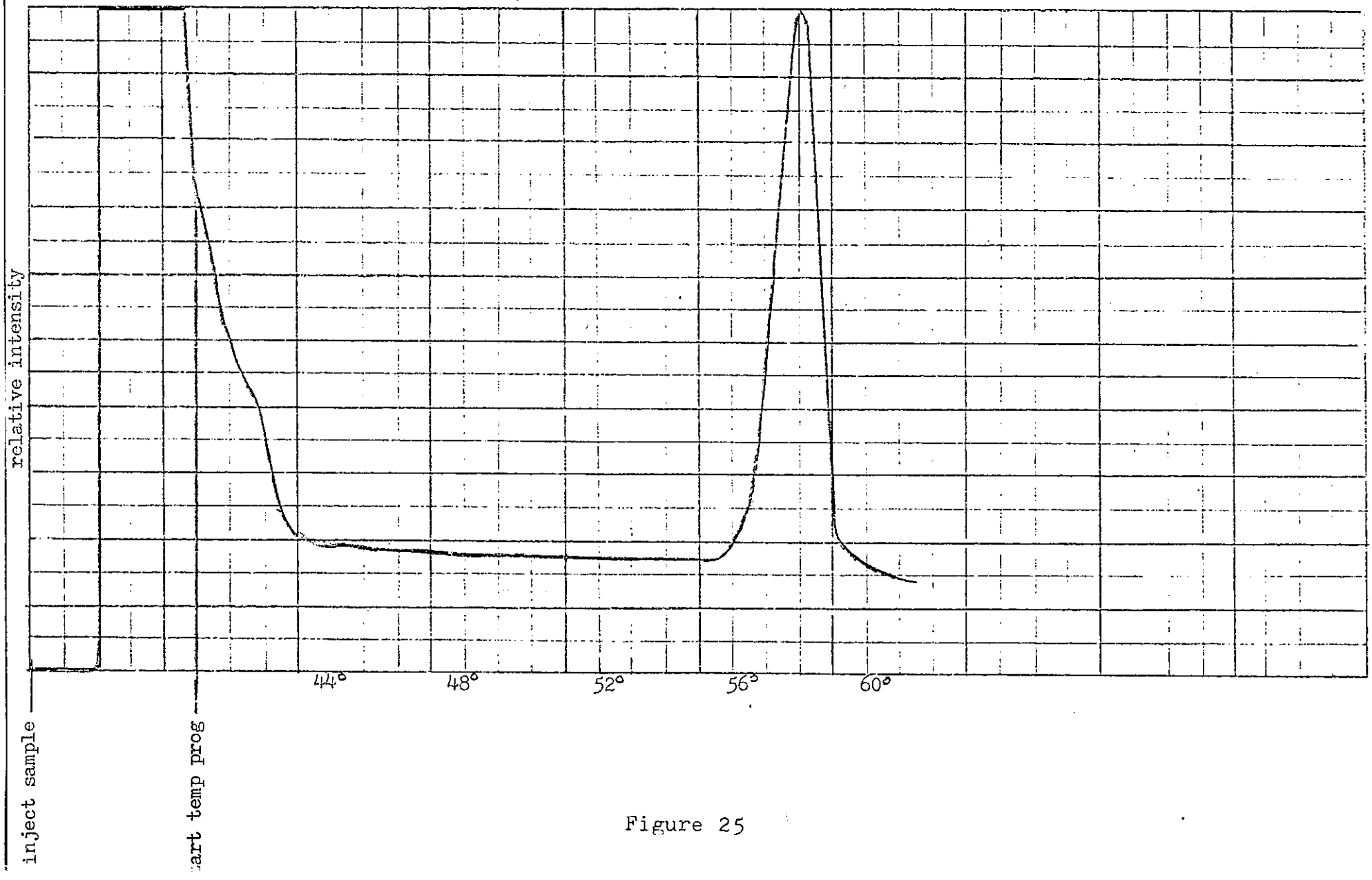


Figure 25

with a methylene chloride blank. After 15 minutes, the results were: two female flesh flies in the +1 (cyclohexanone) section, one female in the $+\frac{1}{2}$ section, two females in the 0 arm, one female in the $-\frac{1}{2}$ section, and five females in the -1 section for a total response of -3.0. Cyclohexanone was probably not the sex pheromone and something else had to be done.

That something else was the use of another, more polar column. Increasing the polarity of the G.C. column should slow the passage of the sex pheromone through the G.C. column if the sex pheromone were polar. A 10% carbowax column was chosen to replace the OV-17 column because it was substantially more polar than the OV-17 material. As with the DEGS columns, "bleeding" is a problem at higher oven temperature with carbowax columns and the maximum oven temperature had to be limited to 200° C. The 10% carbowax column was activated prior to its use.

Reproducibility was initially poor with the 10% carbowax column: peaks would often appear, disappear, then reappear on successive injections of the unpurified 183-6 fraction. Finally, after about ten injections, the column settled down and gave excellent traces with 10 µl injections (see Figures 26 and 27). Notice that there are two peaks before the solvent peak and several peaks before 60°.

After baking the column for several hours, I decreased the temperature program dial to 4°/min and injected seven µl of the unpurified 183-6 into the G.C. The first small peak appeared at 48° C. (see Figure 28a) while a much larger peak comes off the column three degrees later. Another small peak-large peak combination came off the column between 70° and 73°. See Figures 28b and 28c for the rest of this trace. Since there were no large peaks at 73° with the OV-17 column, I had

183-6 Fraction in Methylene Chloride on 10% Carbowax Column

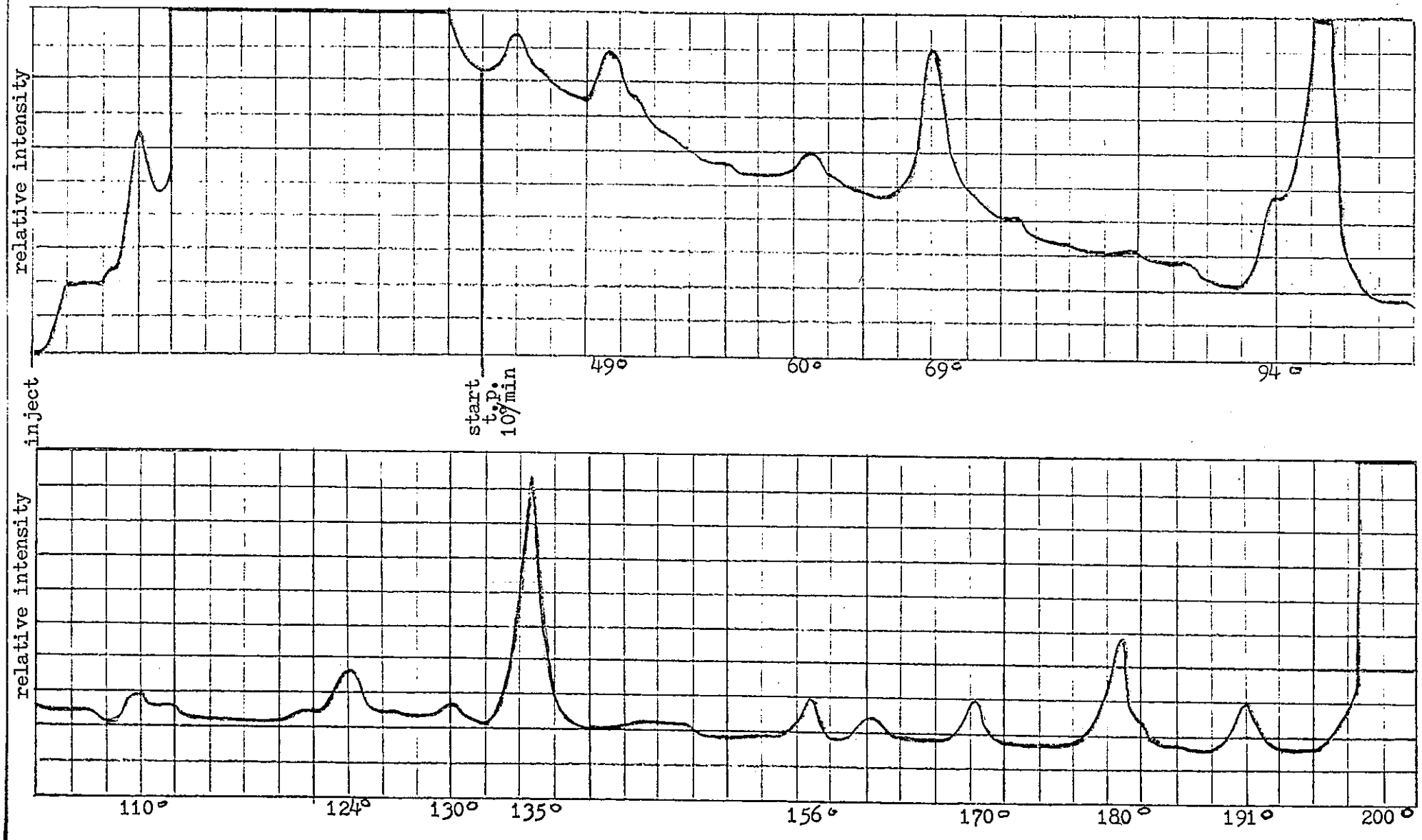


Figure 26

Fraction 183-6 in Methylene Chloride on 10% Carbowax Column

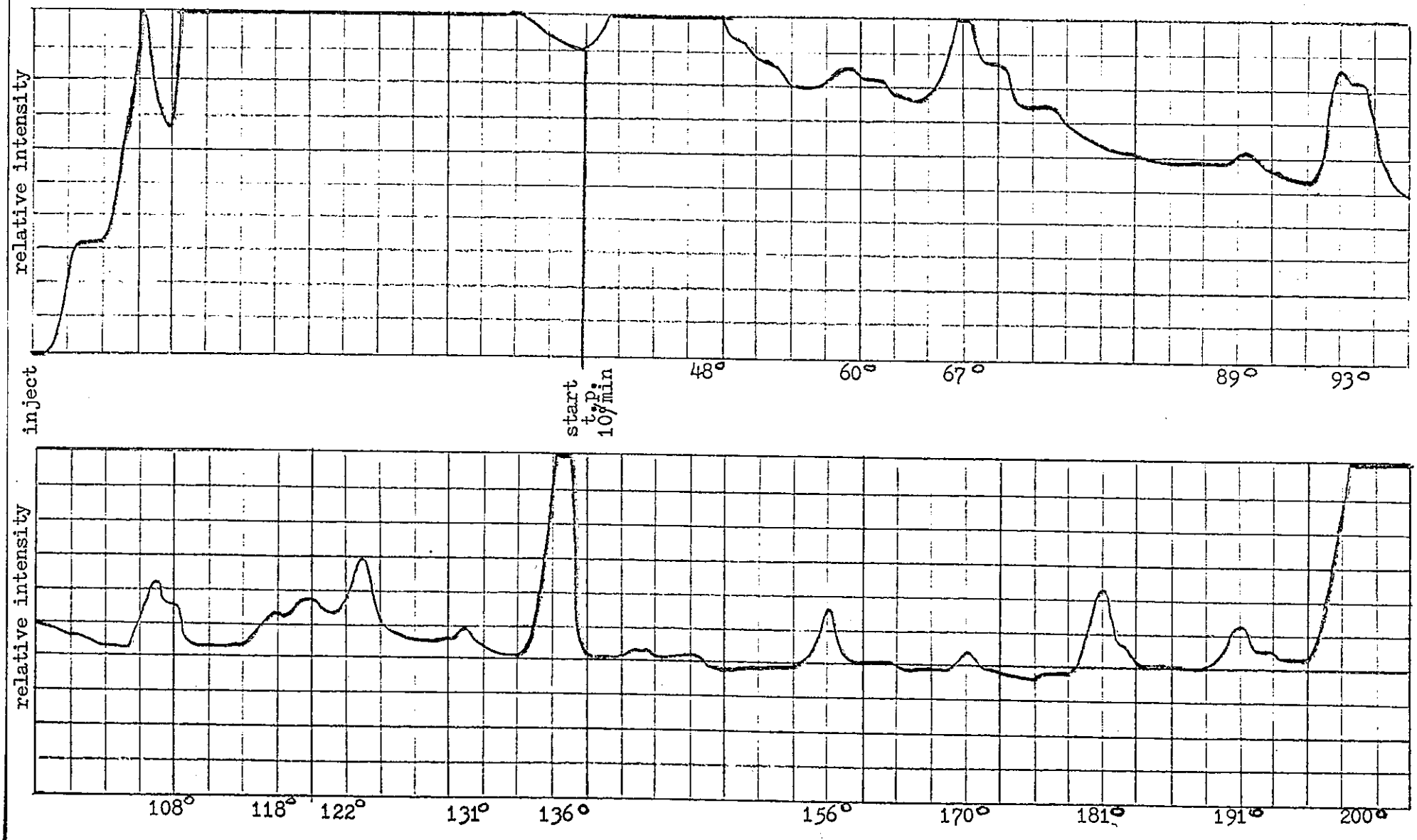


Figure 27

183-6 Fraction in Methylene Chloride on 10% Carbowax Column

Part 1

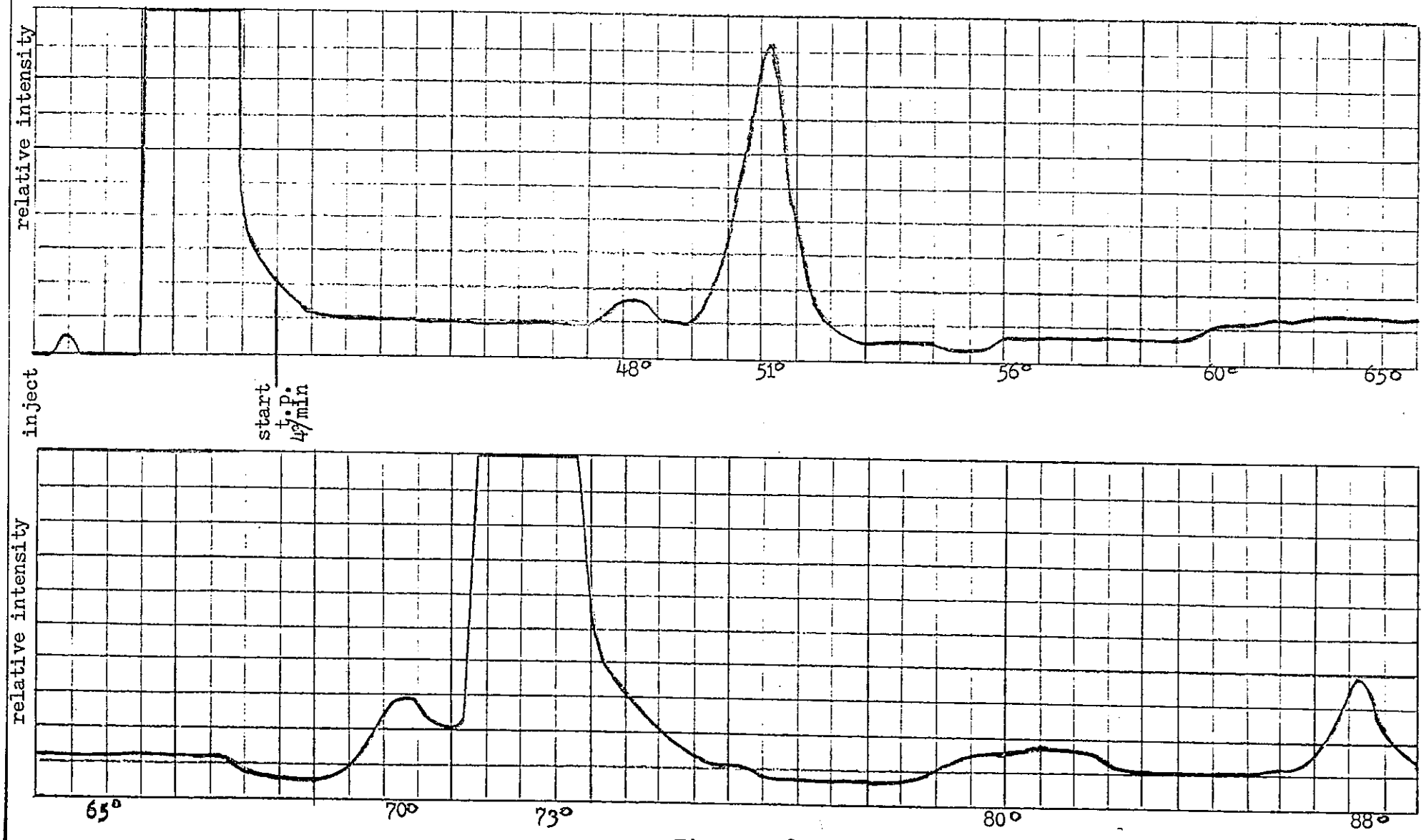
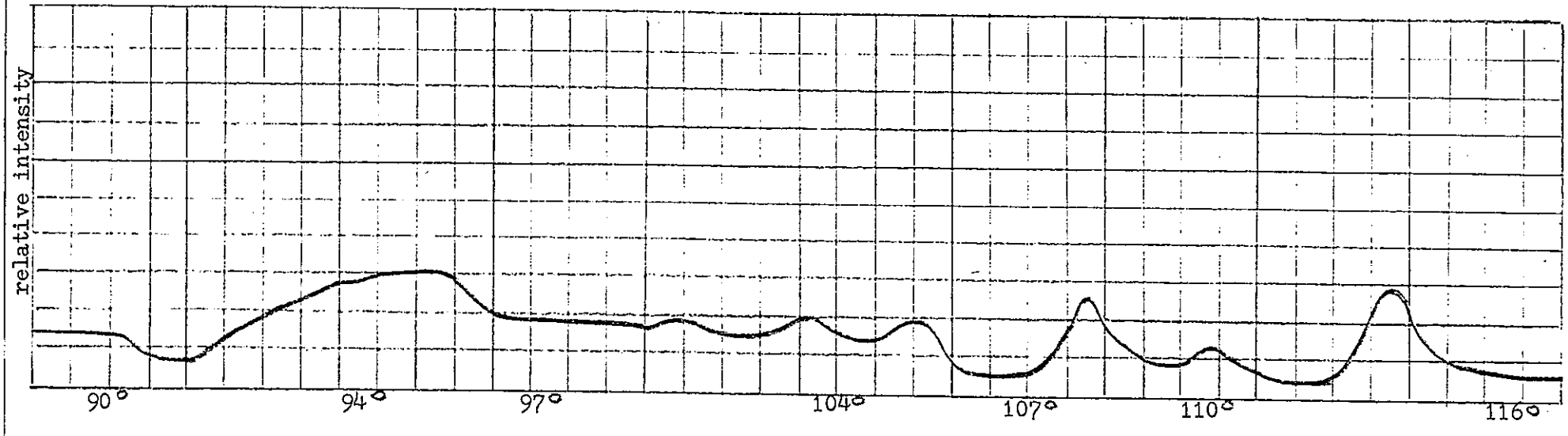


Figure 28a

183-6 Fraction in Methylene Chloride on 10% Carbowax Column

Part 2



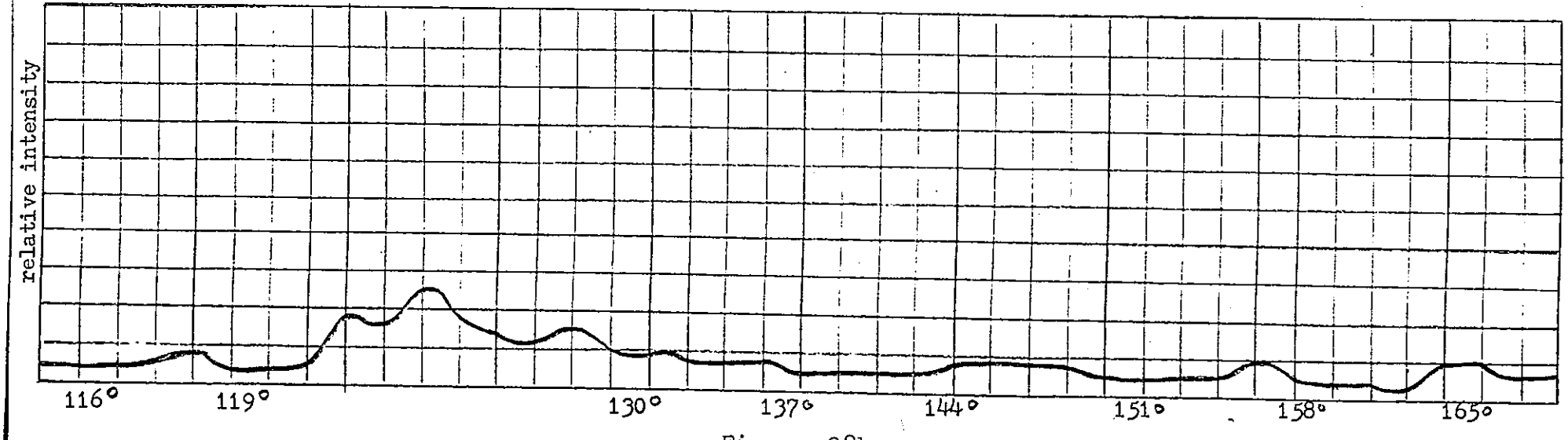
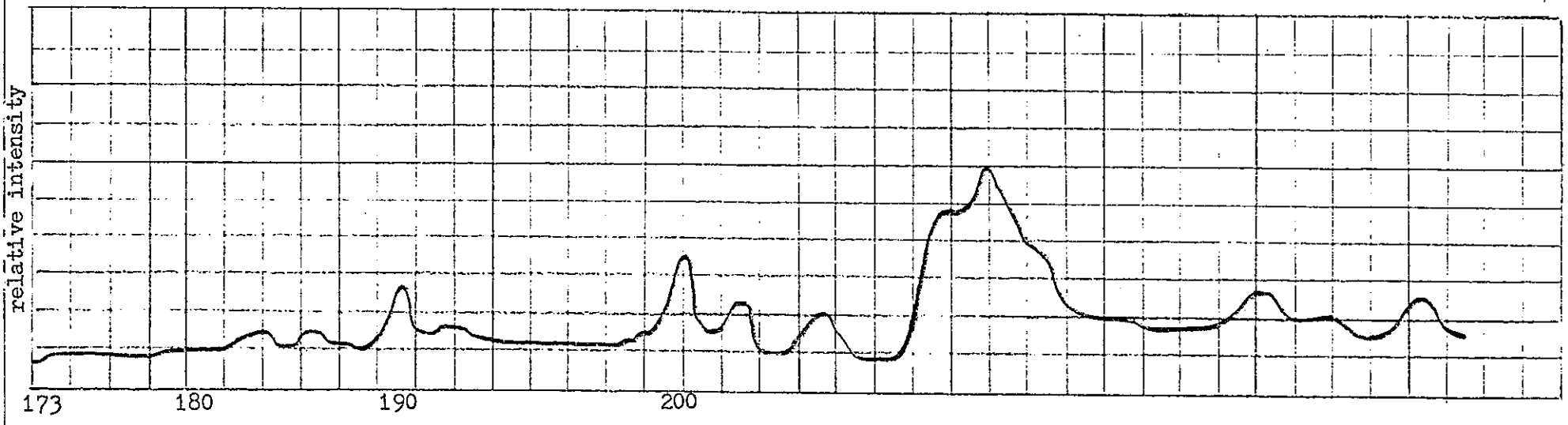


Figure 28b

183-6 Fraction in Methylene Chloride on 10% Carbowax C6lumn

Part 3



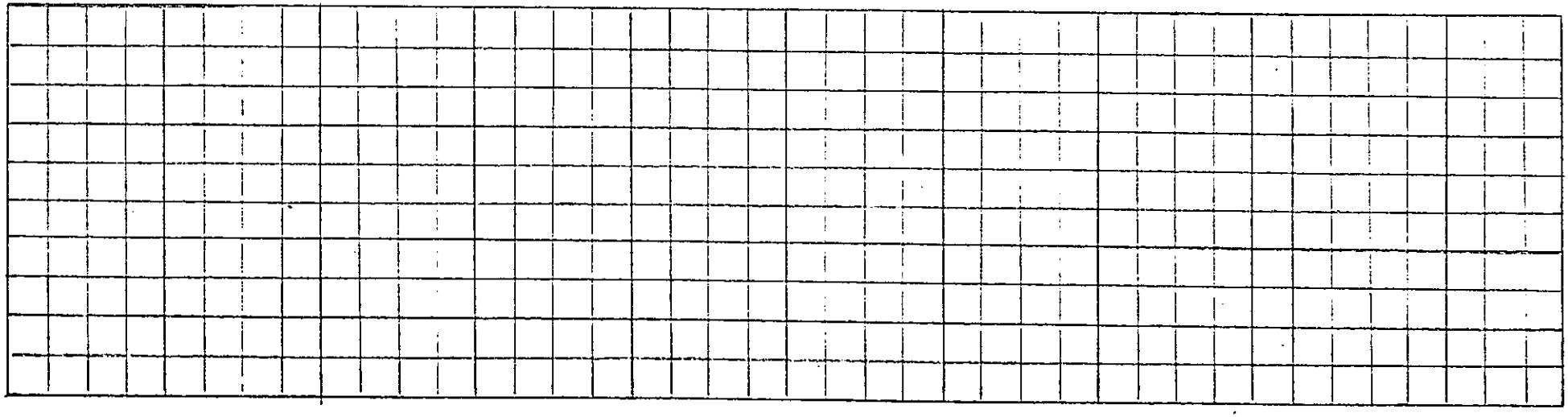
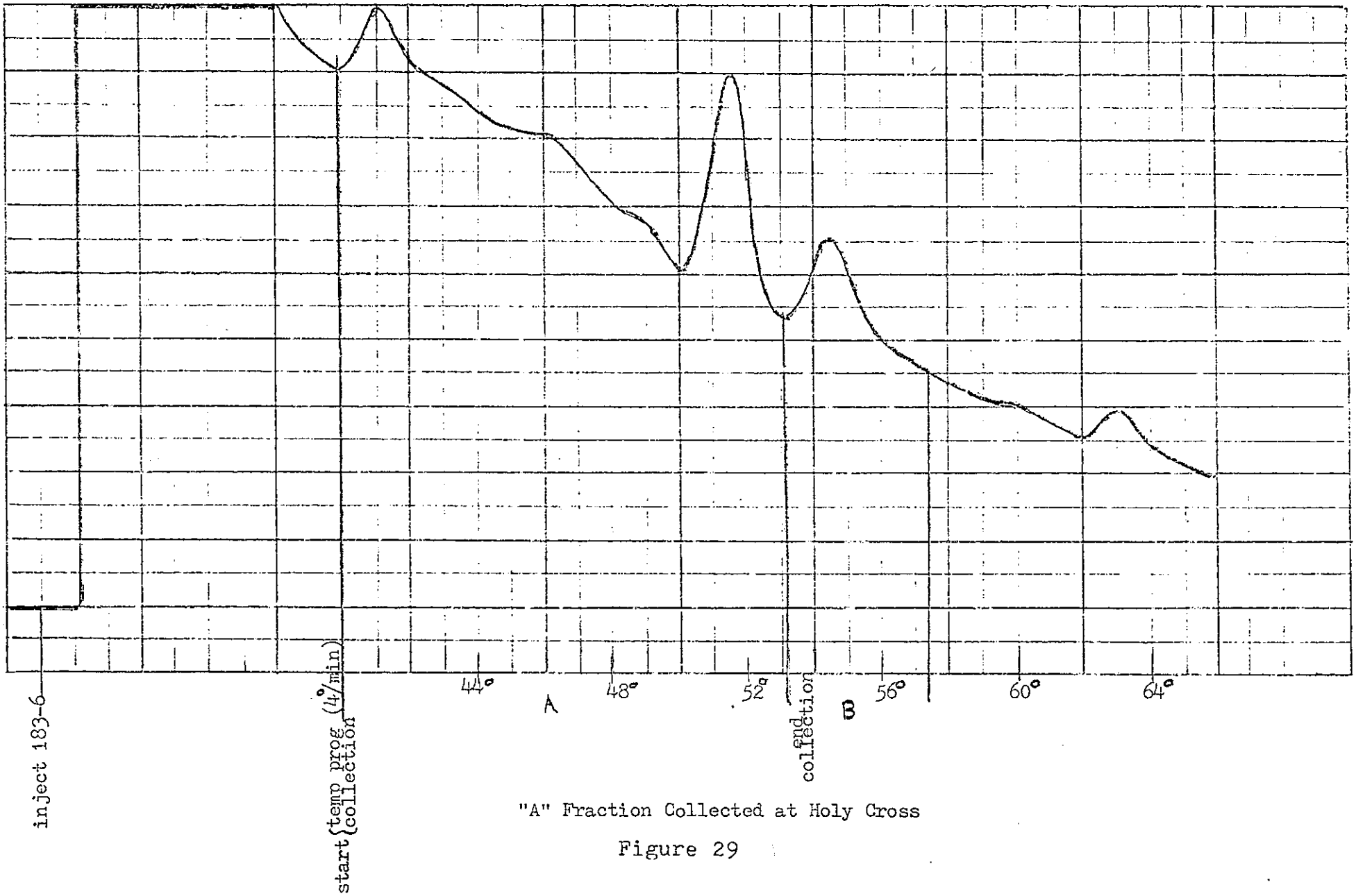


Figure 28c

hoped that this peak was one of the compounds formerly under the peak at 51°. The only way to tell was to collect several samples of the active peak off the OV-17 column and then to inject it into the 10% carbowax column.

The OV-17 column went back into the G.C. But there was a problem - the collecting apparatus was not good enough to capture the first peak coming off the column and had to be changed. With the help of Joe MacDonald, the tip of the collector on the G.C. was removed and replaced with a smaller metal tip. This switch enabled me to use smaller capillary tubes for collecting the peaks. The split ratio, which controls the amount of material that goes to the detector and the collector, was increased to 100-to-1, sending 100 times as much of the effluent gases to the collector as to the detector. The dry ice was not cold enough to quickly condense the material coming off the G.C. and, as a result, much of it escaped. It had to be changed. Joe MacDonald helped me fashion a liquid nitrogen cooled apparatus similar to the one at Pennsylvania State University. Collection of the 48°-57° region could then begin.

The active peak, if it was not under the methylene chloride peak, would probably come off the G.C. somewhere between 40° and 60°. When the unpurified 183-6 was injected into the G.C. fitted with the OV-17 column, a rather large peak appeared at about 51° and a slightly smaller one appeared at about 54° (see Figure 29). I decided to collect two cuts: one between 40° and 52°, and the other between 52° and 57°. Ten collections were made for each cut. One capillary tube containing cut A was opened, rinsed with 15 μl of methylene chloride and injected back into the G.C. Its trace is shown in Figure 30. The peak between 53° and 57° does not appear, indicating that the collection separated



"A" Fraction Collected at Holy Cross

Figure 29

chart speed 1 in/min

Re-injected "A" Fraction of 183-6 Collected at Holy Cross

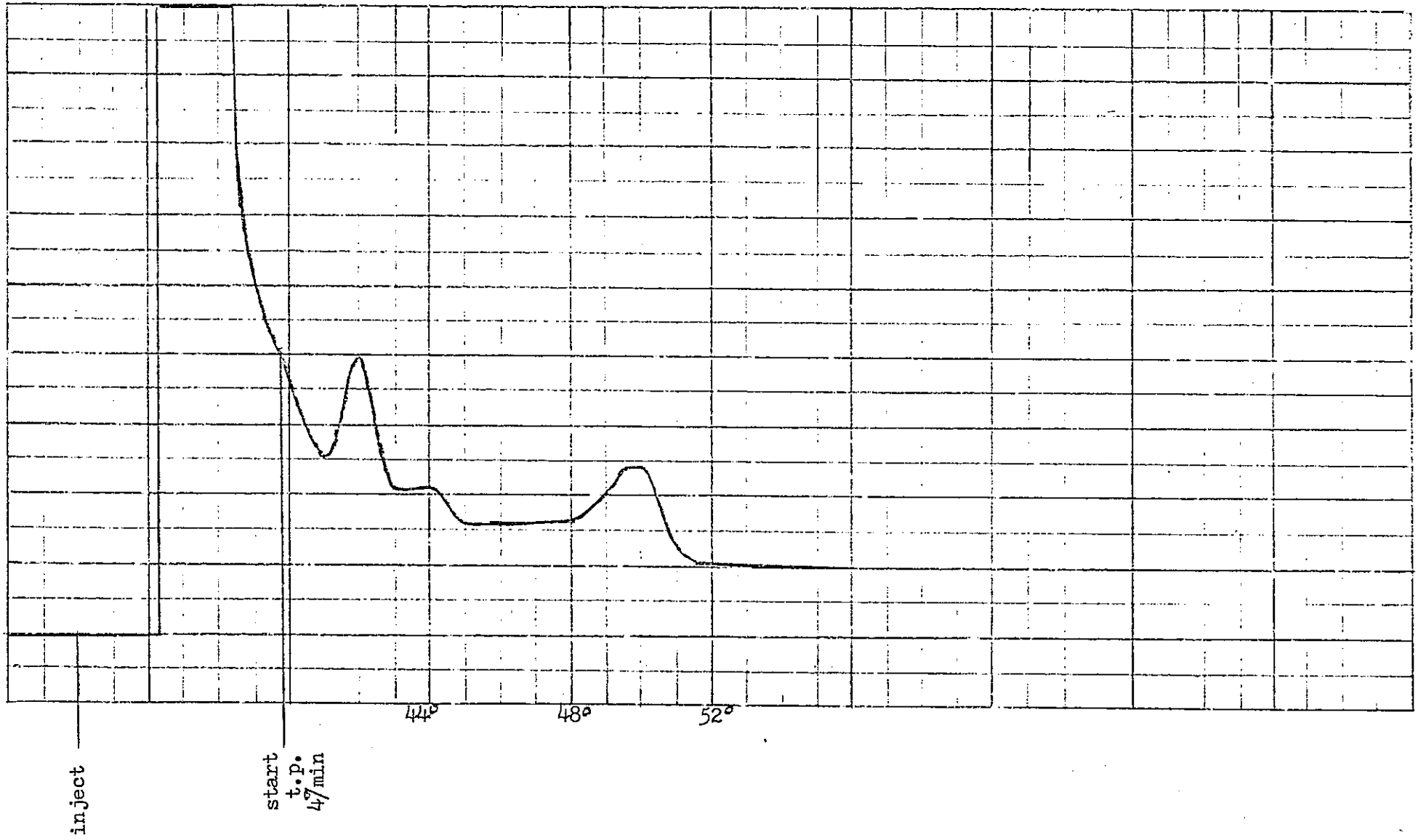


Figure 30

these two peaks fairly well.

The critical test was the bioassay - would the female flesh flies be attracted to either of these two cuts? Cut A was bioassayed first. For each bioassay, the capillary tube was rinsed with about 30 μl of methylene chloride and shot onto a piece of filter paper. An equivalent amount of pure methylene chloride was placed on another piece of filter paper. One of the pieces of filter paper was put in the flask on the right arm of the Y-tube, the other was placed in the flask on the other side. The air flow was set at about 125 ml/min.

The first bioassay with cut A showed some activity:

	A				Blank	
	+1	+½	0	-½	-1	Total
5 min	9	0	2	0	4	+5.0
15 min	6	0	3	0	6	0

as did the second:

	A				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	1	3	0	3	+2.5
15 min	7	0	2	0	3	+4.0

The third bioassay with cut A gave results very similar to the first one:

	A				Blank	
	+1	+½	0	-½	-1	Total
5 min	7	2	2	0	3	+5.0
15 min	5	2	2	2	3	+2.0

Cut B was then bioassayed. The first three bioassays seemed to rule out the possibility of the presence of the pheromone in cut B. All the results are after 15 min:

	B				Blank	
	+1	+½	0	-½	-1	Total
Bioassay #1	6	1	5	0	3	+3.5
#2	4	2	4	2	3	+1.0
#3	5	3	1	0	6	+.5

The fourth bioassay with B, however, showed some activity:

	B				Blank	
	+1	+½	0	-½	-1	Total
5 min	8	0	2	0	7	+1.0
15 min	10	0	2	0	5	+5.0

Since the other cuts of B were inactive and time was running out, I decided to concentrate on collecting the A peak and to ignore peak B.

To determine whether or not the 10% carbowax column could separate the compounds under peak A, I installed the column in the G.C. The five remaining 'A' capillary tubes were washed with 10 µl of pure methylene chloride and injected into the G.C. The trace showed a double peak at 45° and a single peak at 60°, along with numerous small peaks scattered about the 122°-200° region. Although the 10% carbowax column did appear to separate, at least partially, the compounds under peak A, the collecting efficiency was so low (about 6%) that it would be nearly impossible to collect significant amounts of each peak from injections with the previously collected A cuts. The use of the unpurifi 183-6 fraction would have given larger peaks for collections but also would have created some additional problems so I decided to drop the 10% carbowax column and return to the OV-17 column.

With the OV-17 column back in place, I resumed collecting the A peak. However, to increase the concentration of the pheromone in the 183-6 sample, I took 15 ml of the unpurified 183-6 and reduced it to a volume of 1 ml with the aid of a nitrogen gas tank. Any further

reduction in the sample volume would cause several compounds to precipitate out of solution.

Forty-eight collections of this A peak were made. In many of the capillary tubes, a tiny dot, consisting of some yellow-brown viscous material, was visible to the naked eye. The sealed capillary tubes were stored in the freezer.

Once all 48 collections were finished, it was time for another trip - this time to the General Electric Research Labs in Schenectady, New York. I brought all 48 capillary tubes with me, along with the remaining portion of the unpurified 183-6. Although it was packed carefully, the sealed ampoule containing the 183-6 broke during the trip and was empty when I arrived in Schenectady.

An OV-17 column was installed in the Varian Mat 11 G.C.-M.S. and baked at 250° for 30 minutes. One of the capillary tubes was opened and washed with about 10 μl of methylene chloride and then injected into the G.C.-M.S. As with the G.C.-M.S. at Pennsylvania State University, most of the methylene chloride had to be diverted away from the M.S. ionizer. No peaks appeared after the methylene chloride peak. When it was tried again with three capillary tubes instead of one, three peaks appeared on the G.C. trace but an examination of their Mass Spectra revealed that these peaks were caused by the packing material trapped within the capillary tubes during the collection of peak A. Peak A evidently was coming off the G.C. with the methylene chloride so something had to be done.

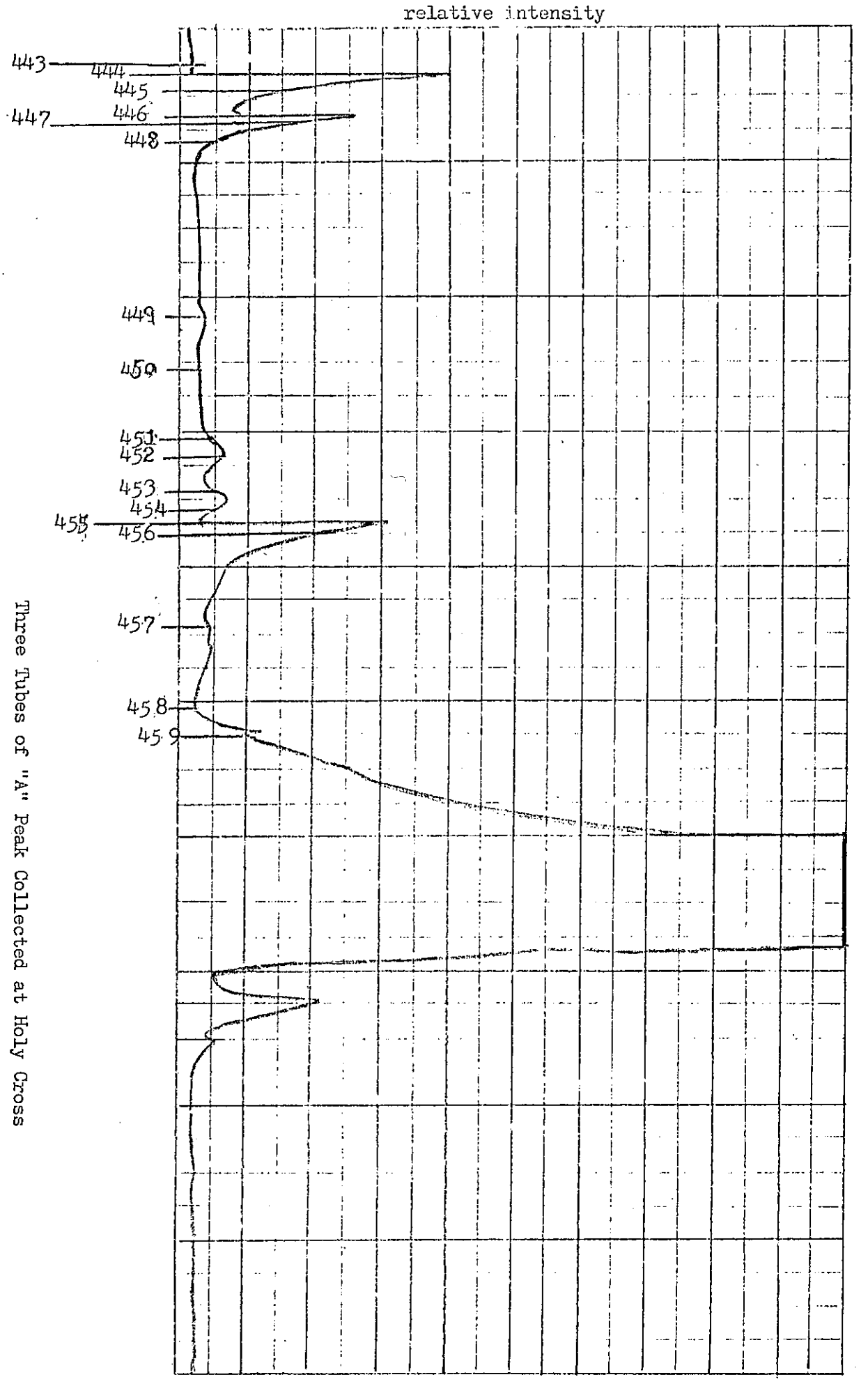
We first tried changing the column on the G.C. A special column, an Iso 180 Porapak Q, replaced the OV-17 one. This column can separate the volatile compounds which normally come off an OV-17 column near solvents like methylene chloride. Compounds usually coming off an

OV-17 column later than toluene (about 90°C.) normally get trapped within an Iso 180 Porapak Q column. It was hoped that this column would slow down the passage of the compounds under the A peak and thereby enable us to obtain their Mass Spectra. It was tried but it did not work: still no peaks after the solvent peak was diverted. The OV-17 column went back into the G.C.-M.S.

Since changing the column did not work, we then tried changing the solvent. If a solvent could be found which would come off the OV-17 column much later than methylene chloride, not only would all peaks under the solvent peak become visible but also the solvent peak would not be diverted away from the M.S. until after all the important peaks had come off. This would remove any chance that the active peak was being diverted with the methylene chloride. Dichlorobenzene seemed to come off the OV-17 column quite a bit later than methylene chloride and so was chosen.

Three capillary tubes with the A peak were rinsed with about 10 μl of dichlorobenzene and this solution was injected into the G.C.-M.S. The G.C. trace appears in Figure 31. The numbers (444, 445, etc.) refer to the Mass Spectra taken at that point.

The Mass Spectra of the first small peak (see Figure 32) suggests that it was merely an air bubble: a predominant peak at m/e 28 ($_7N_2^{14}$), smaller peaks at m/e 14 ($_7N^{14}$), 16 ($_8O^{16}$), 18 (H_2O), and 32 ($_8O_2^{16}$). The second peak also turned out to be air (see Figures 33 and 34). Mass Spectra #446 (Figure 35) also appeared to be an air bubble in the dichlorobenzene. However, the middle of that third peak (Spec #447 - Figure 36) was definitely not air. In fact, the very prominent pair of double peaks on the Mass Spectra (m/e 84, 86, and 49, 51) makes the assignment of this Spectra very easy - it is methylene chloride.



Three Tubes of "A" Peak Collected at Holy Cross

Figure 31

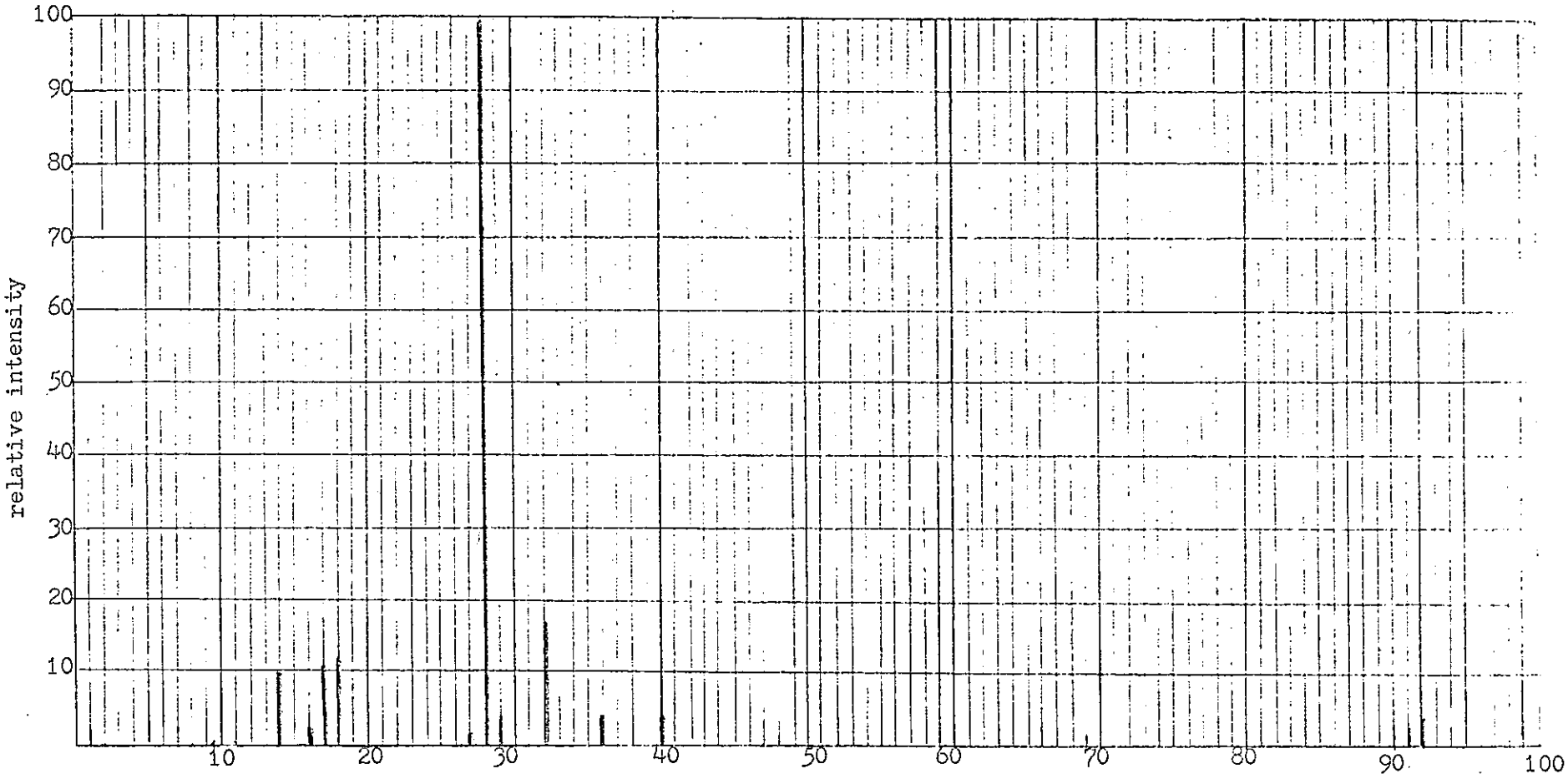
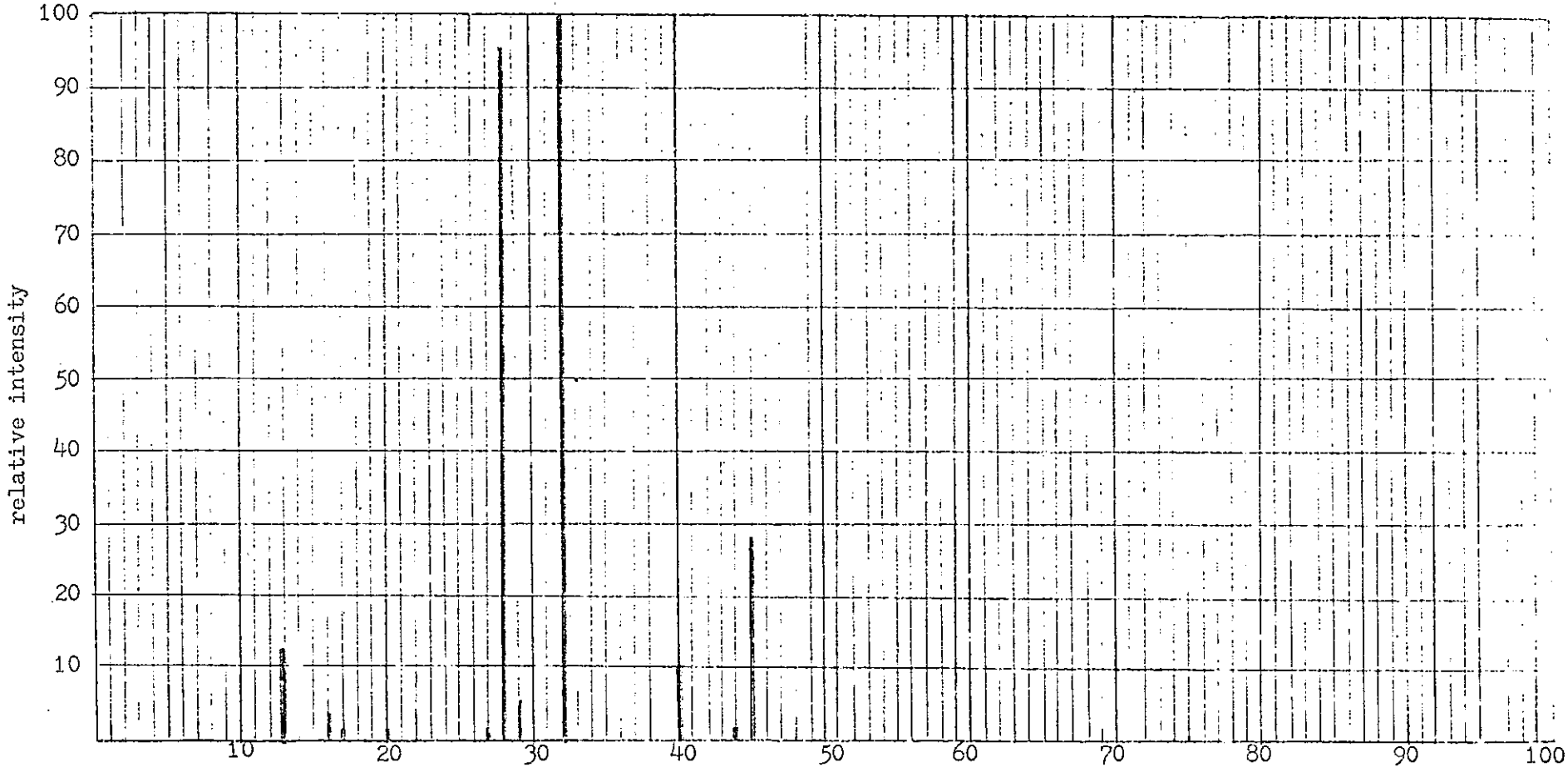


Figure 32

AIR



Spec #444 (EI)

Figure 33

AIR

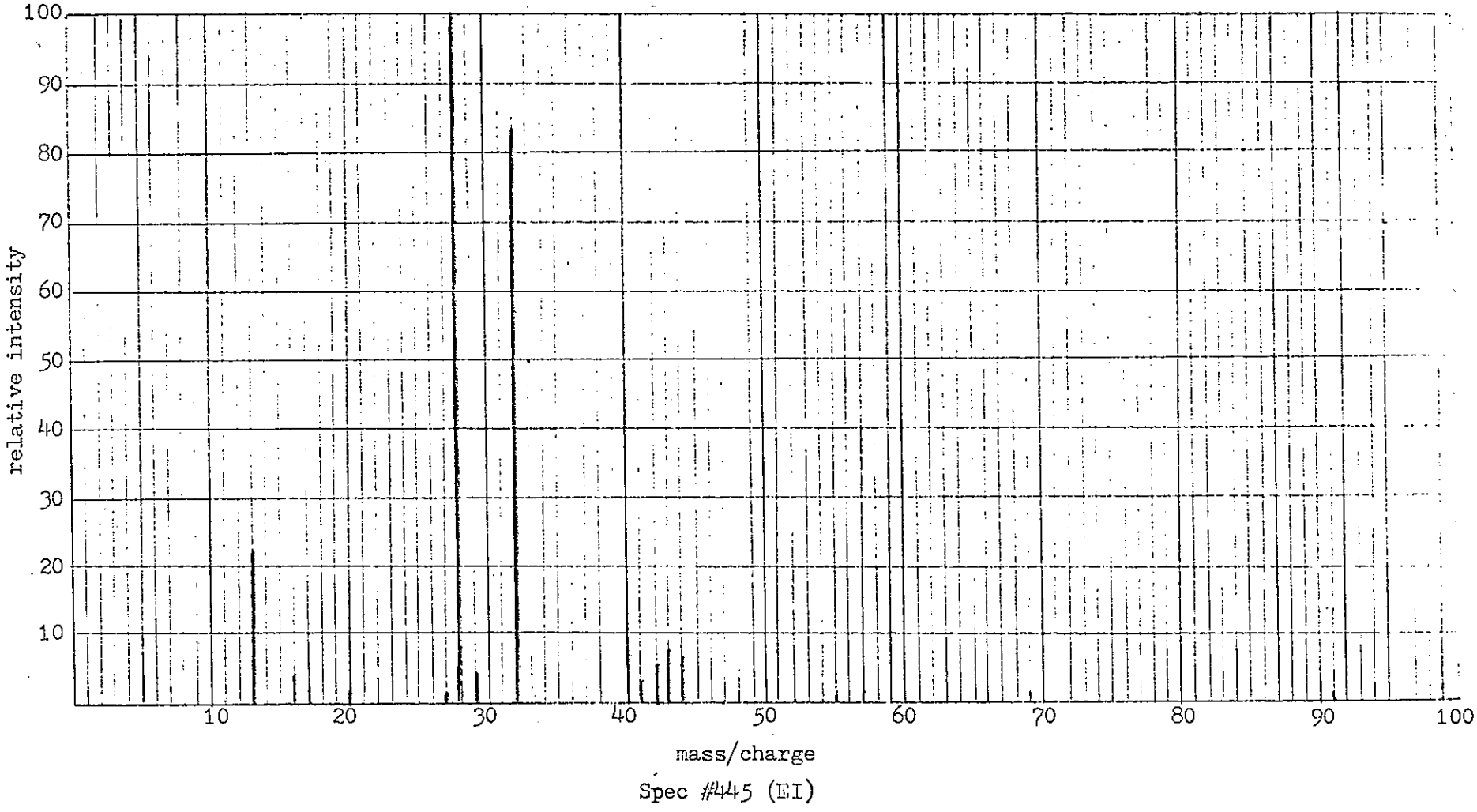

relative intensity
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
mass/charge
Spec #445 (EI)

Figure 34

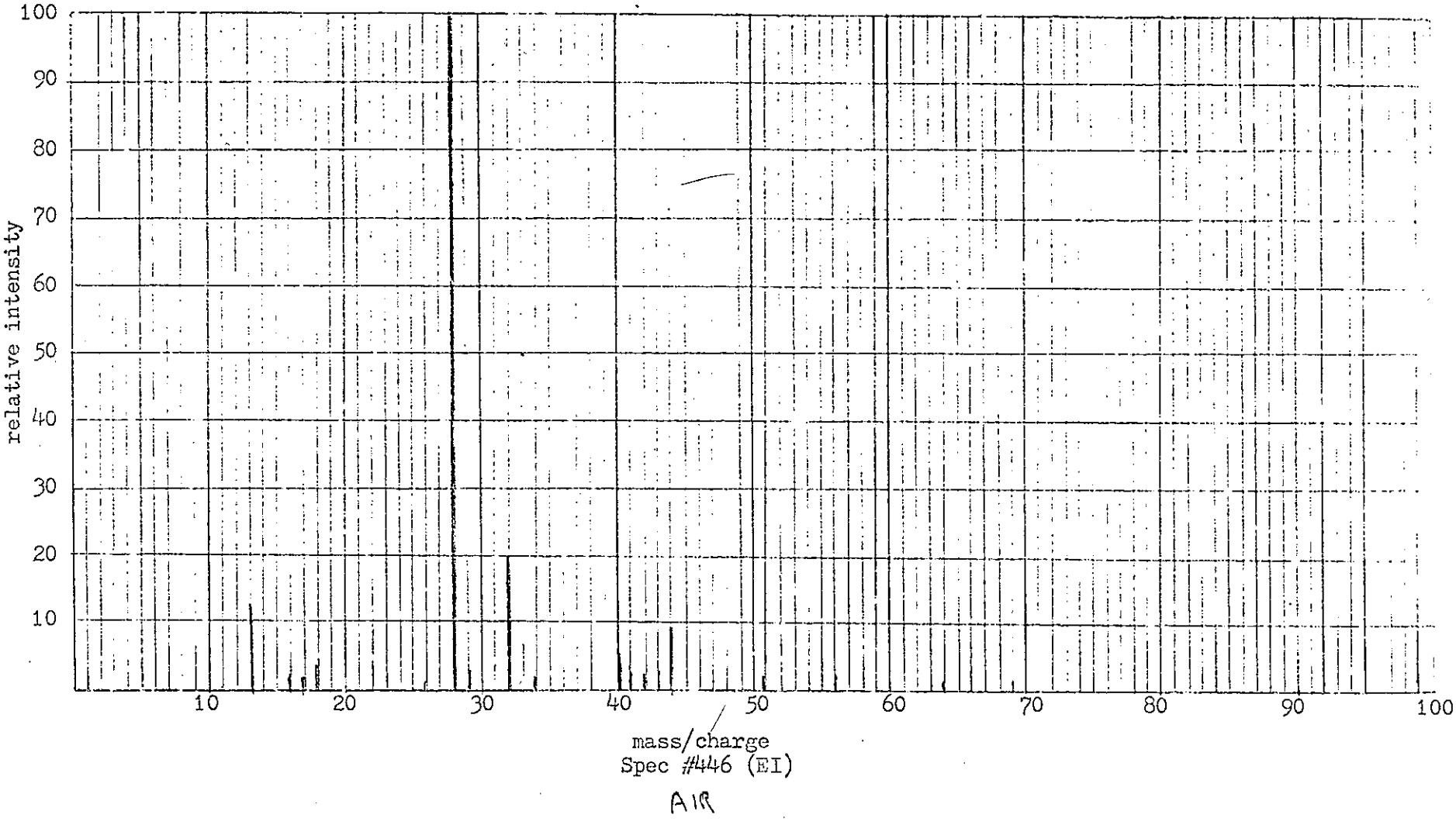

100
90
80
70
60
50
40
30
20
10
relative intensity
10
20
30
40
50
60
70
80
90
100
mass/charge
Spec #446 (EI)
AIR

Figure 35

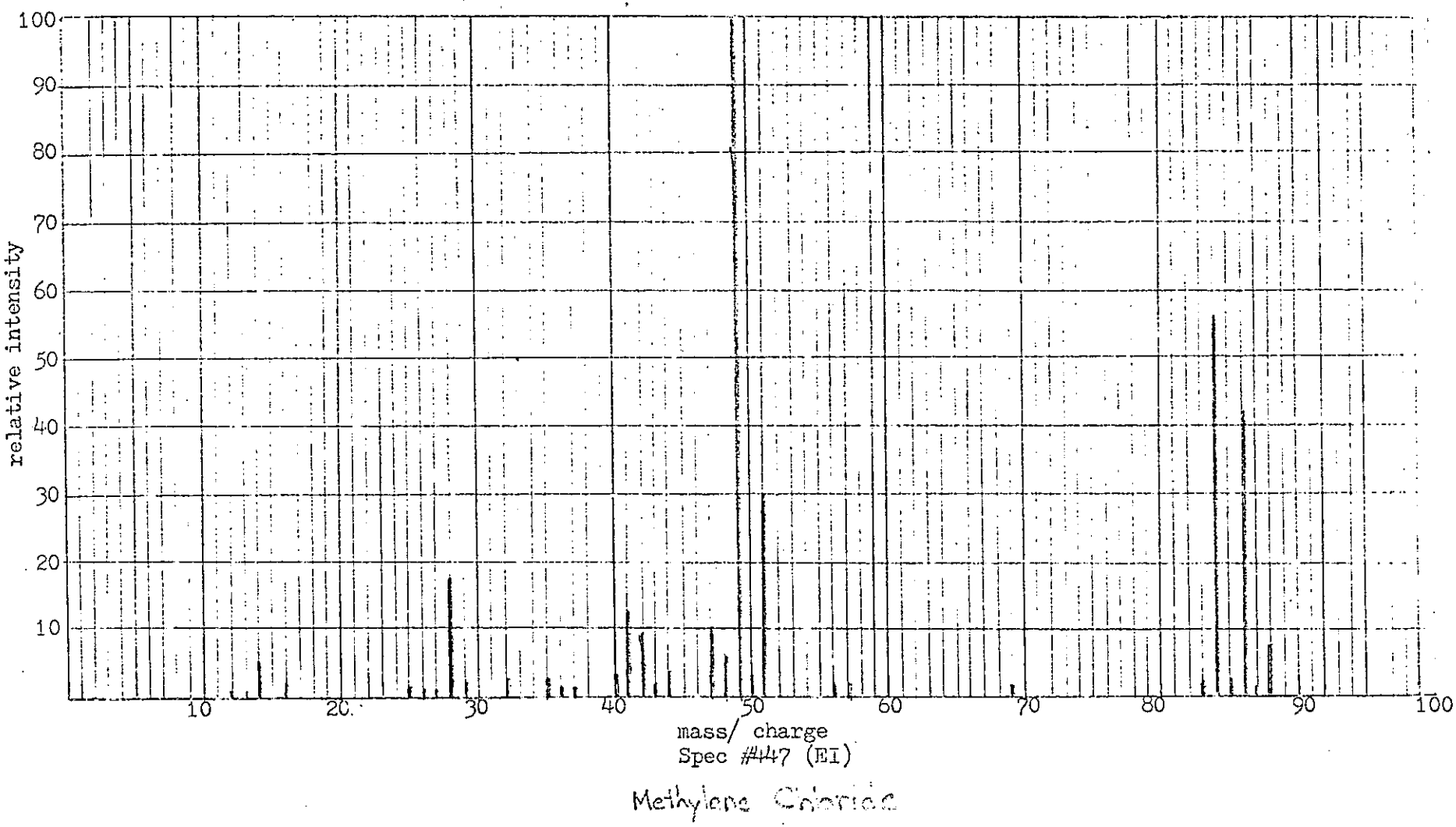
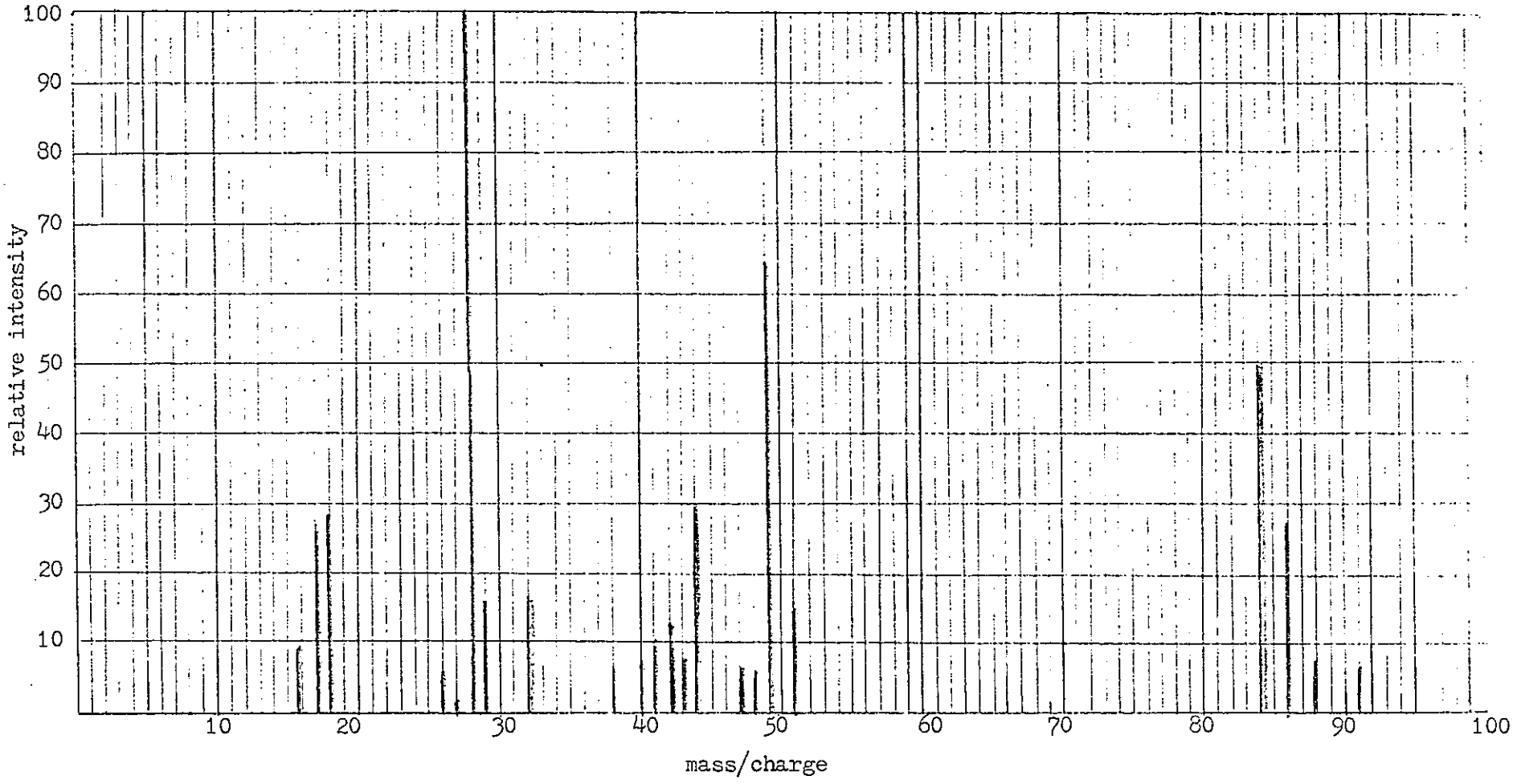

relative intensity
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
mass/ charge
Spec #447 (EI)
Methylene Chloride

Figure 36

The parent peak is at m/e 84. The peak at m/e 86 comes from methylene chloride molecules possessing one naturally-occurring $_{17}Cl^{37}$ atom. Methylene chloride molecules with a molecular weight of 84 amu can lose a $_{17}Cl^{35}$ atom to give a peak at m/e 49. Methylene chloride molecules with a molecular weight of 86 amu can lose a $_{17}Cl^{35}$ atom to give a peak at m/e 51 or can lose a $_{17}Cl^{37}$ atom to also give a peak at m/e 49. By the end of that peak (Spectra #448, Figure 37), the methylene chloride molecules were still around, as evidenced by the prominent peaks at m/e 49, 51, 84, and 86, but there was some other unidentified compound present which was responsible for the peaks on the Mass Spectra below m/e 49.

Mass Spectra #449 (Figure 38) probably does not represent a real compound. Since the Mass Spectra #450 was subtracted from all the other peaks (to eliminate "background" chemicals) and since the peak on the G.C. for #449 is only slightly above the base line of #450, the Mass Spectra of #449 and #450 were probably very similar. When the two were subtracted, it probably yielded a Spectra very unlike either #449 or #450. The base peak of Mass Spectra #449 is at m/e 16 - which is very, very imporbable for any molecule containing C, H, and O.

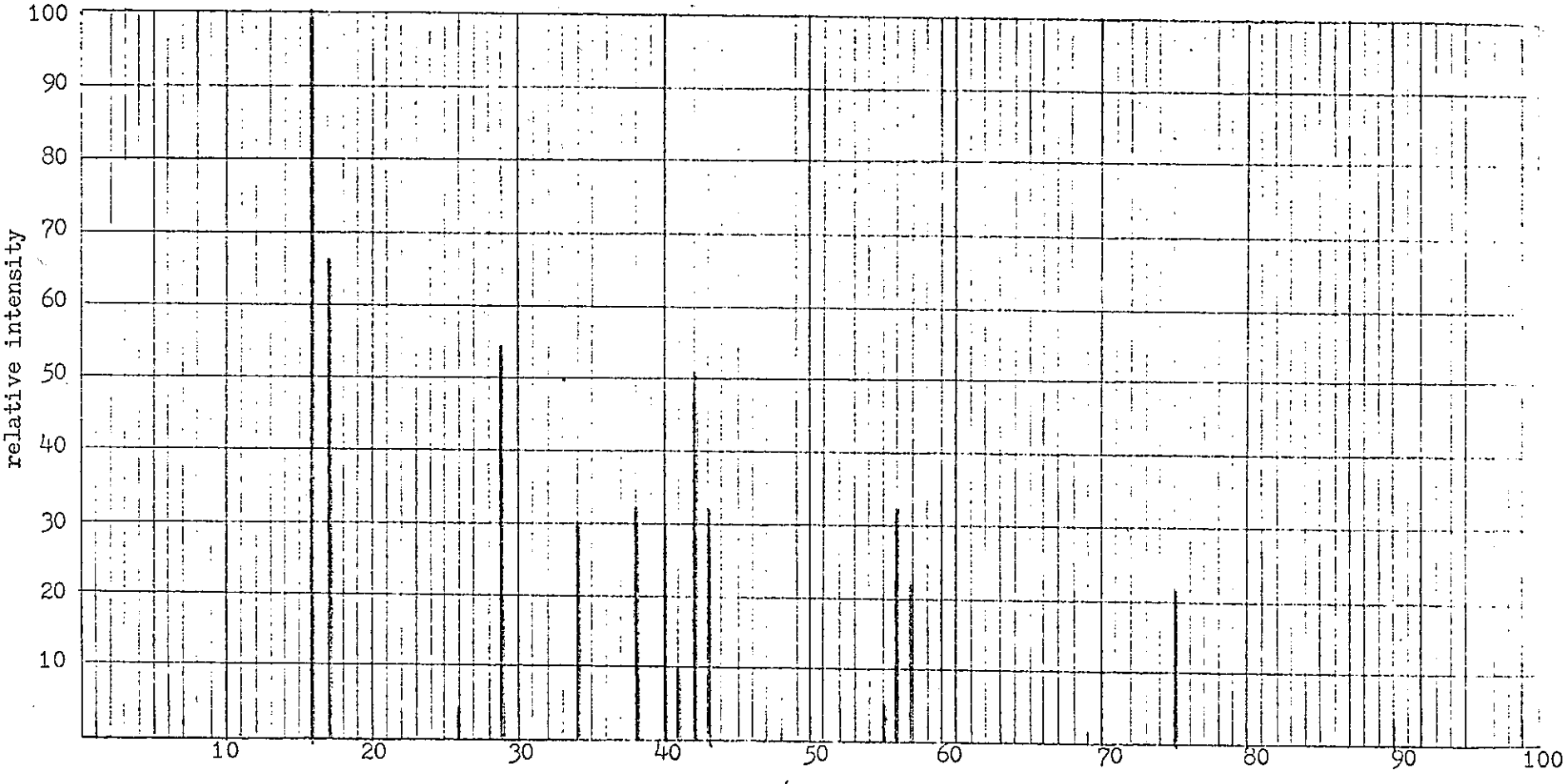
The next peak off the G.C. gave Mass Spectra #451 (Figure 39) and #452 (Figure 40). At first, it was very confusing - what would account for the peaks at m/e 82 and m/e 72 - an apparent loss of 10 amu (the peak at m/e 91 was ignored because it was found in many of the Spectra)? It was decided that either one of these two peaks was spurious (a result of the subtraction) or that more than one compound was present. In either case, the assignment of a structure(s) for this compound(s) would be difficult, if not impossible, and so was ignored for the time being. It was to be re-examined very closely



Spec #448 (EI)

Methylene Chloride + unidentified compound(s)

Figure 37

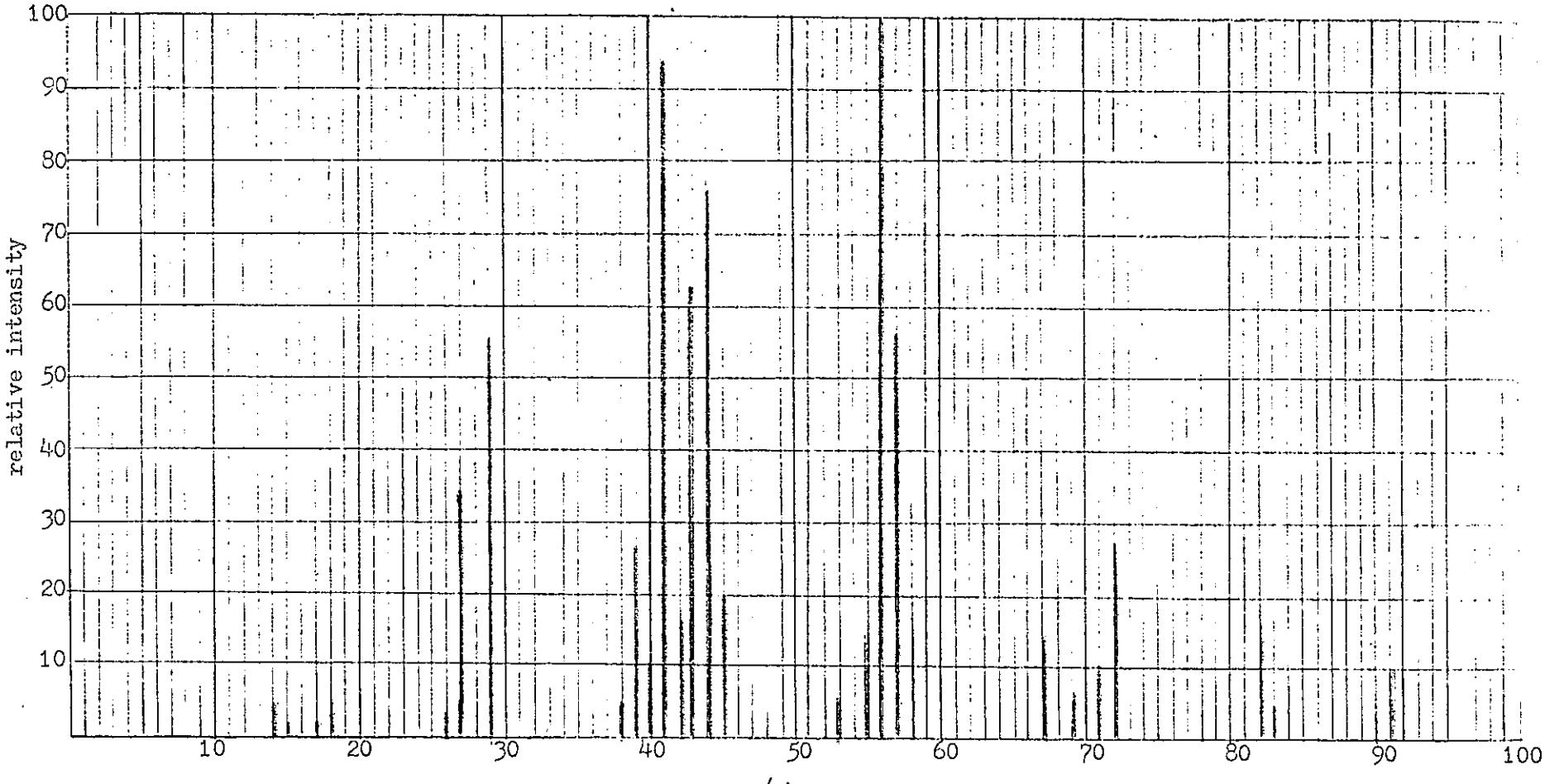


Note: Also peak at 207 (30%)

Spec #449 (EI)

Unidentified but probably not real

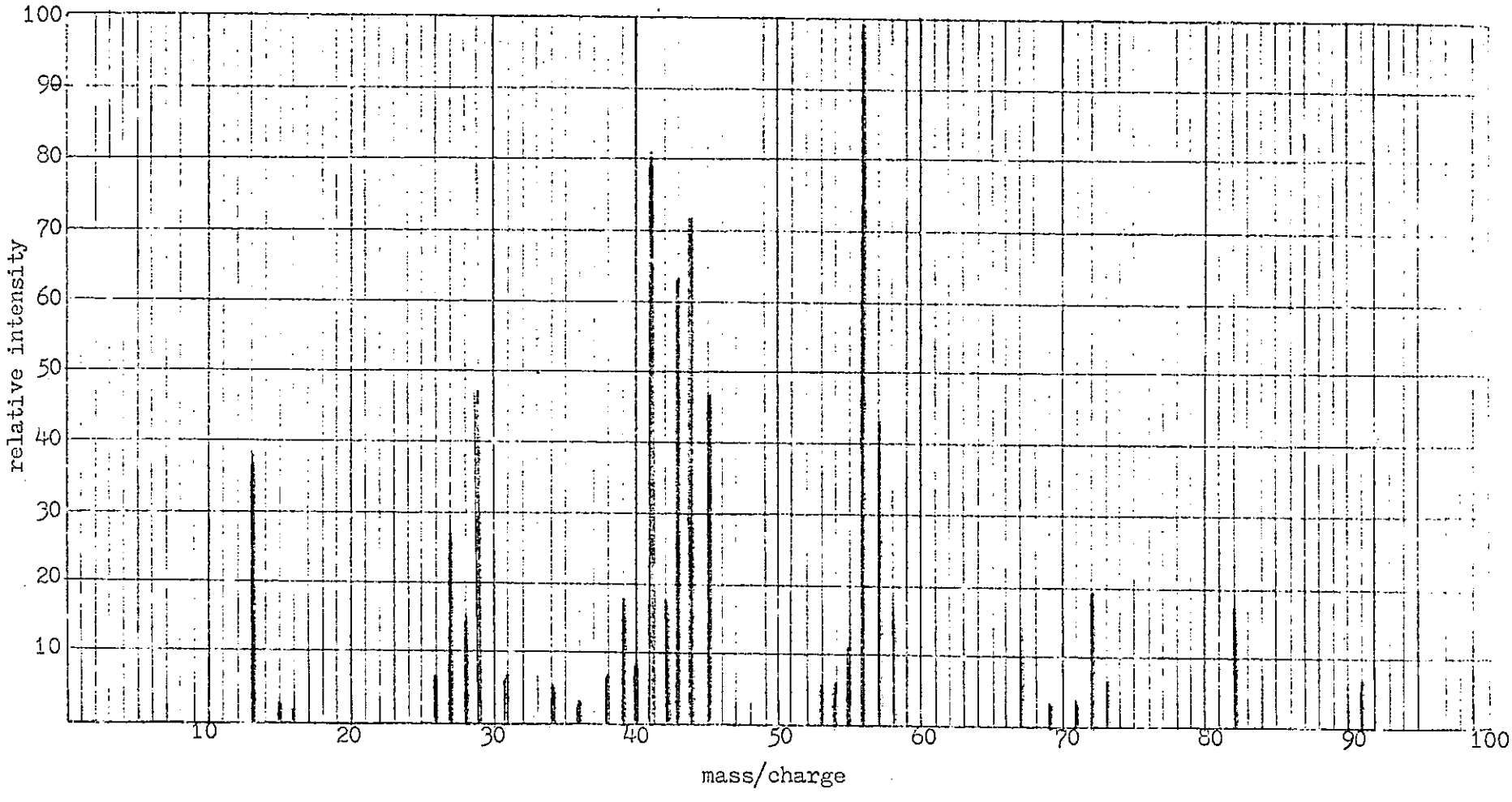
Figure 38



Spec #451 (EI)

HEXANAL

Figure 39



Spec #452 (EI)

HEXANE

Figure 40

a little later.

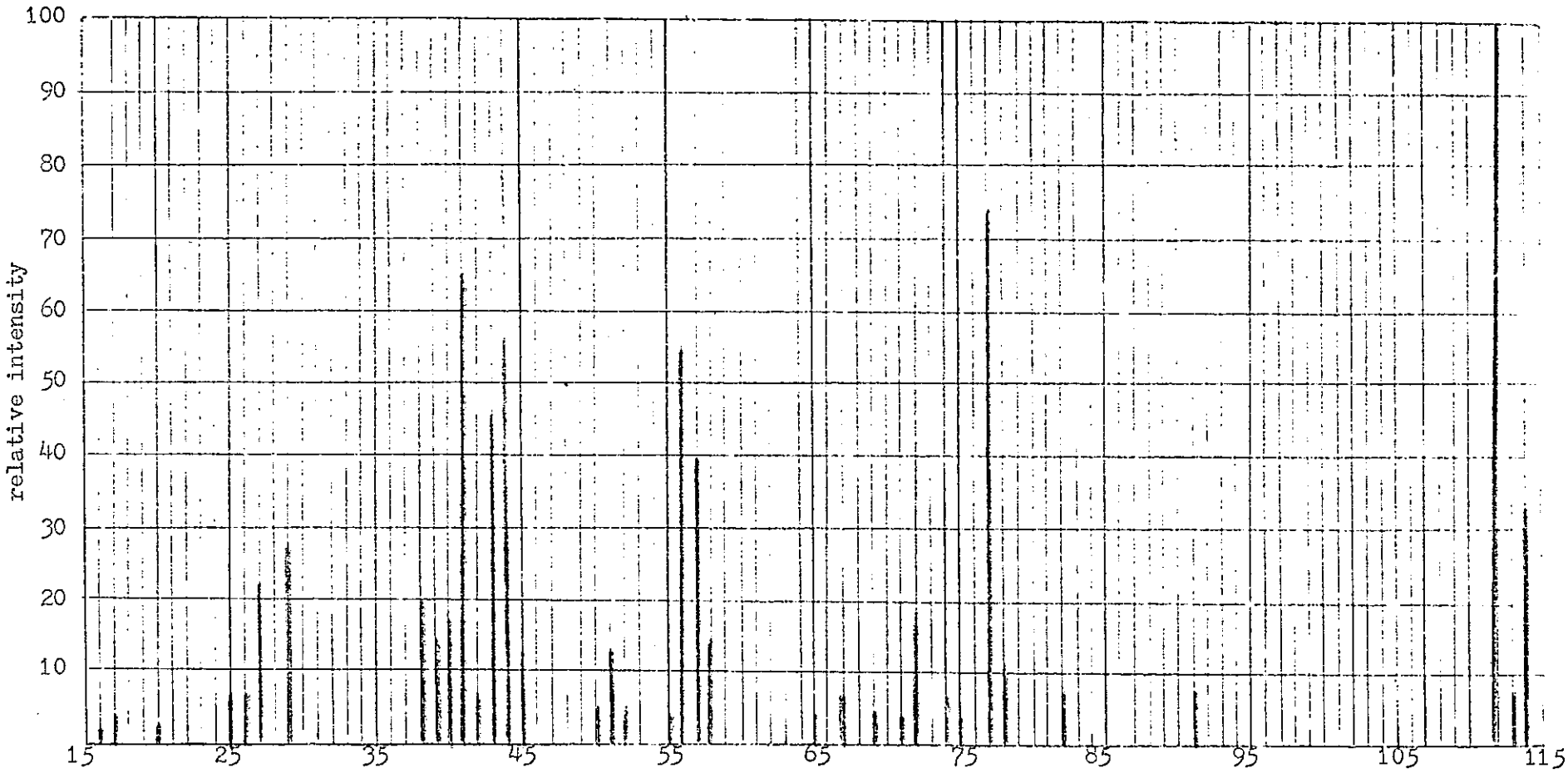
The next small peak coming off the OV-17 column was probably an impurity in the dichlorobenzene. Note its size in relation to the peak immediately preceding it (Figure 31). Mass Spectra #453 reveals what the compound is: monochlorobenzene. The M (m/e 112) and M+2 (m/e 114) peaks betray the presence of a chlorine-containing compound ($_{17}Cl^{35}$ and $_{17}Cl^{37}$) while the peak at m/e 77 suggests an aromatic ring (C_6H_5): see Figure 41.

The next peak came from a compound with a very high molecular weight: in Spectra #455 (Figure 42), the base peak had a m/e of 281. In all probability, this was more of the packing material noticed in the earlier Mass Spectra.

The final two Mass Spectra for this run - #457 (Figure 43) and #458 (Figure 44) were difficult to identify. They may be a result of an overlap of several different compounds.

None of the identified compounds seemed likely pheromone candidates. However, since the peaks were very small on the G.C. (about one microgram of a chemical would cause full scale defection), we took five capillary tubes containing the A peak, rinsed it with 10 μl of dichlorobenzene and shot it into the G.C.-M.S.. The trace of this injection had considerably larger peaks (see Figure 45).

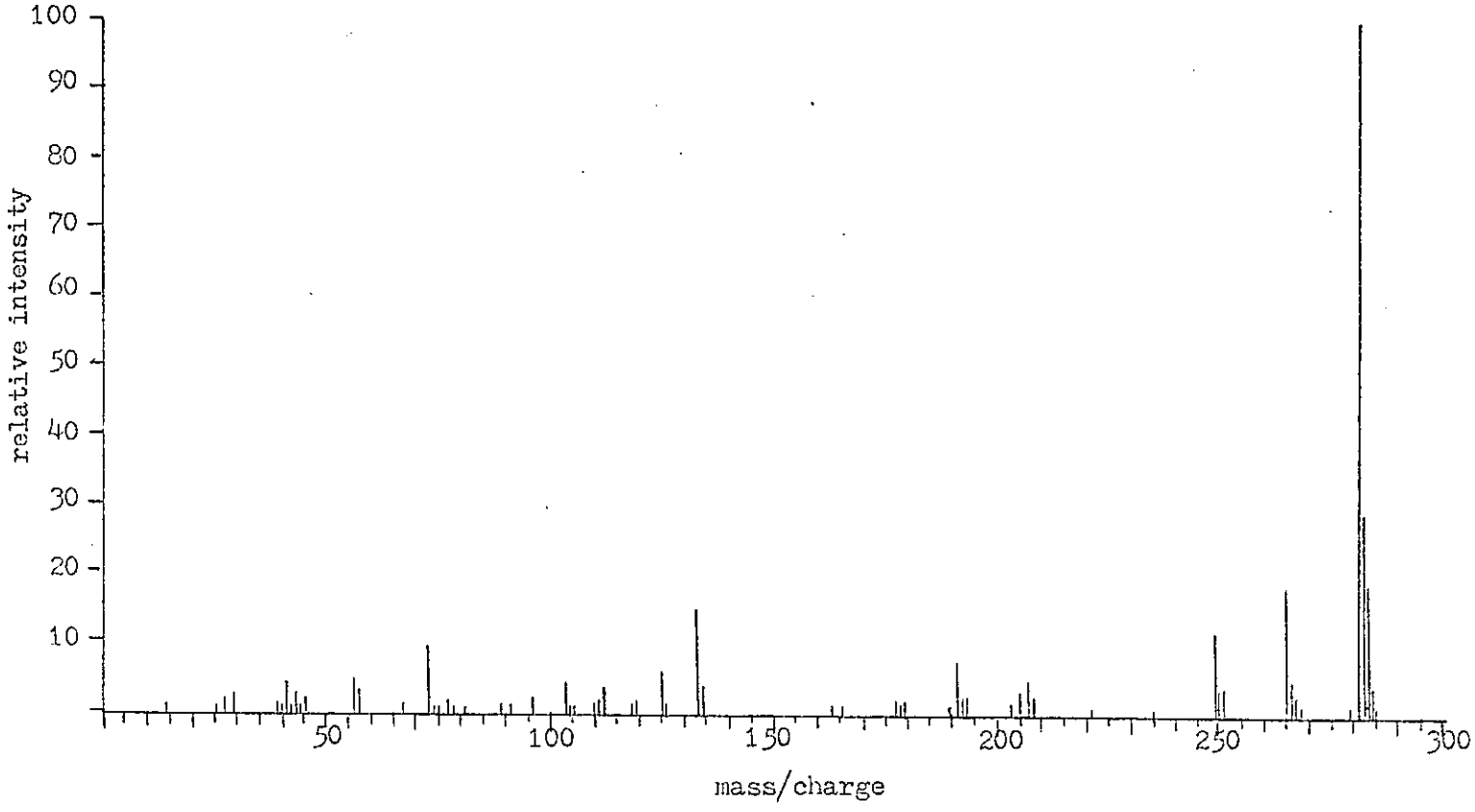
The first peak coming off the G.C. this time was probably air (see Figure 46) although the peak on the Mass Spectra at m/e 44 is abnormally intense. The next Mass Spectra, #478 (Figure 47), from the tiny peak following the air peak on the G.C., has not yet been identified although the very prominent peak at m/e 13 makes it suspect. Perhaps it has been distorted from the subtraction of Mass Spectra #485 from it. The next peak is from the tiny bit of methylene



Spec #453 (EI)

Monochlorobenzene

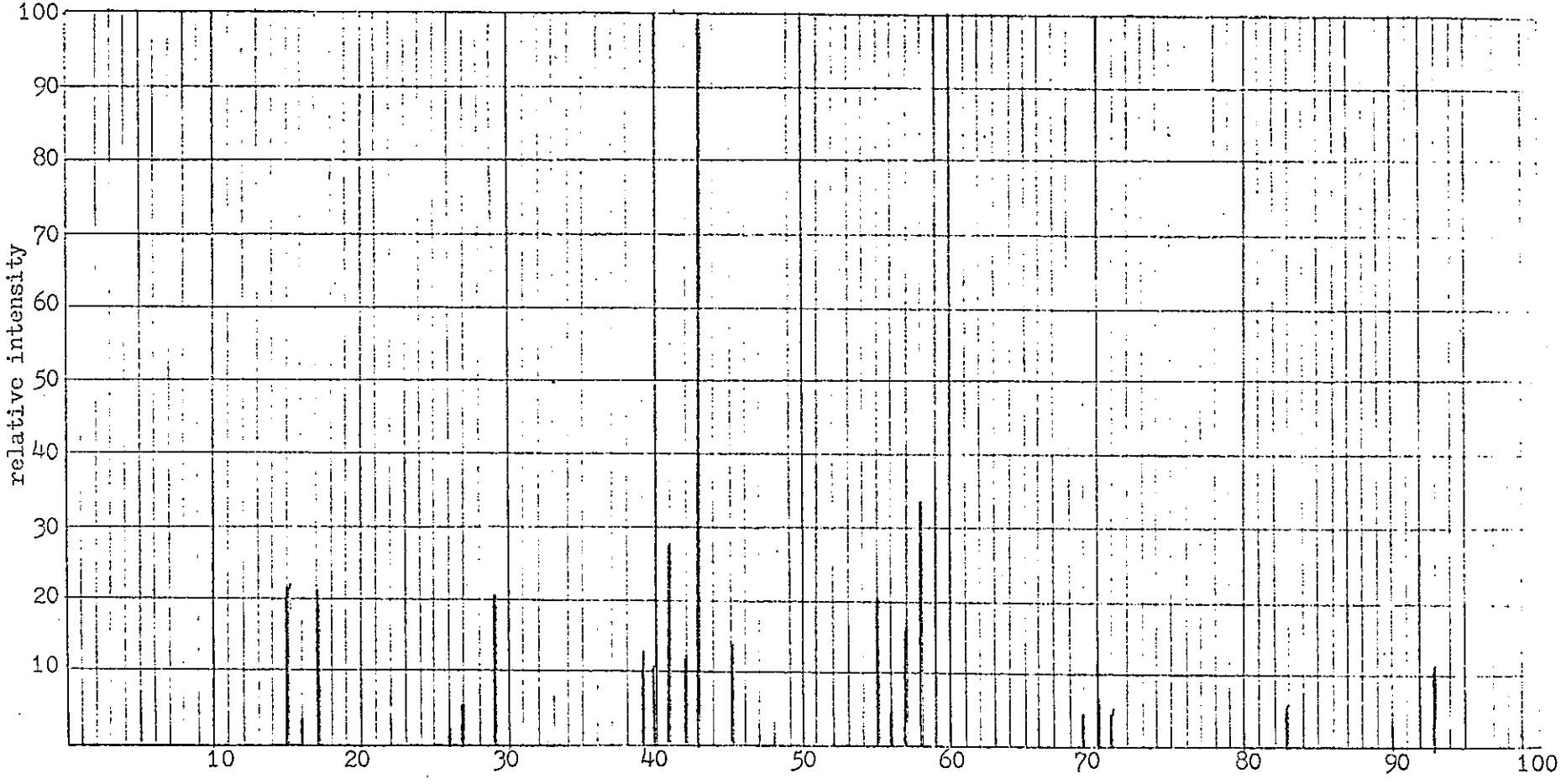
Figure 41



Spec #455 (EI)

PACKING [illegible]

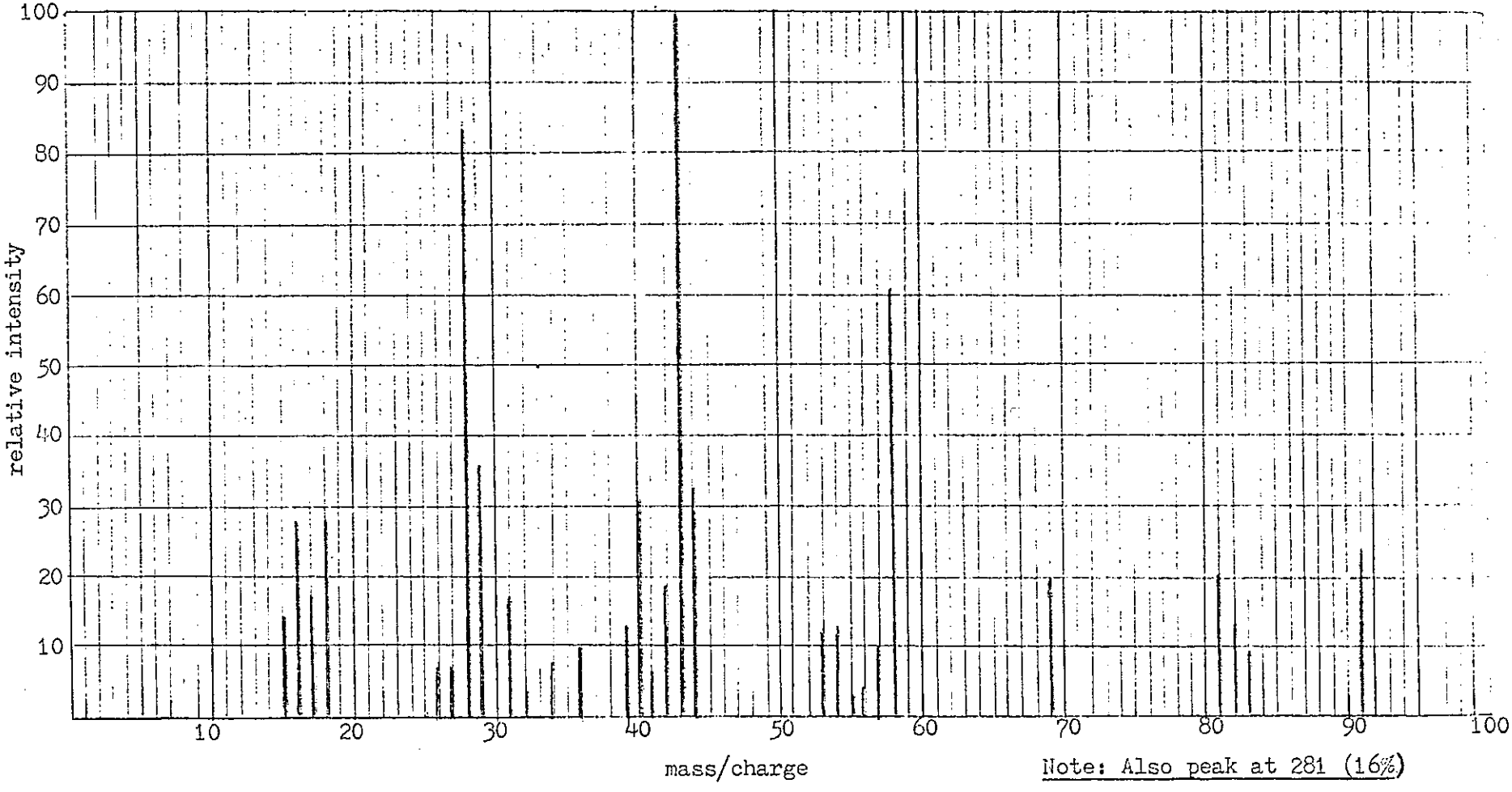
Figure 42



Note: Peaks at 249 (6%)
271 (16%)

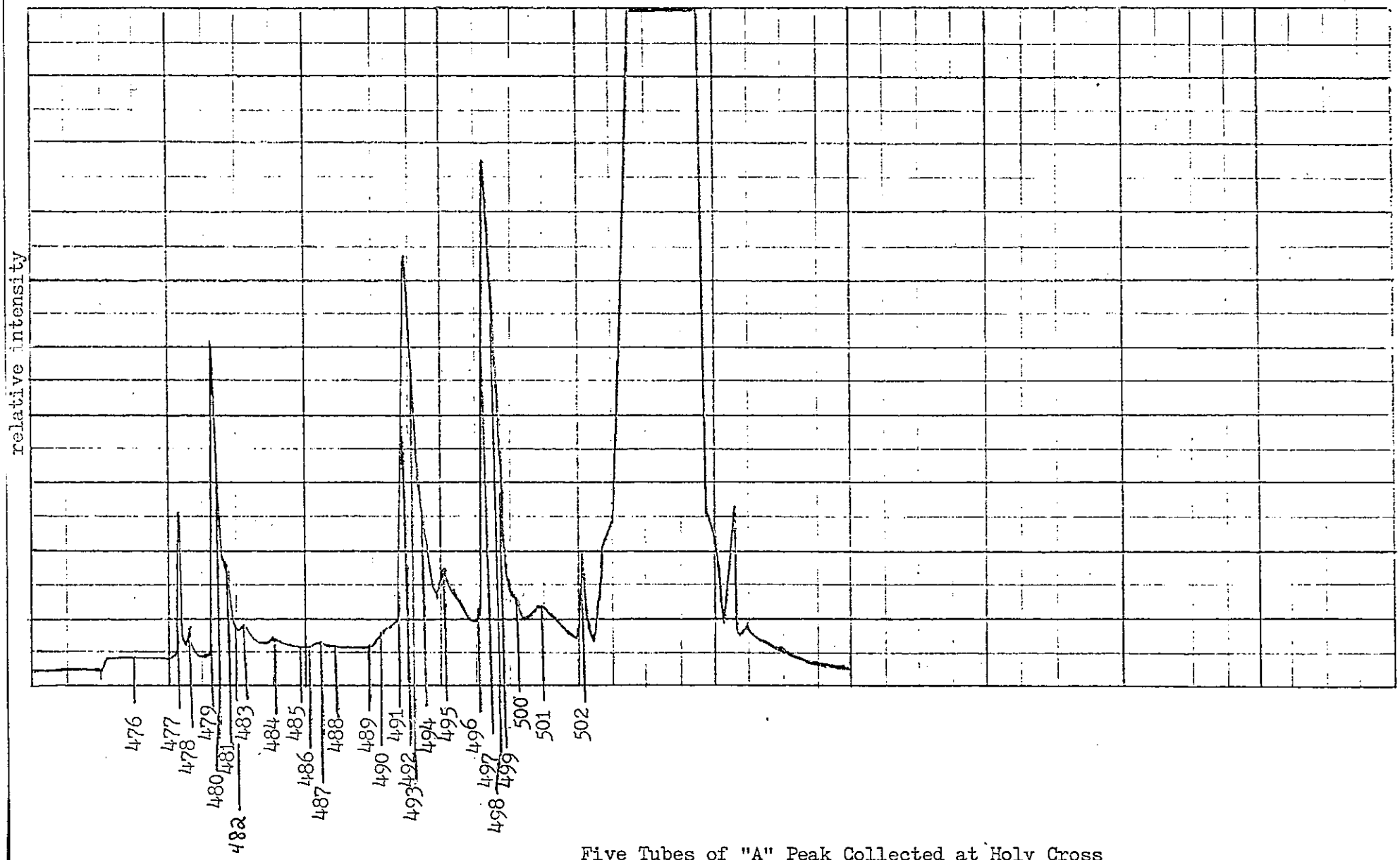
Spec #457 (EI)
Unidentified

Figure 43

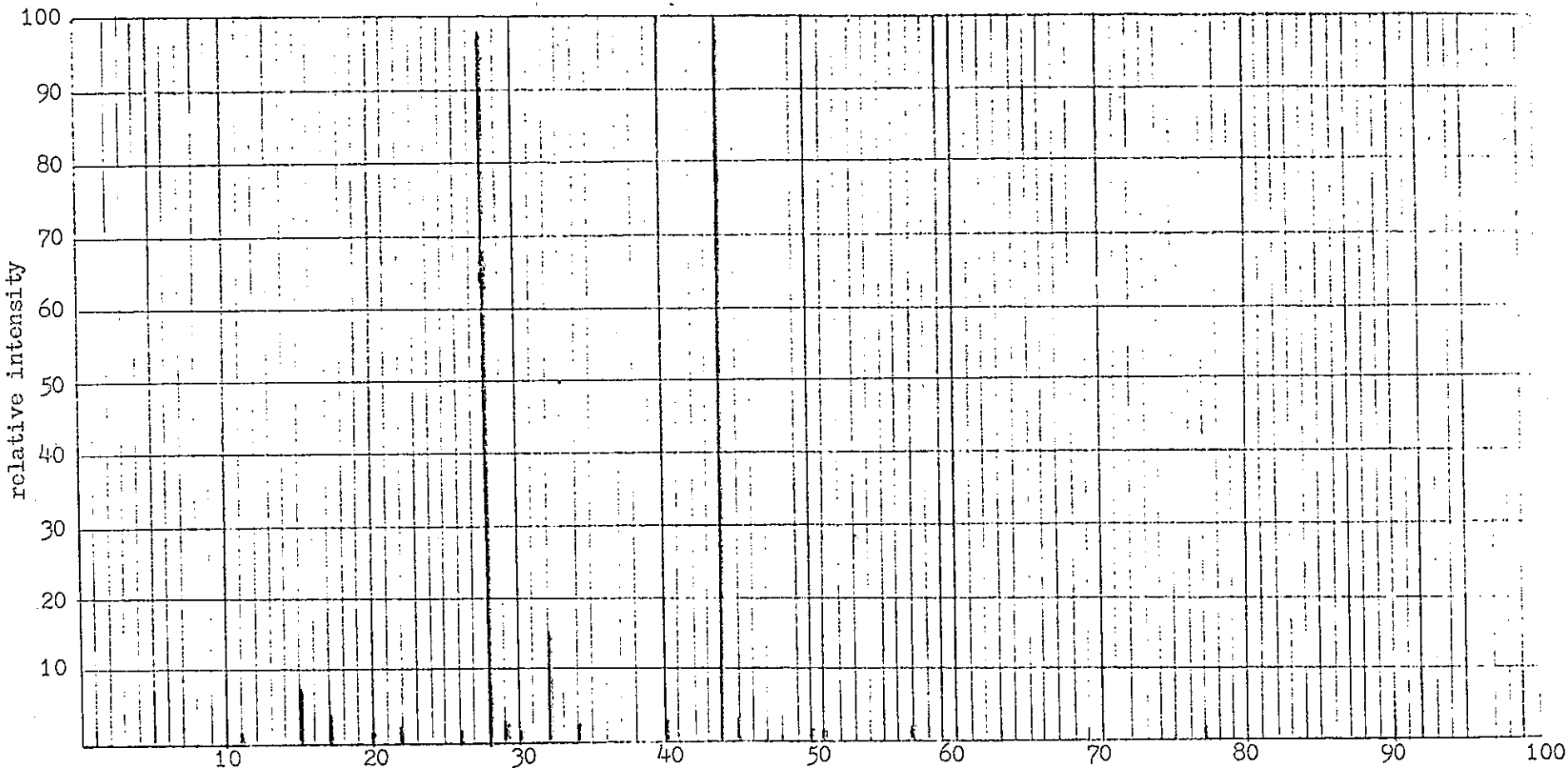


Spec #458 (EI)
Unidentified

Figure 44



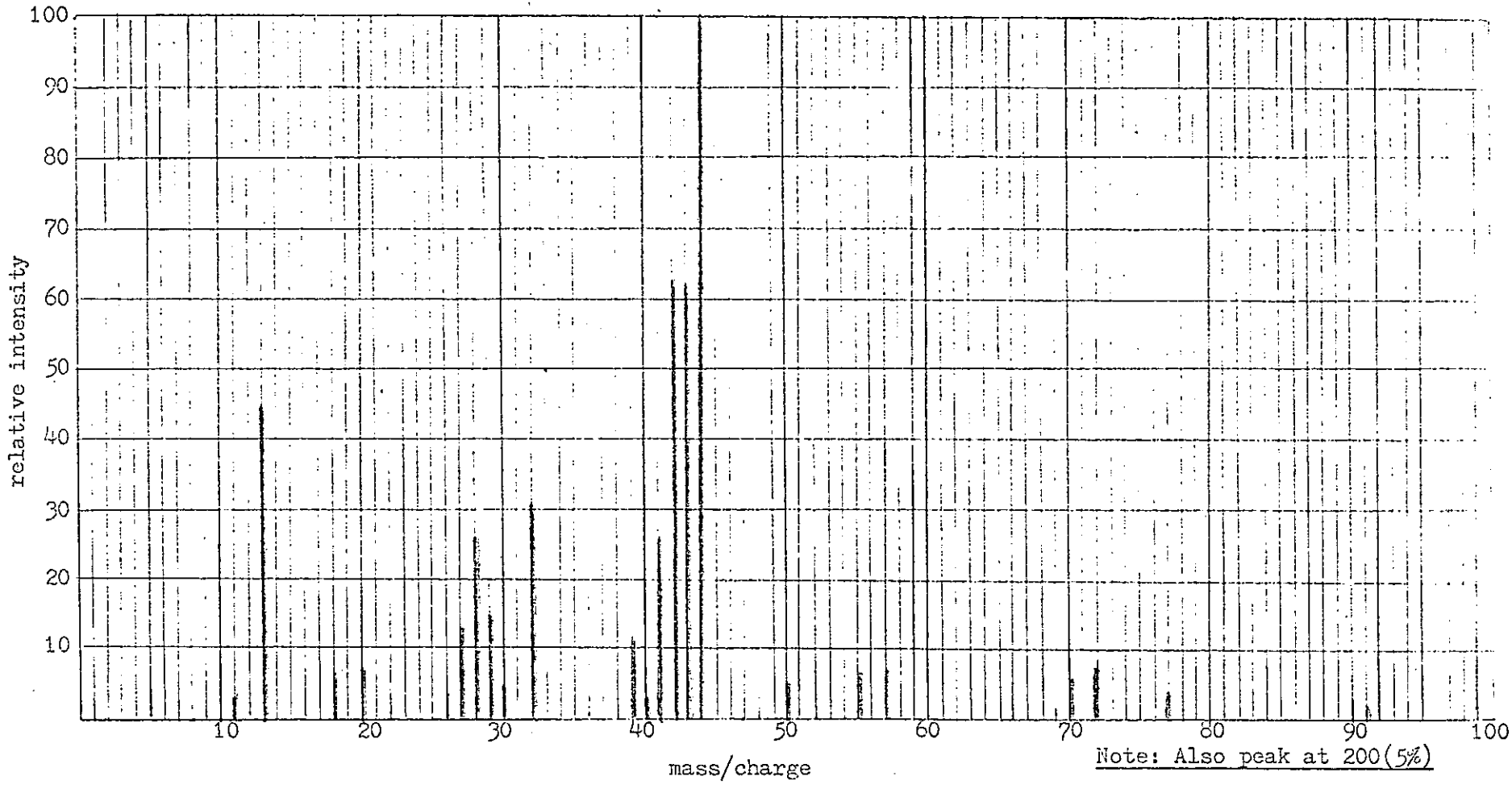
Five Tubes of "A" Peak Collected at Holy Cross
Figure 45



Spec #477 (EI)

AIR

Figure 46



Spec #478 (EI)

Questionable

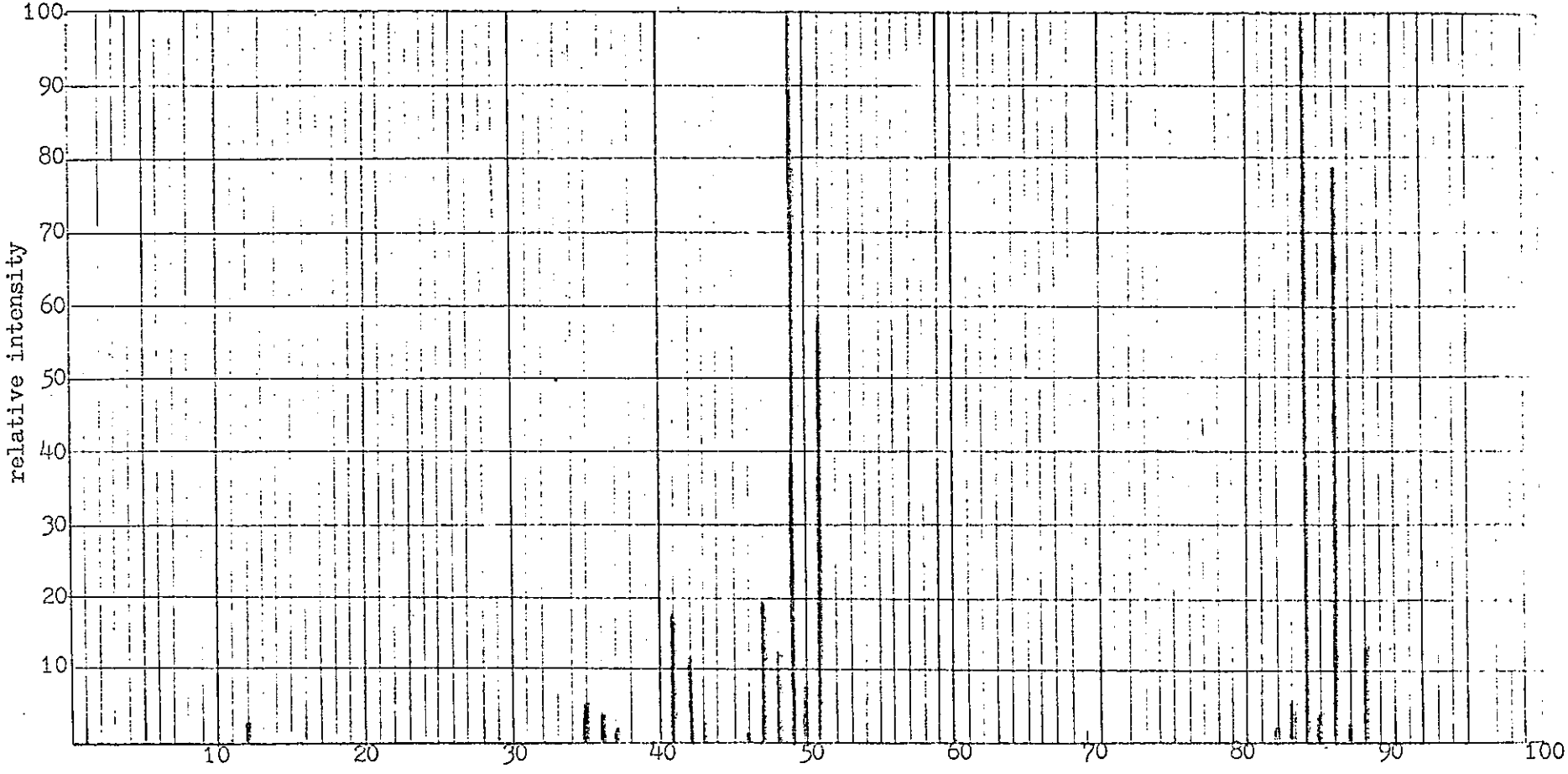
Figure 47

chloride collected along with peak A: the Mass Spectra shows peaks at m/e 49, 51, 84, 86. But the tail end of the methylene chloride peak on the G.C. contained at least one other compound: the methylene chloride peaks in the M.S. (m/e 49, 51, 84, 86) decrease while there is an increase in several peaks below m/e 49 (see Figures 49 and 50). This other compound(s) was not identified.

The small peaks coming off the G.C. continued to be difficult to identify - it was often difficult to determine whether the Mass Spectra was of a pure compound, a mixture of compounds, or distorted by the subtraction of Mass Spectra #485. The peak immediately following the methylene chloride peak on the G.C. (Spectra #483, Figure 51) still had traces of methylene chloride (peaks at m/e 49, 51, 84, 86) but also contained something else capable of giving a very intense peak at m/e 78. Benzene may be the chemical responsible for this m/e 78 peak - after all, a 50:50 methanol-benzene solvent was used to elute the 183-6 fraction from the Sephadex column.

None of the Mass Spectra between #484 and 490 (Figures 52-57) have been identified although the peaks at m/e 32 might be from trace amounts of methanol.

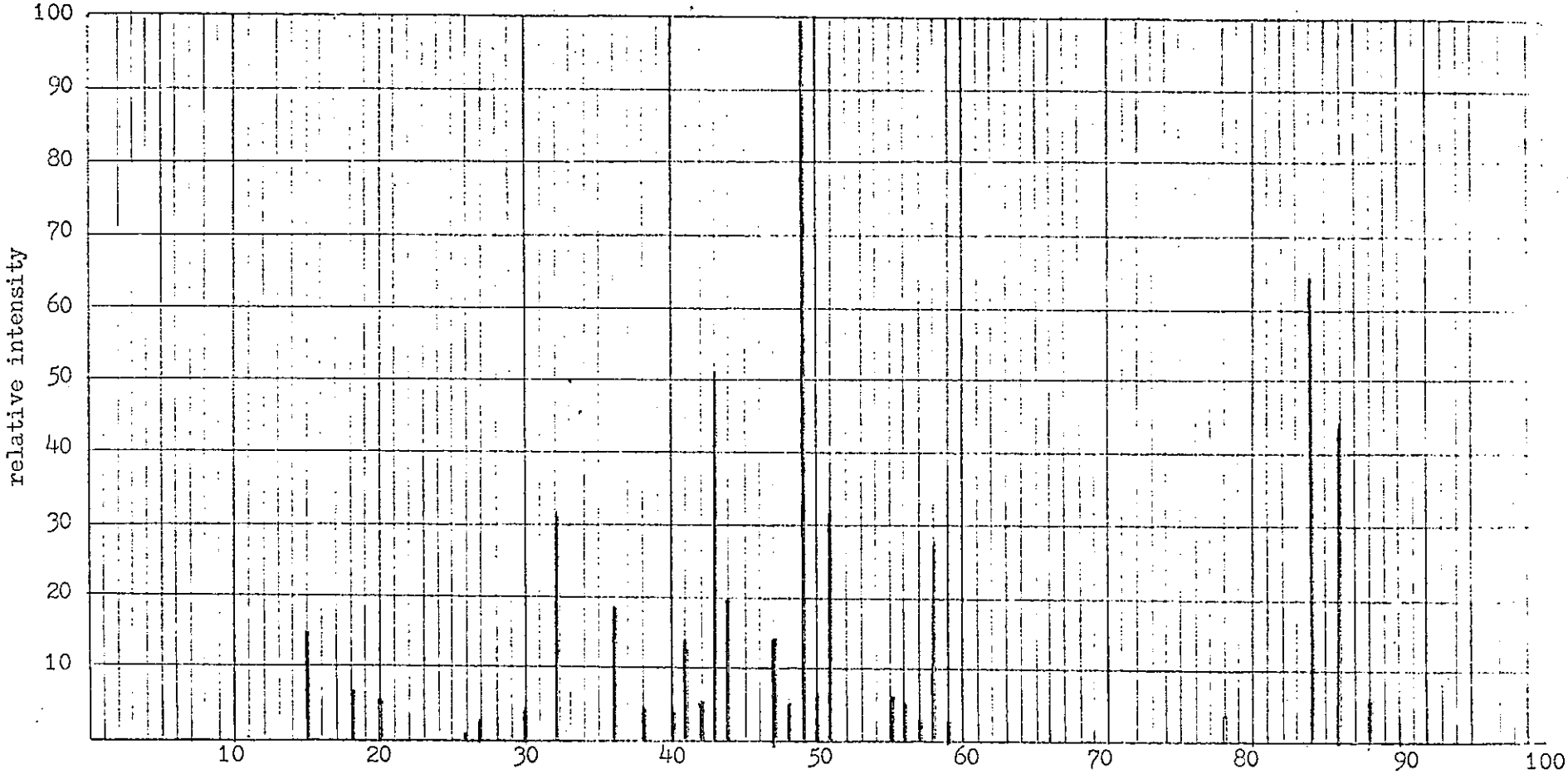
Mass Spectra #491 was passed over in favor of Mass Spectra #492 (Figure 58) because the latter one was near the center of the peak on the G.C. Intense peaks at m/e 29, 43, 55, and 98 revealed the structure of the compound responsible for the Spectra. A m/e of 29 is often indicative of an aldehyde or ketone, while the peak at m/e 98 suggested a compound with a molecular weight of 98 amu, in agreement with the results of the first G.C.-M.S. work done at Pennsylvania State University. Mass Spectra #492 closely matched the published



Spec #479 (EI)

Methylene Chloride

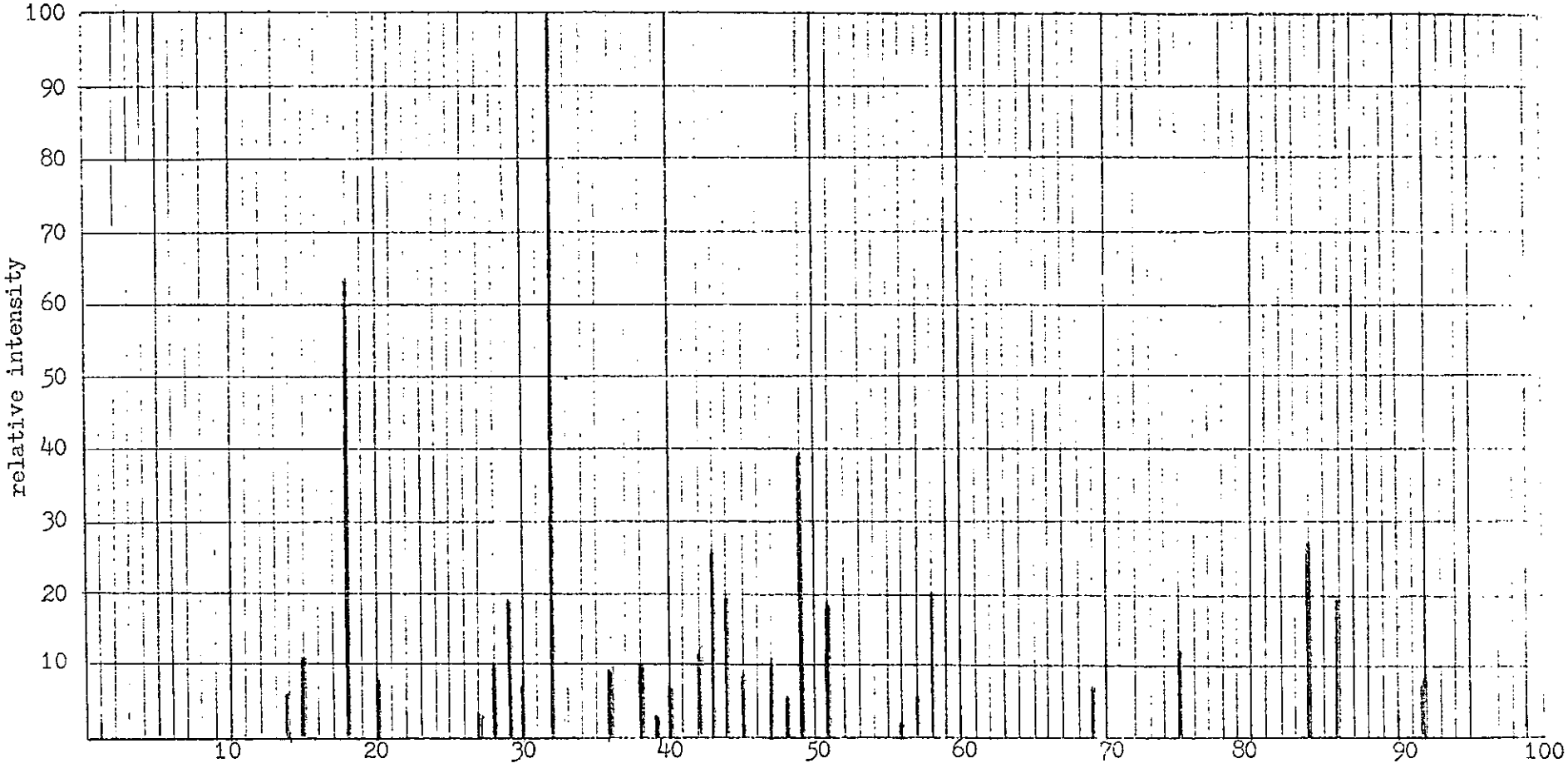
Figure 48



Spec #481 (EI)

Methylene Chloride + unidentified compound(s)

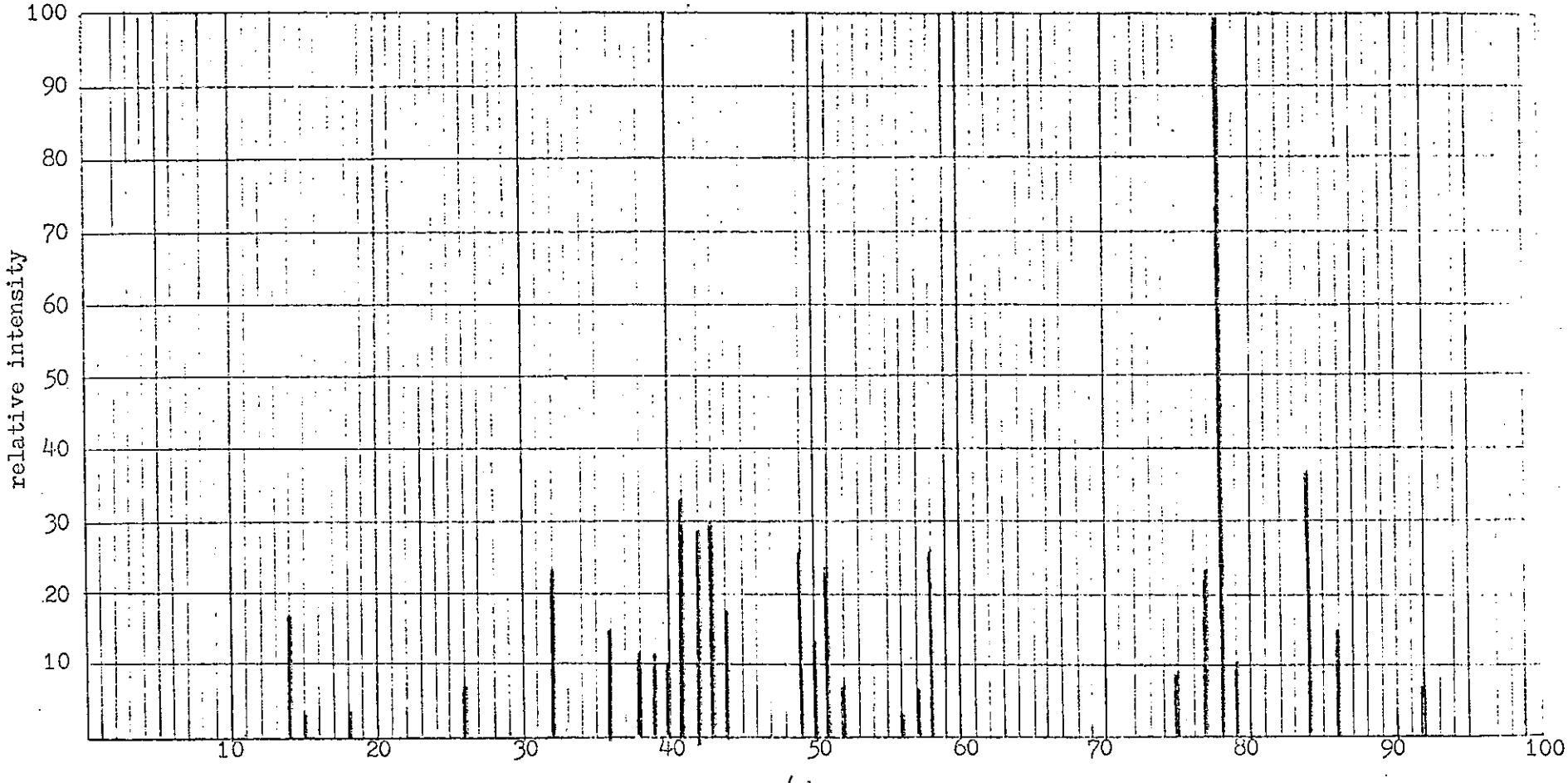
Figure 49



Spec #482 (EI)

Methylene Chloride + unidentified compounds.
(Methanol may give peaks at m/e 32 and 18)

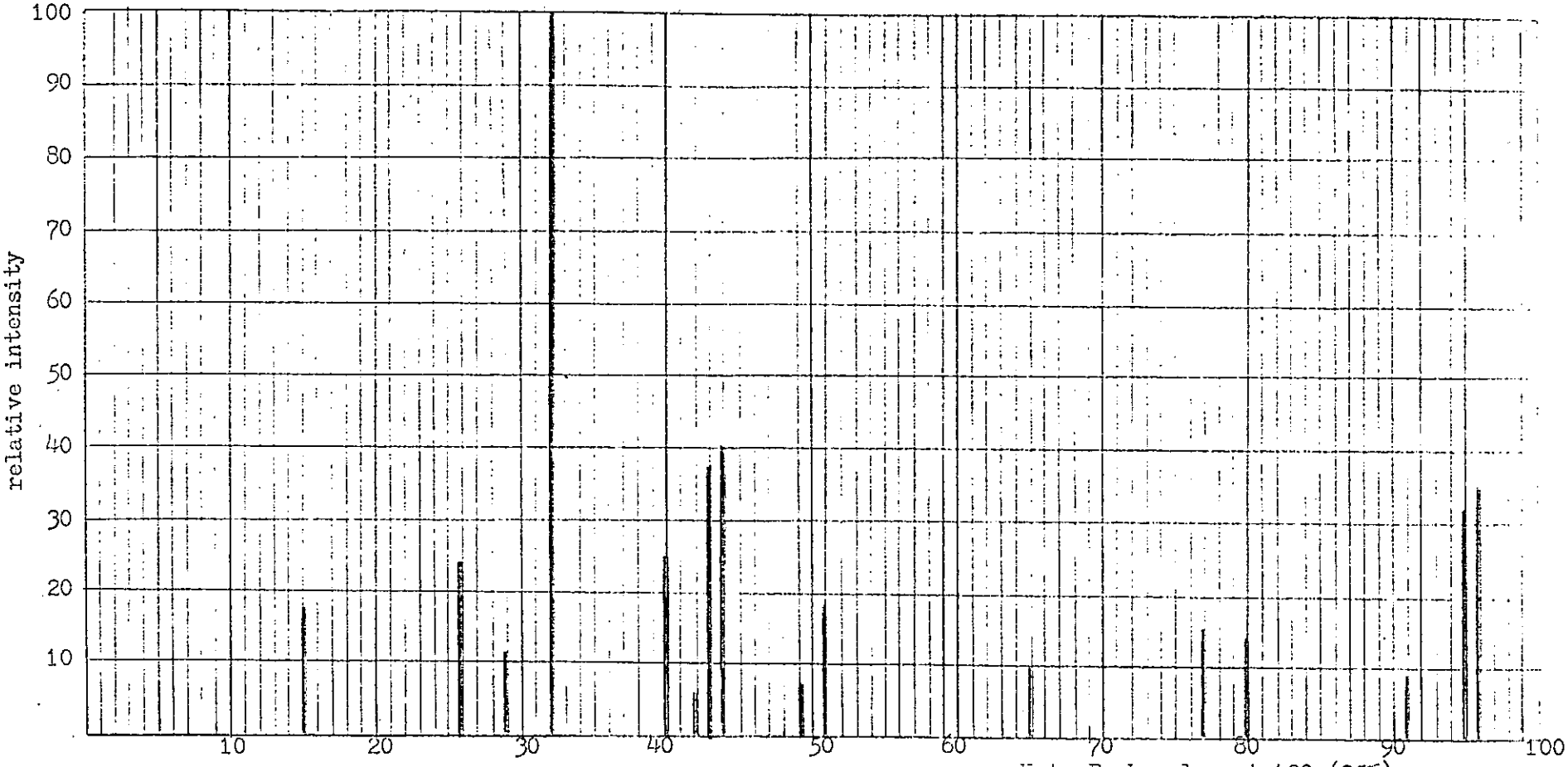
Figure 50



Spec #483 (EI)

Methylene Chloride + Benzene(?)

Figure 51

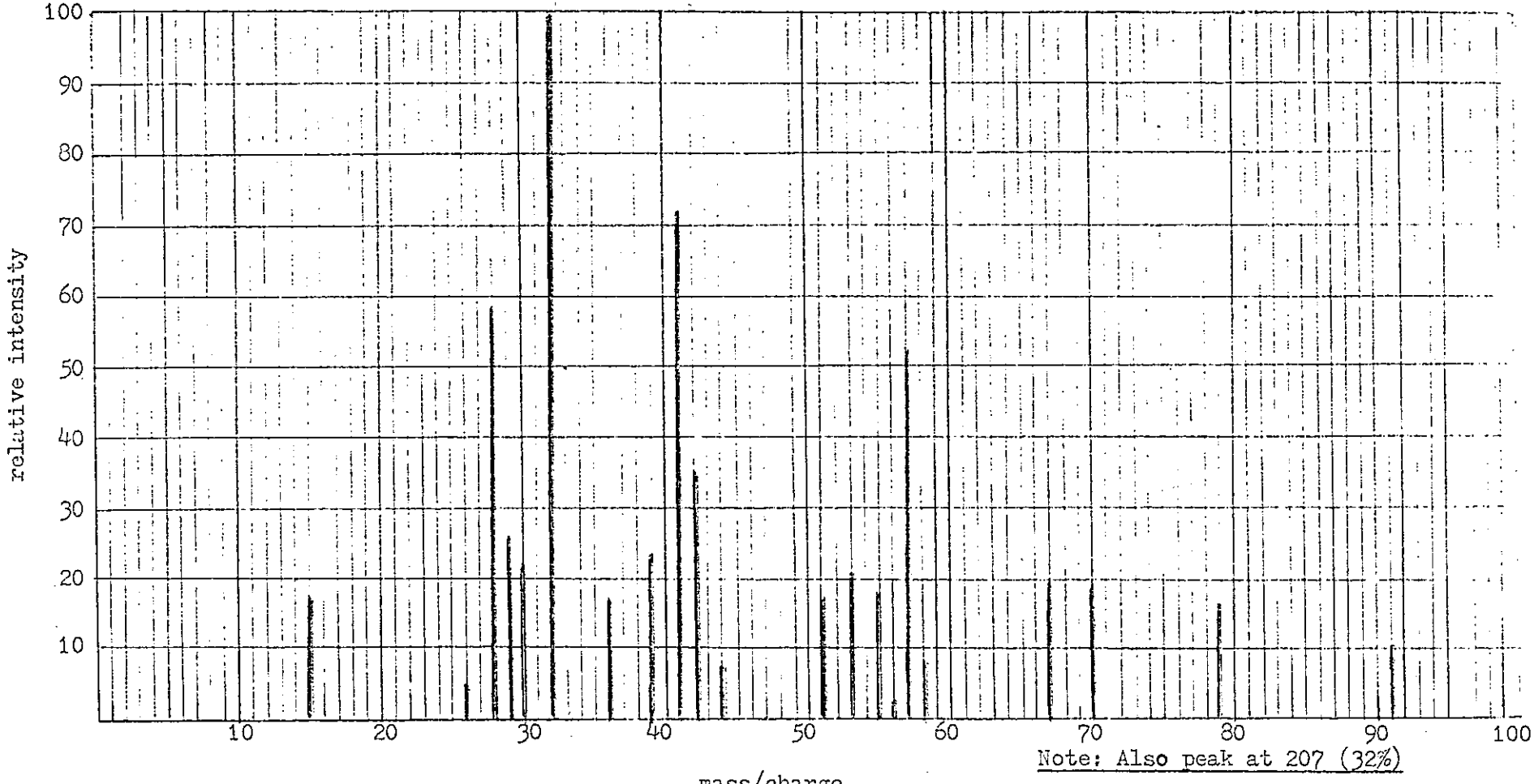


Note: Peaks also at 130 (35%)
132 (27%)

Spec #484 (EI)

Unidentified
(May be Methanol at m/e 32)

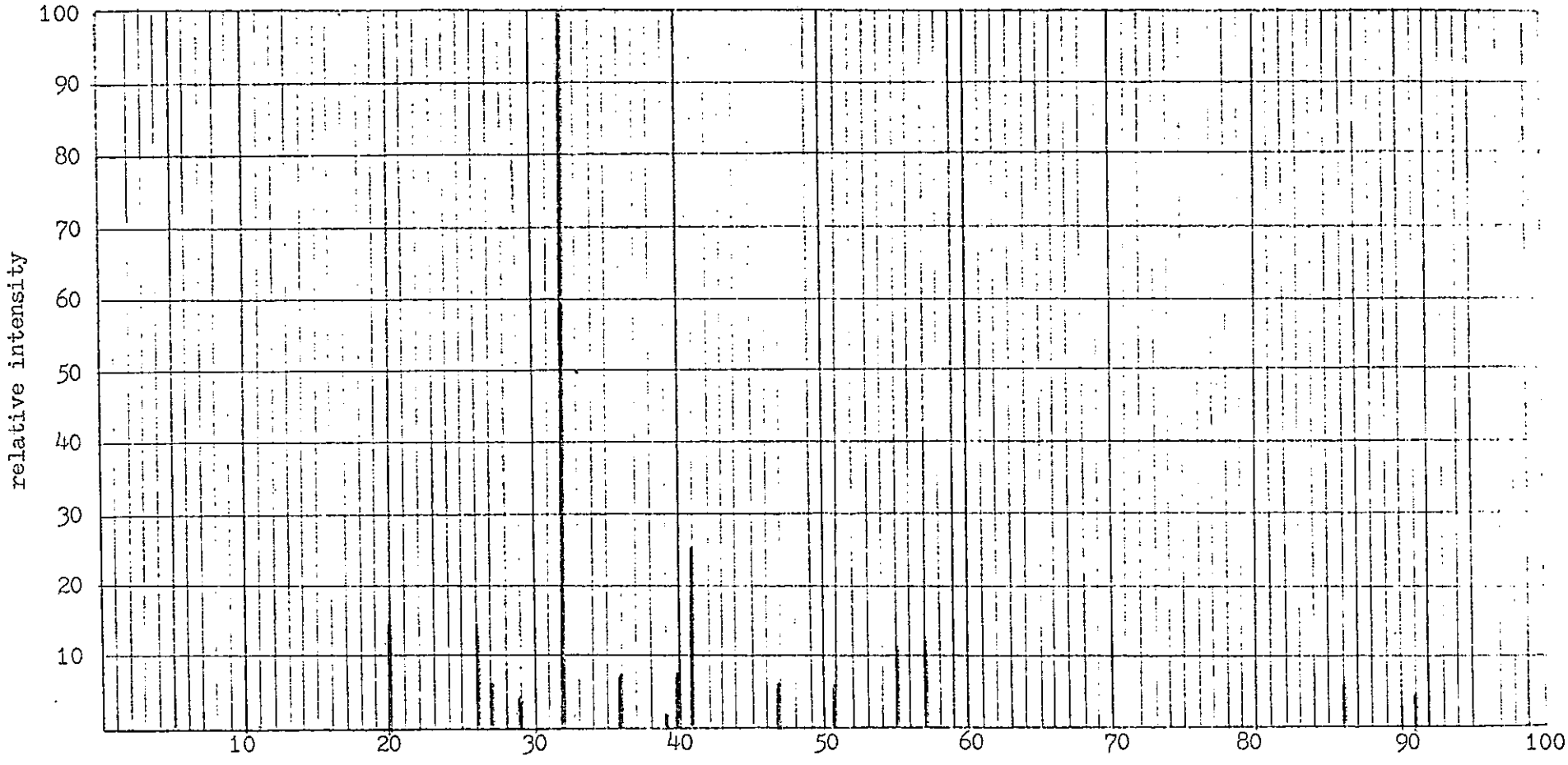
Figure 52



Spec #486 (EI)

unidentified
(May be Methanol at m/e 32)

Figure 53

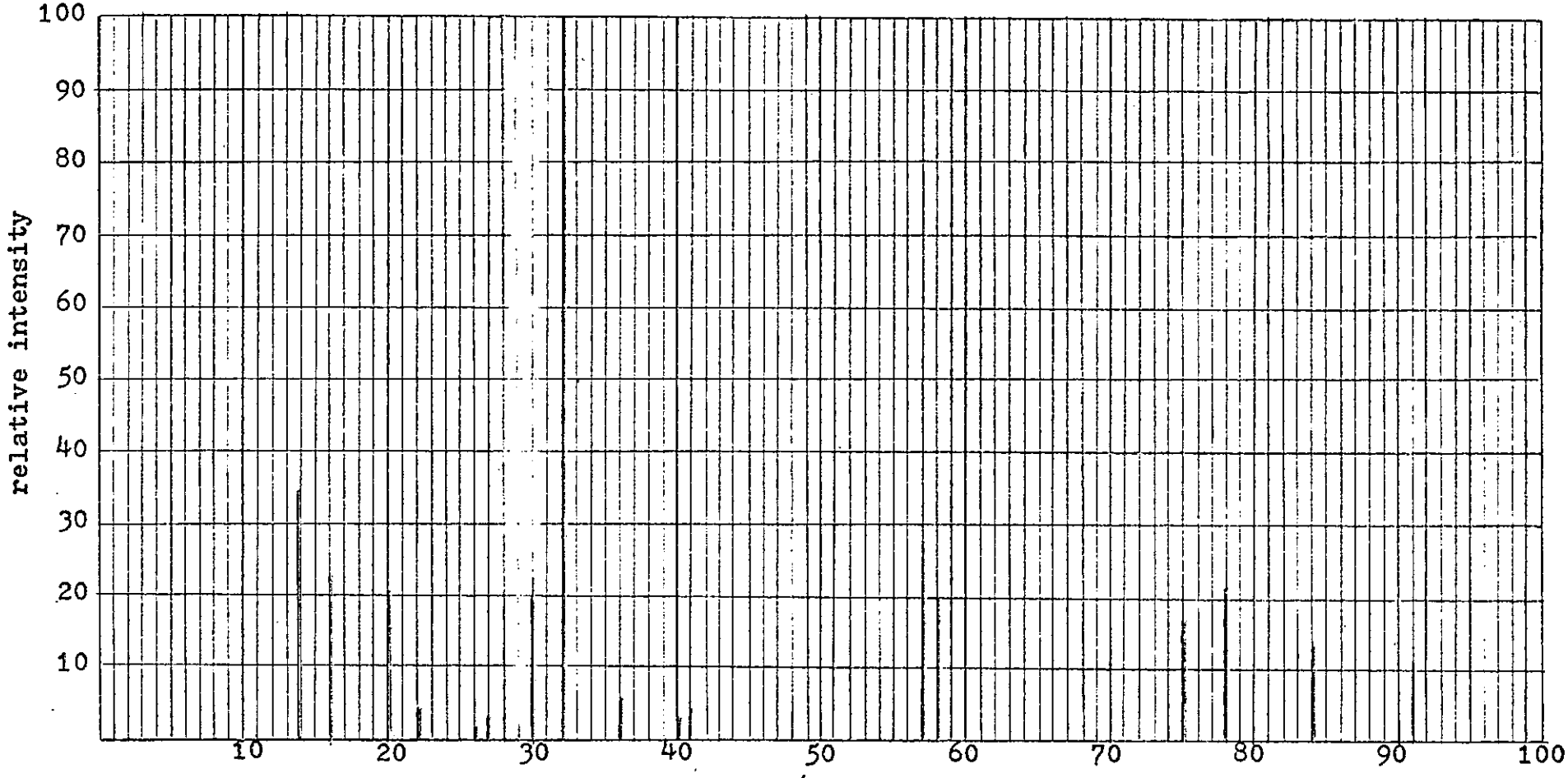


Note: Peaks also at 205 (6%)
207 (7%)

Spec #487 (EI)

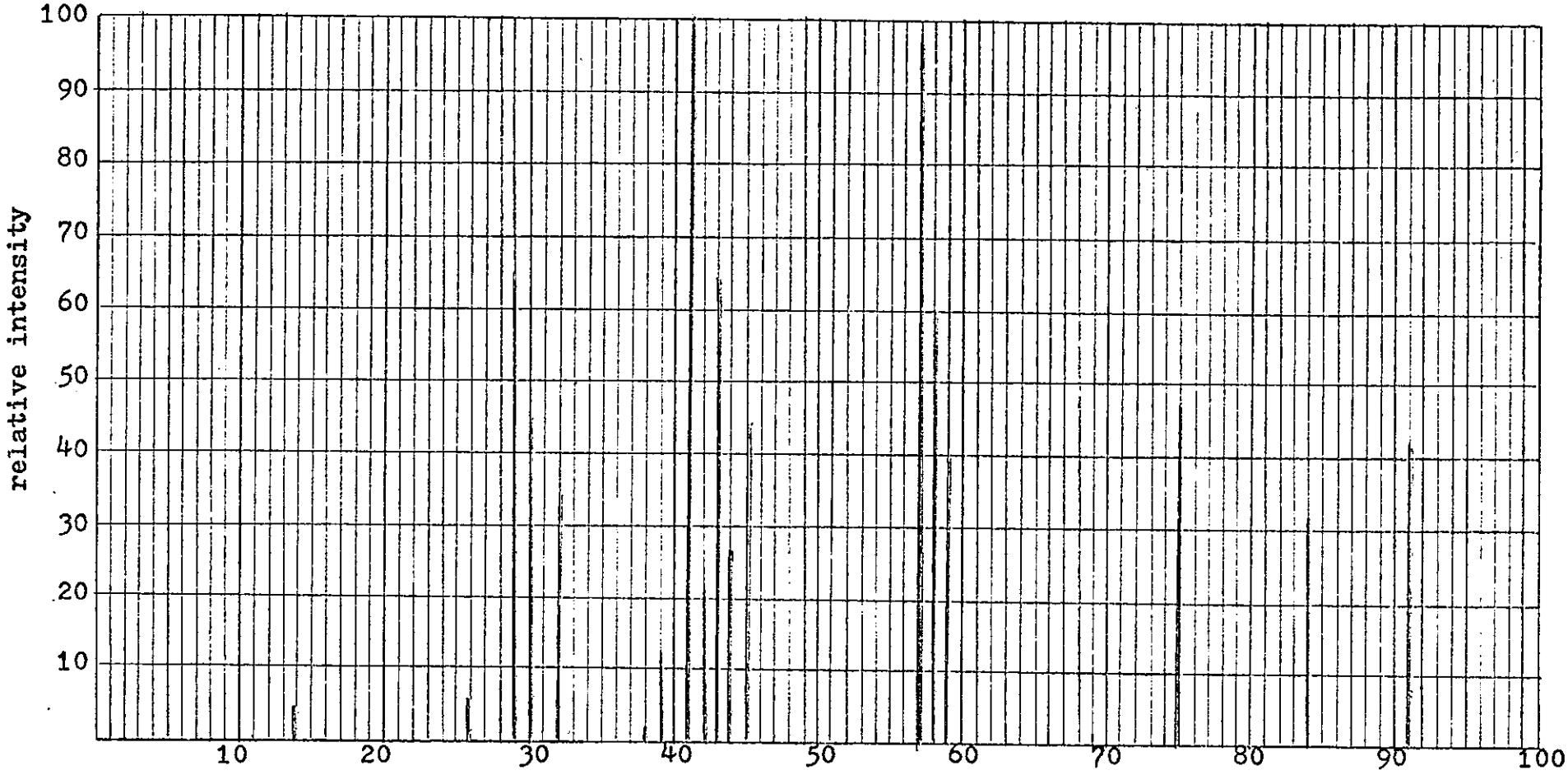
Unidentified
(may be methanol at m/e 32)

Figure 54



Unidentified
(may be methanol at m/e 32)

Figure 55



Spec #489 (EI)

Unidentified

Figure 56

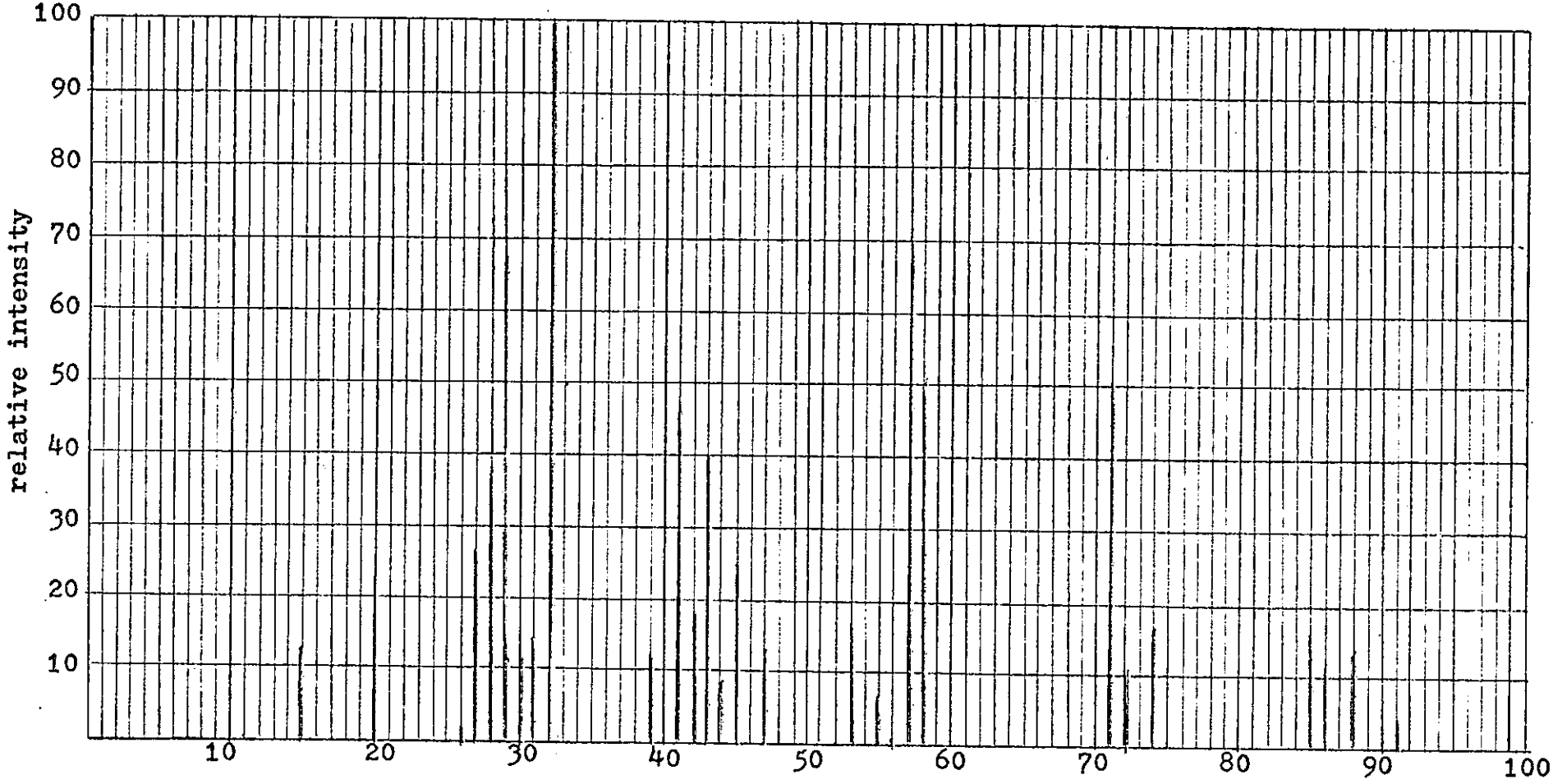
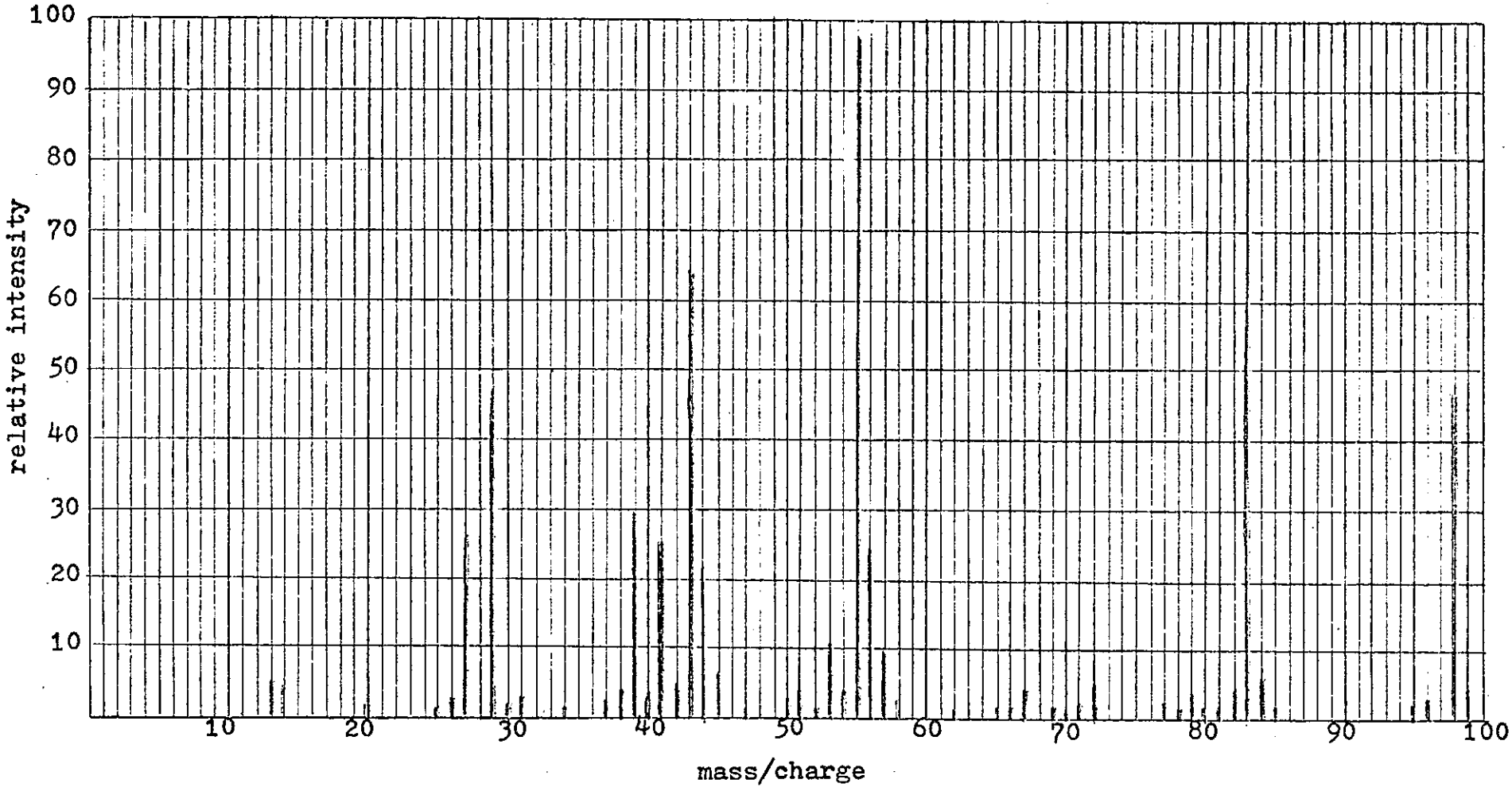

100
90
80
70
60
50
40
30
20
10
relative intensity
10
20
30
40
50
60
70
80
90
100
mass/charge
Spec #490 (EI)
Unidentified

Figure 57


100
90
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60
50
40
30
20
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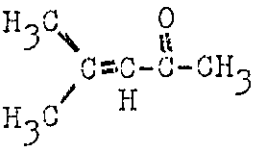
relative intensity
10
20
30
40
50
60
70
80
90
100
mass/charge
Spec #492 (EI)
Mesityl Oxide

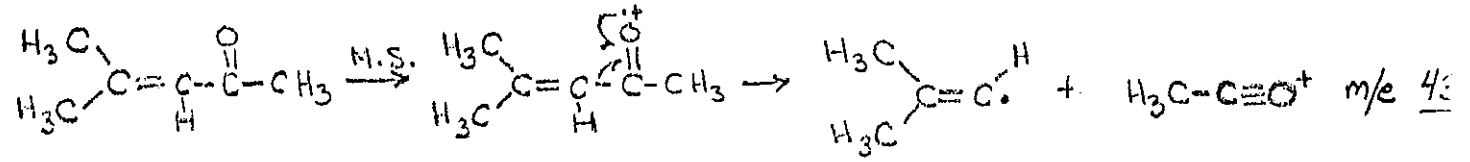
Figure 58

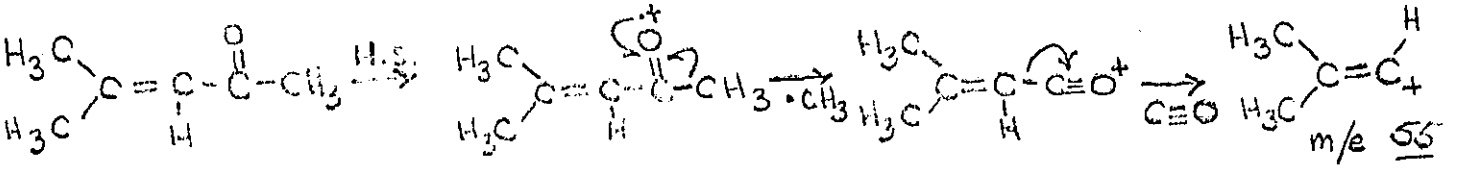
Mass Spectra of 4-Methylpent-3-en-2-one (Figure 59), or Mesityl Oxide:

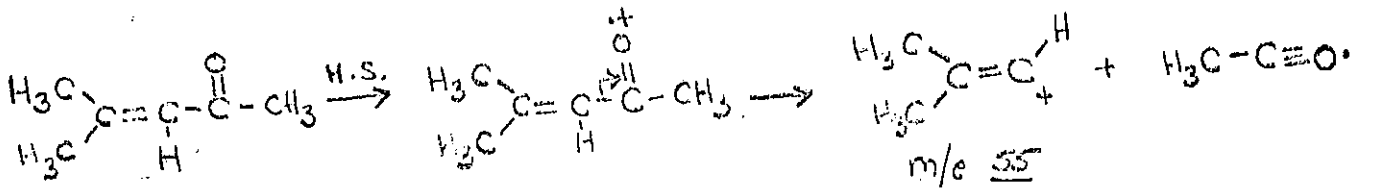


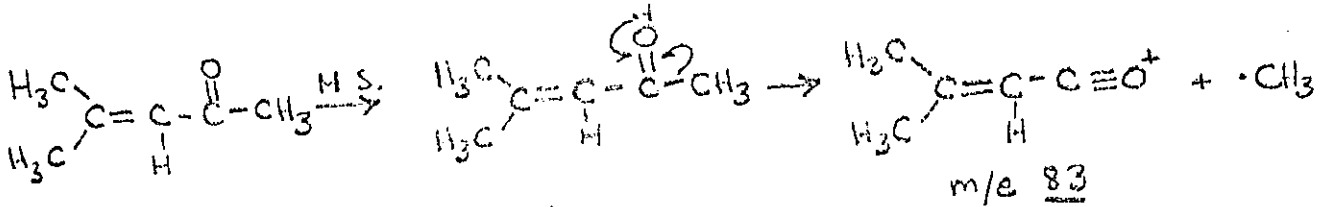
The major peaks on the Mass Spectra may be a result of the following:

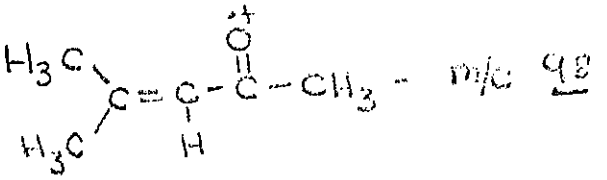
$HC\equiv O^+$ - m/e 29











Mass Spectra #493 (Figure 60) and #494 (Figure 61) also appear to be that of Mesityl Oxide.

4-Methylpent-3-en-2-one (Mesityl Oxide)

Source: Mass Spectrometry Data Center
AWRE Aldermaston, Berkshire
MSDC 2624

Figure 59

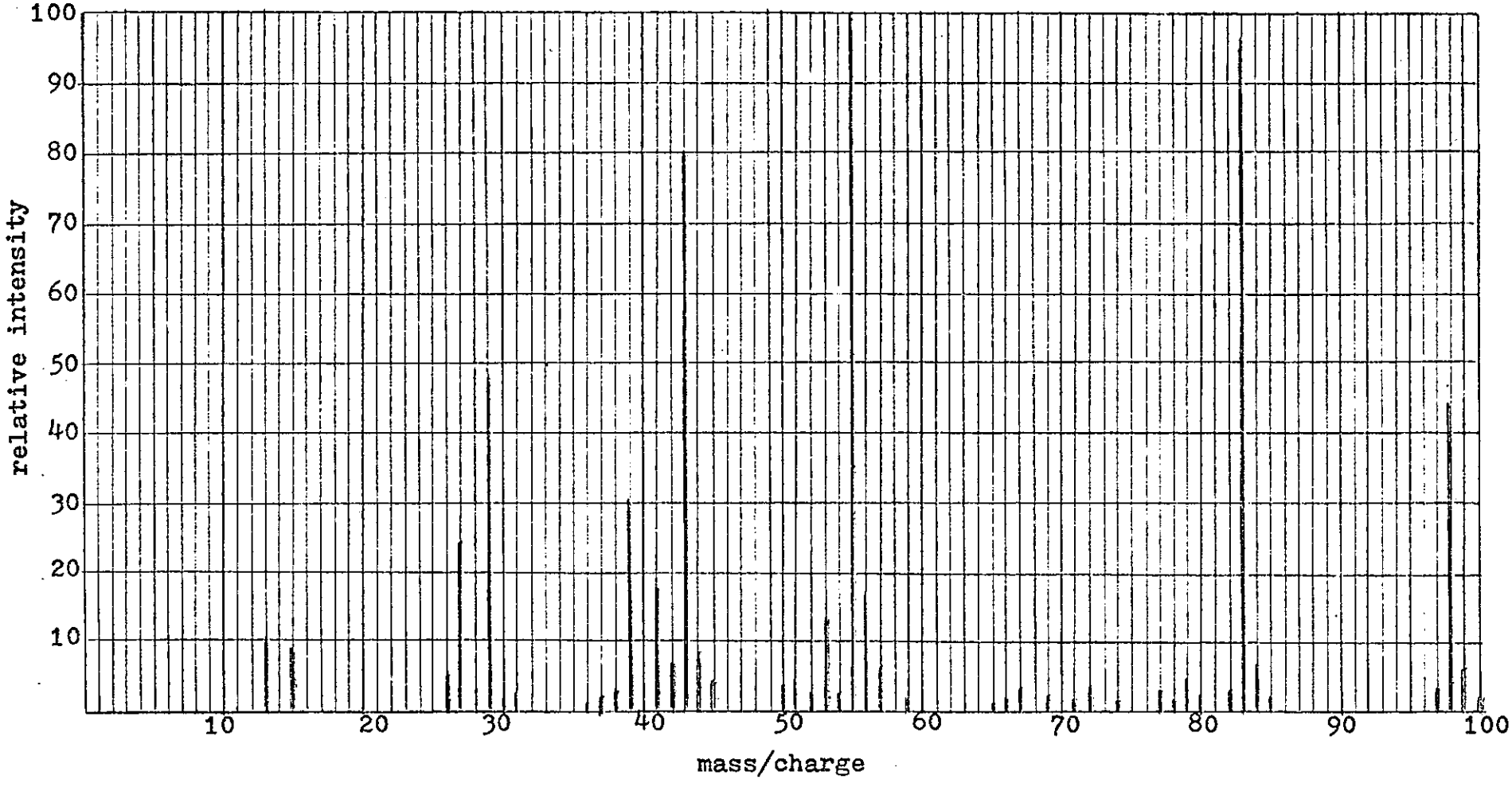

100
90
80
70
60
50
40
30
20
10
relative intensity
10
20
30
40
50
60
70
80
90
100
mass/charge
Spec #493 (EI)

Figure 60

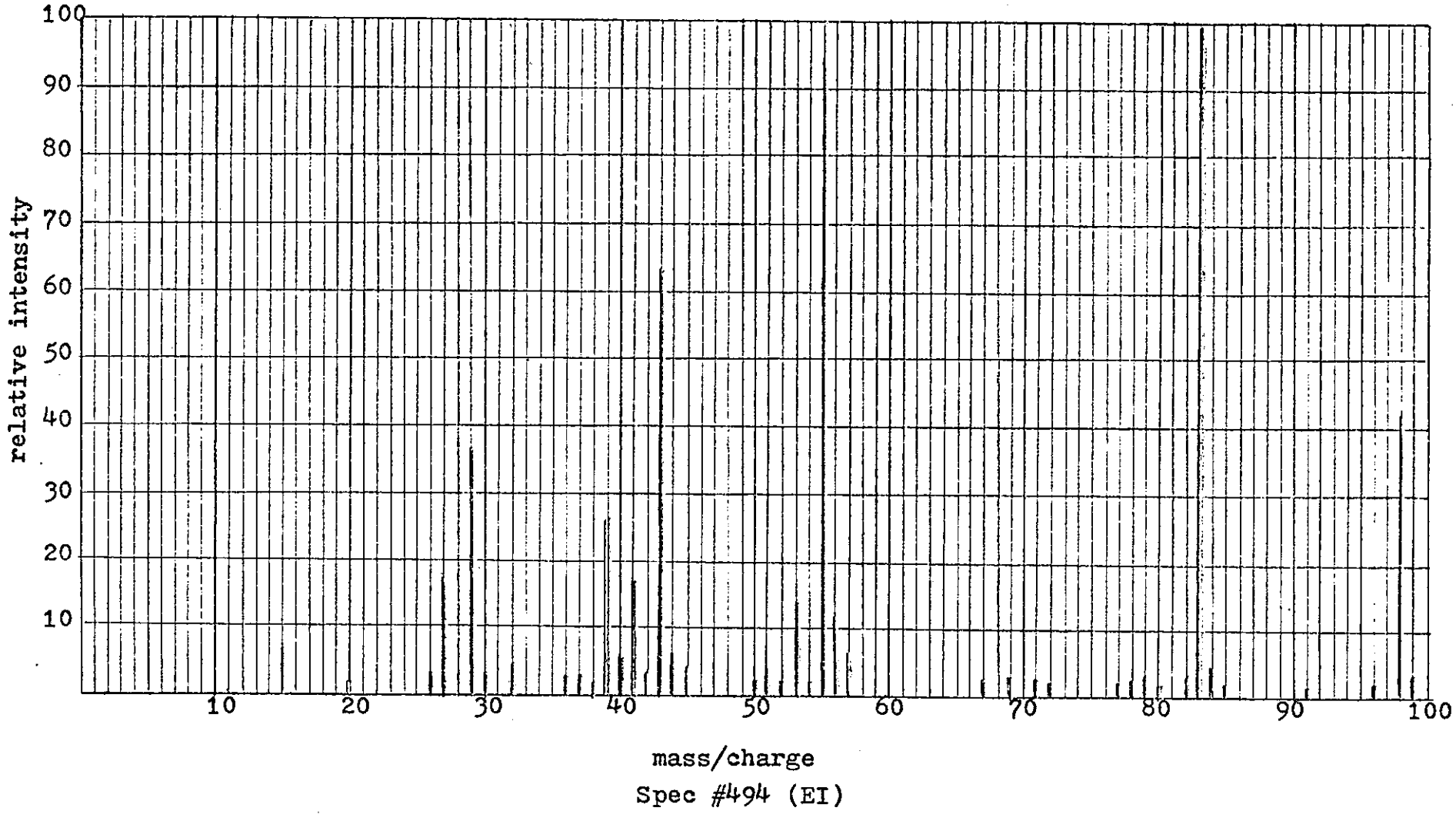

relative intensity
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
90
100
mass/charge
Spec #494 (EI)

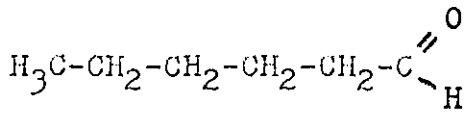
Figure 61

Mass Spectra #495 (Figure 62), from the peak immediately following the Mesityl Oxide peak on the G.C., has the familiar M and M+2 peaks at m/e 112 and 114, and is probably monochlorobenzene, an impurity in dichlorobenzene (the solvent).

The largest peak in the G.C. trace - aside from the solvent peak - is probably from the packing material. Mass Spectra #497 (Figure 63) had a base peak at m/e 281. The Mass Spectra from the last two G.C. peaks before the solvent peak (Figures 64 and 65) were not identified.

Dr. Girard and I were about ready to quit when we noticed Mass Spectra #491 (Figure 66). There were peaks at m/e 82, 72, 57, 56, 44, 43, 41, and 29 - just as in Spectra #451 and 452. The similarities between these spectra were too close to be coincidental: some compound probably did give peaks at m/e 72 and 82. This meant that its molecular weight had to be greater than 82 amu. It also had to fragment easily - otherwise its parent ion would be visible. Many aldehydes and alcohols lose a molecule of water fairly easily with EI M.S., so we searched for an alcohol or aldehyde which could give a Mass SPectra similar to that in #491.

After checking several references, we decided that Spectra #491 was that of n-Hexanal:



Some published Mass Spectra for n-Hexanal appear in Figures 67-71 (they differ slightly - perhaps a result of different ion accelerating potentials).

So I returned to Holy Cross with two candidates for the sex

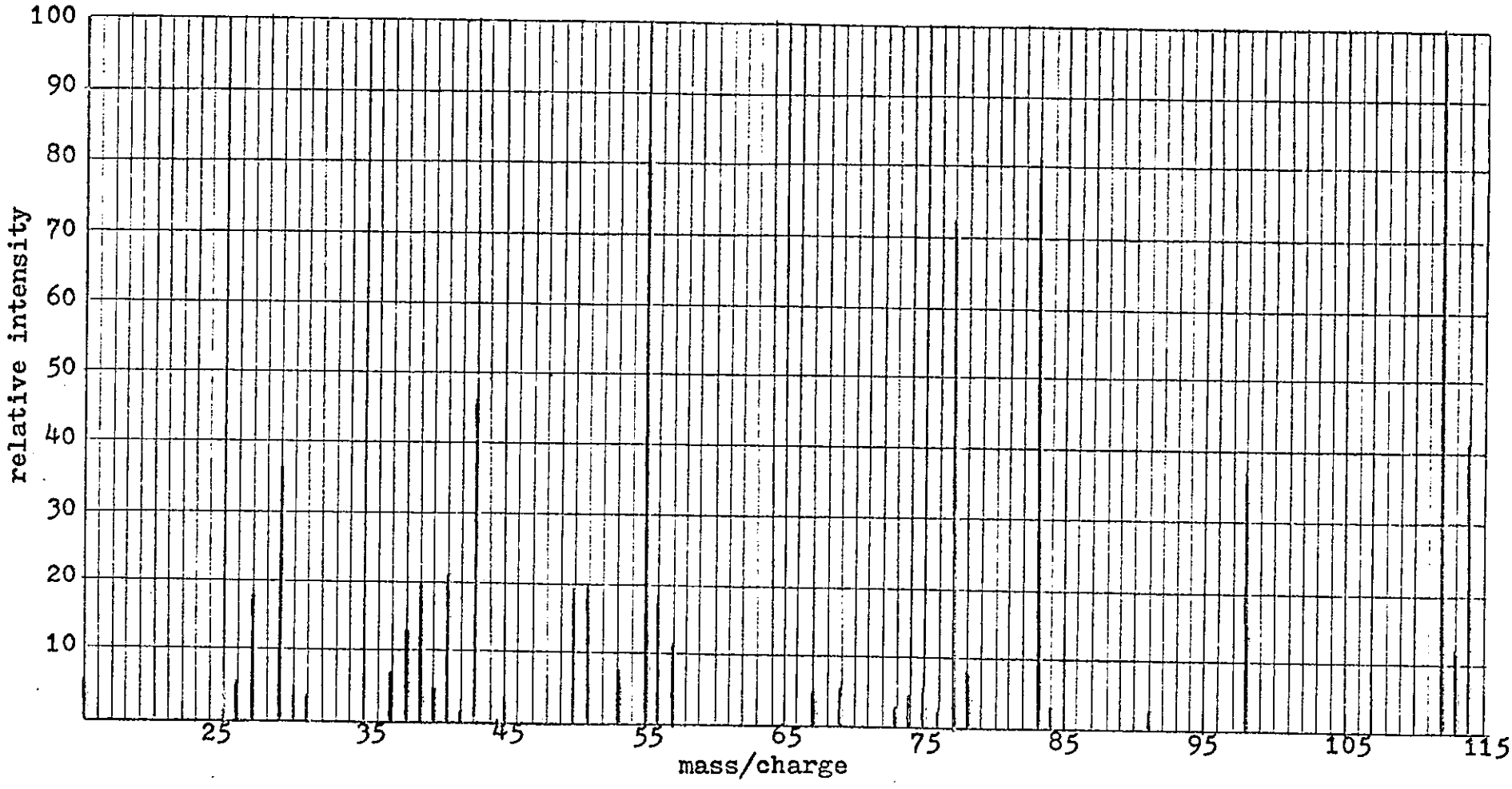
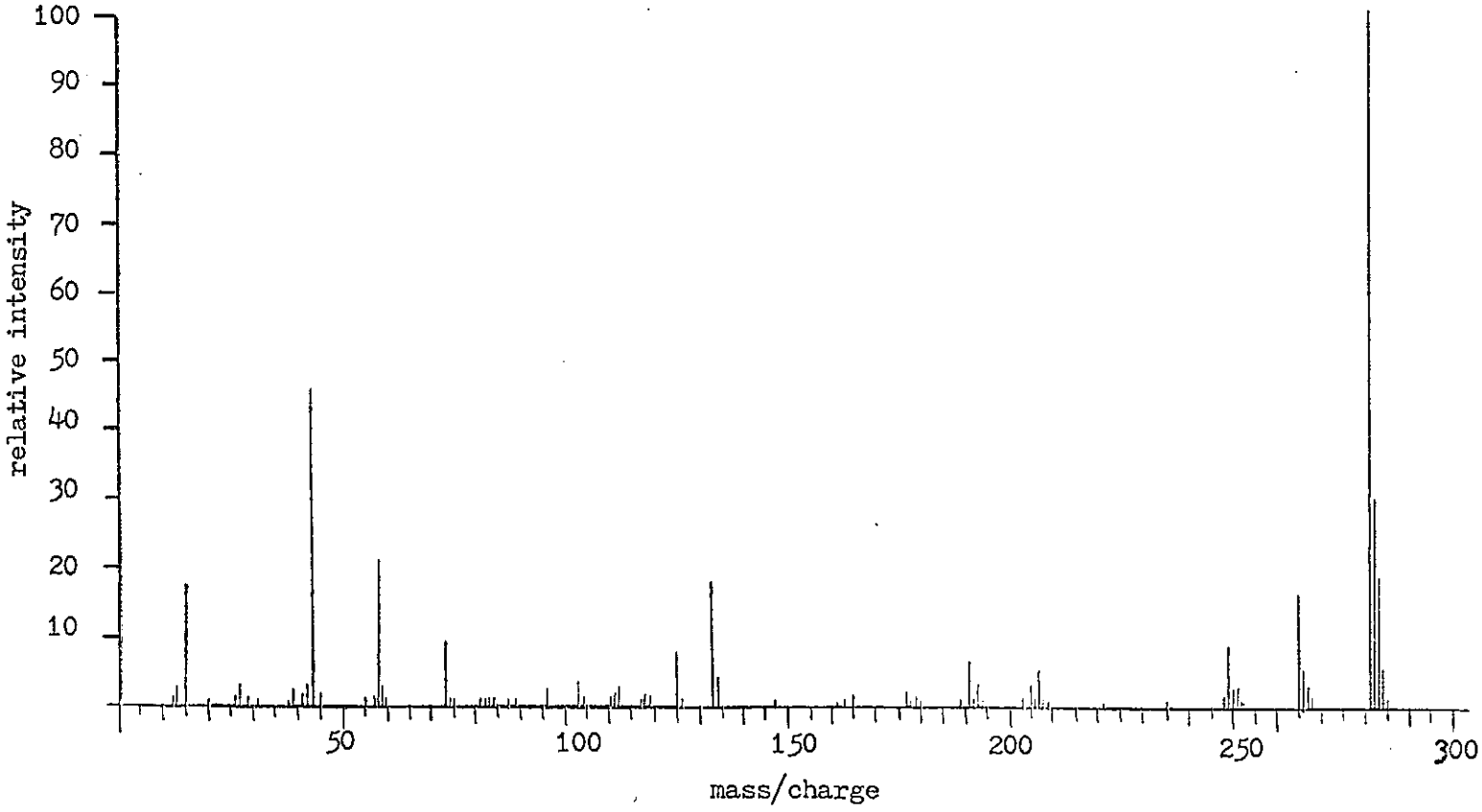

100
90
80
70
60
50
40
30
20
10
relative intensity
25
35
45
55
65
75
85
95
105
115
mass/charge
Spec #495 (EI)
Mono chlorobenzene

Figure 62



Spec #497 (EI)

PACKING MATERIAL

Figure 63

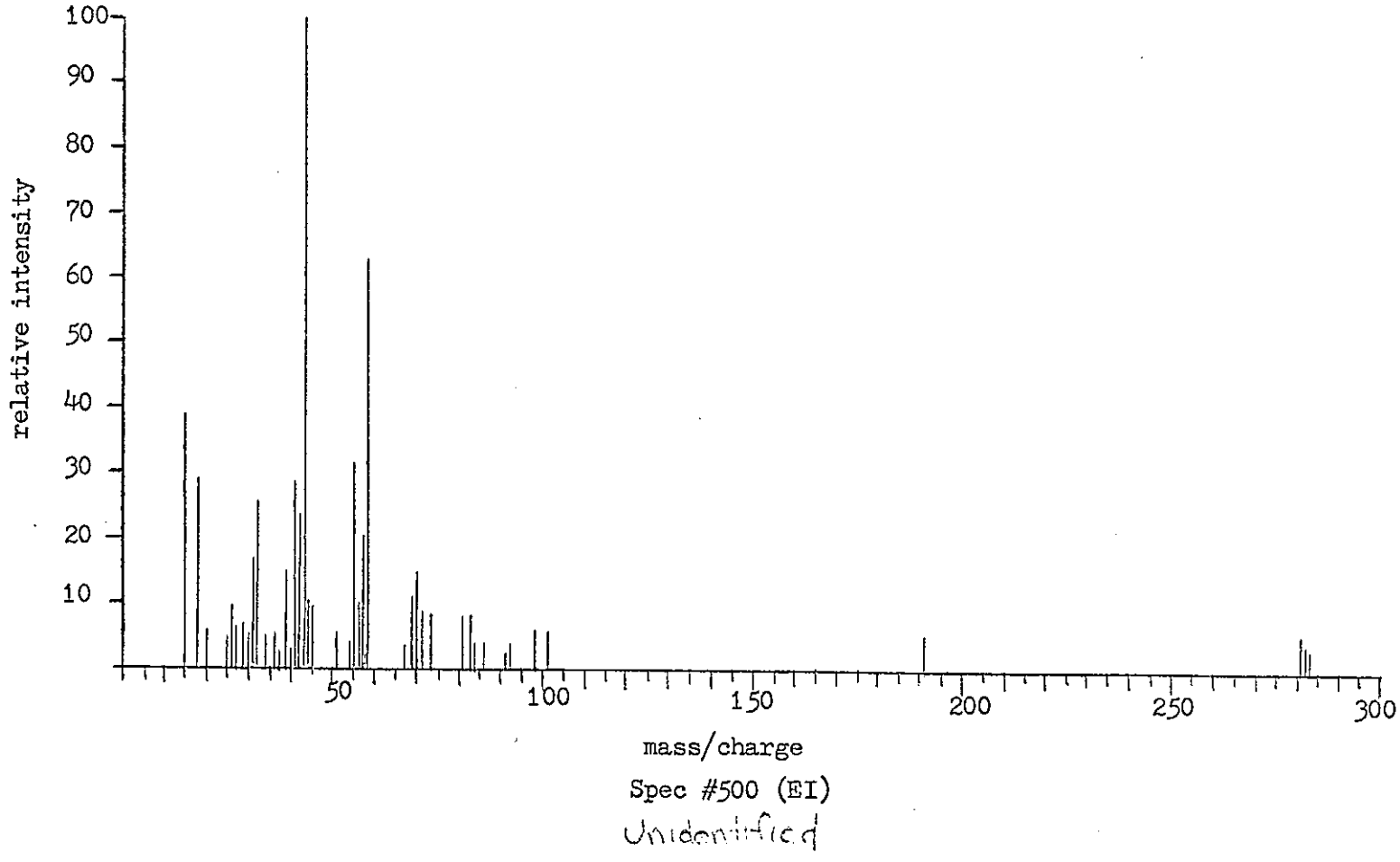
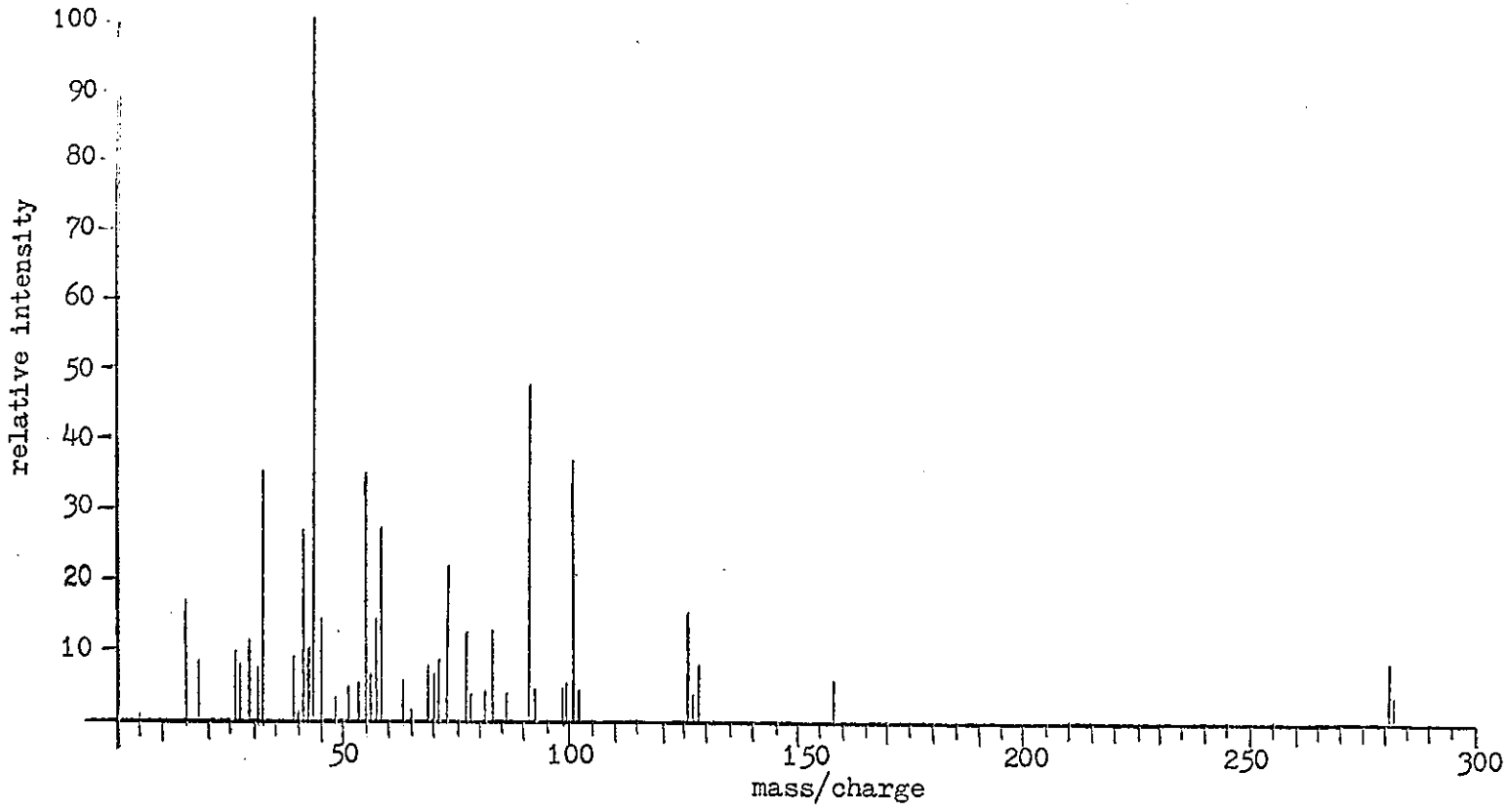

relative intensity
100
90
80
70
60
50
40
30
20
10
50
100
150
200
250
300
mass/charge
Spec #500 (EI)
Unidentified

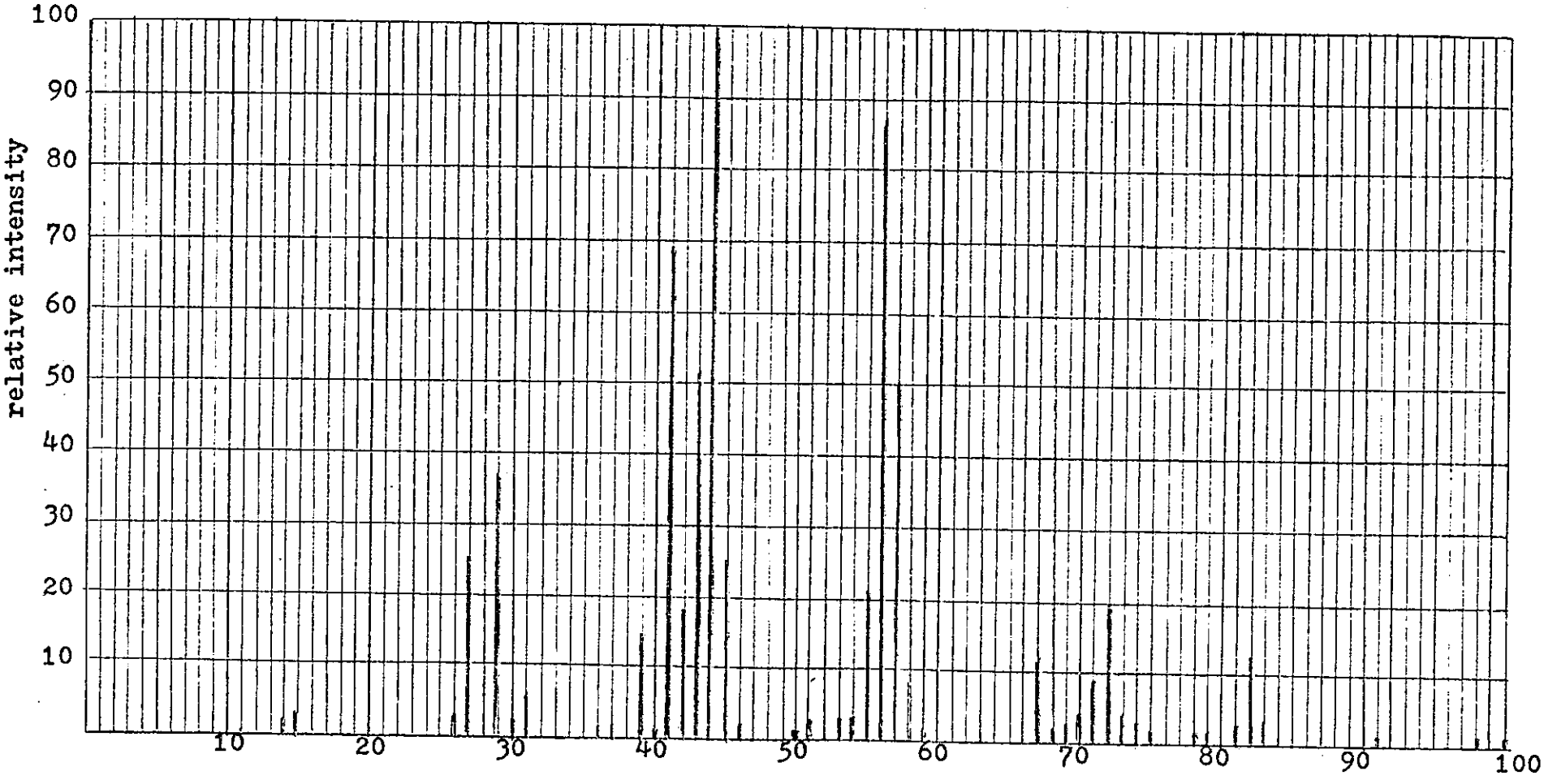
Figure 64



Spec #501 (EI)

unidentified

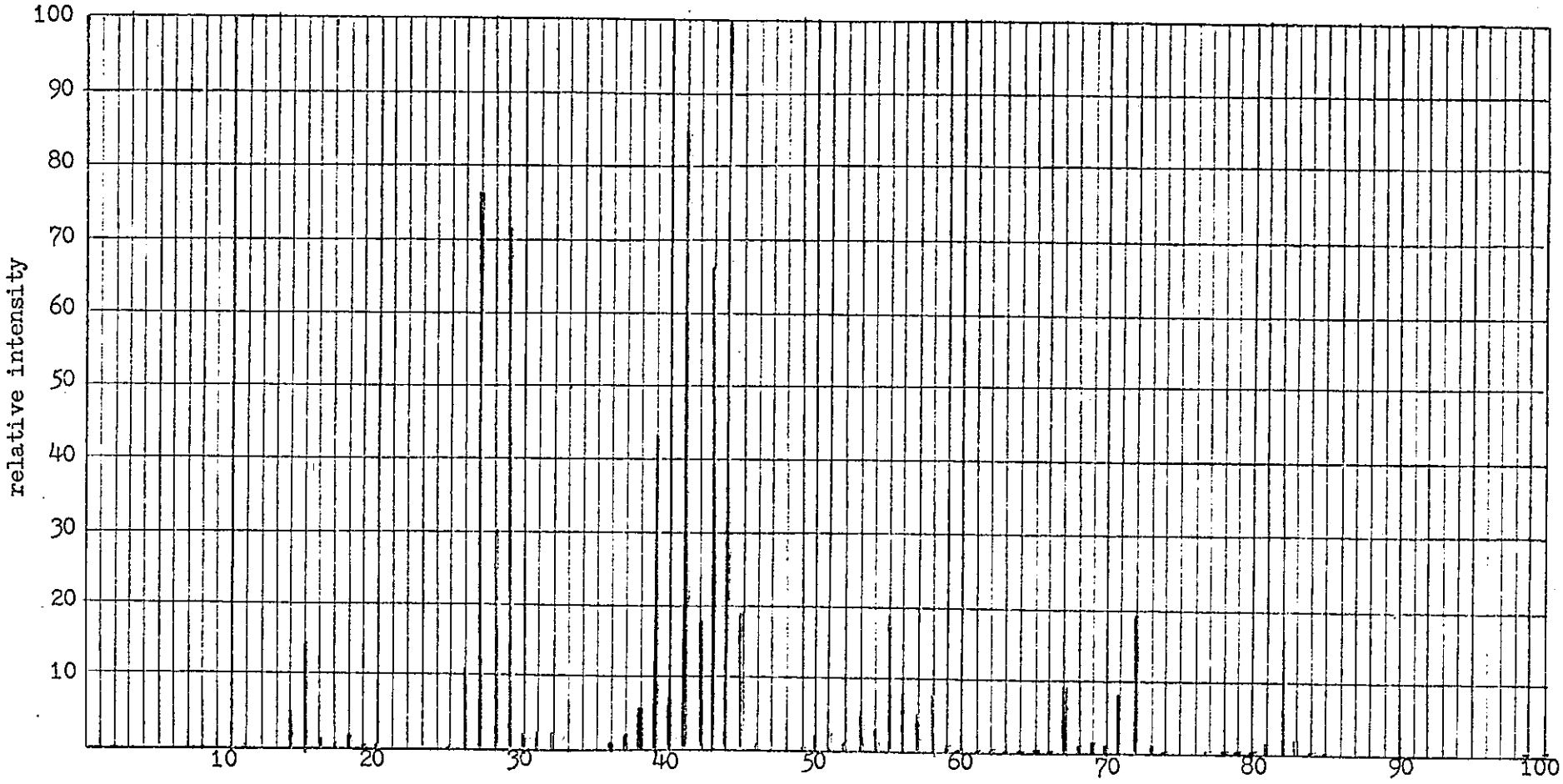
Figure 65



Spec #491 (EI)

Hexane

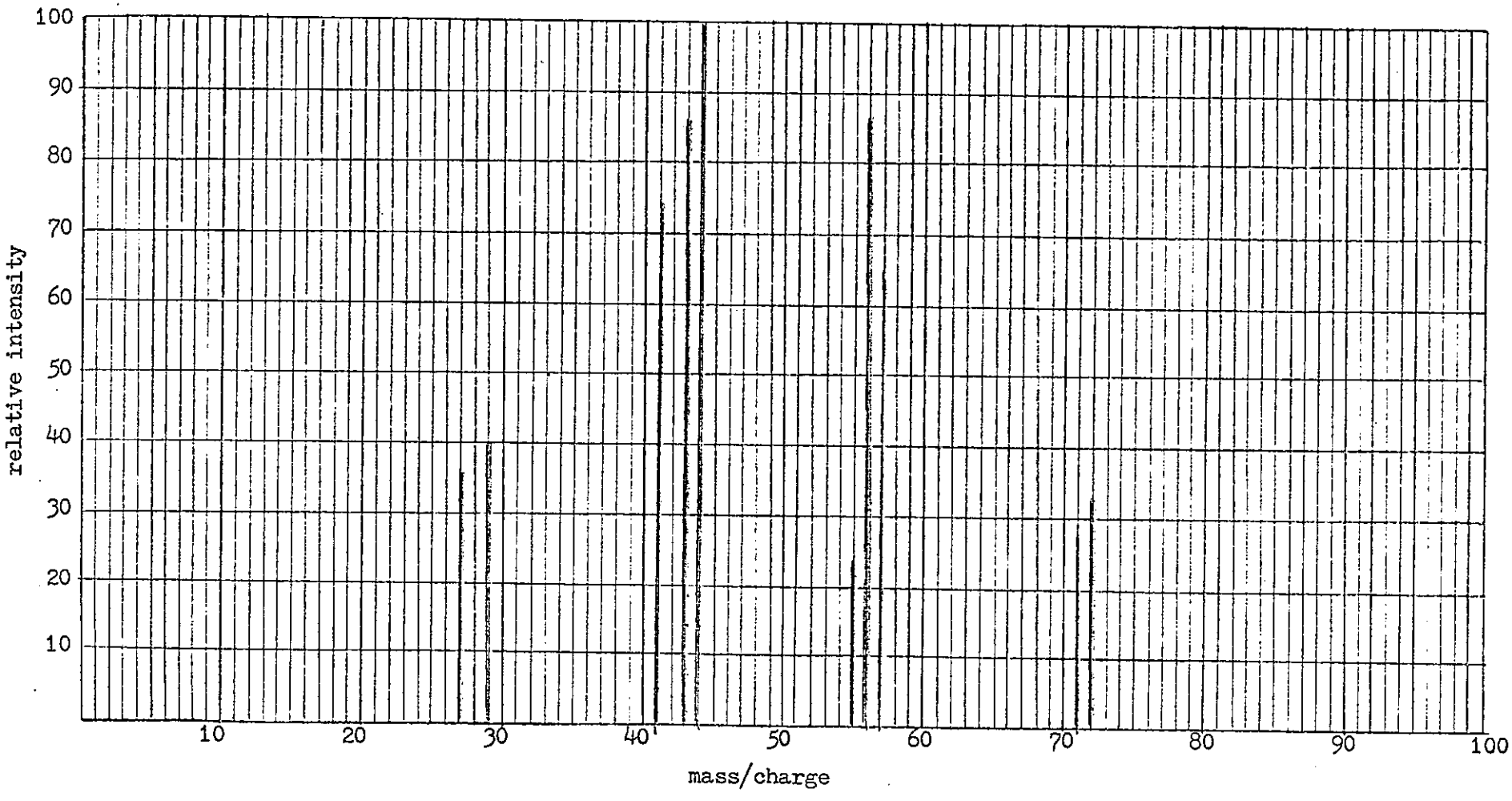
Figure 66



HEXANAL

Source: Mass Spectrometry Data Center
AWRE Aldermaston, Berkshire
MSDC 4420

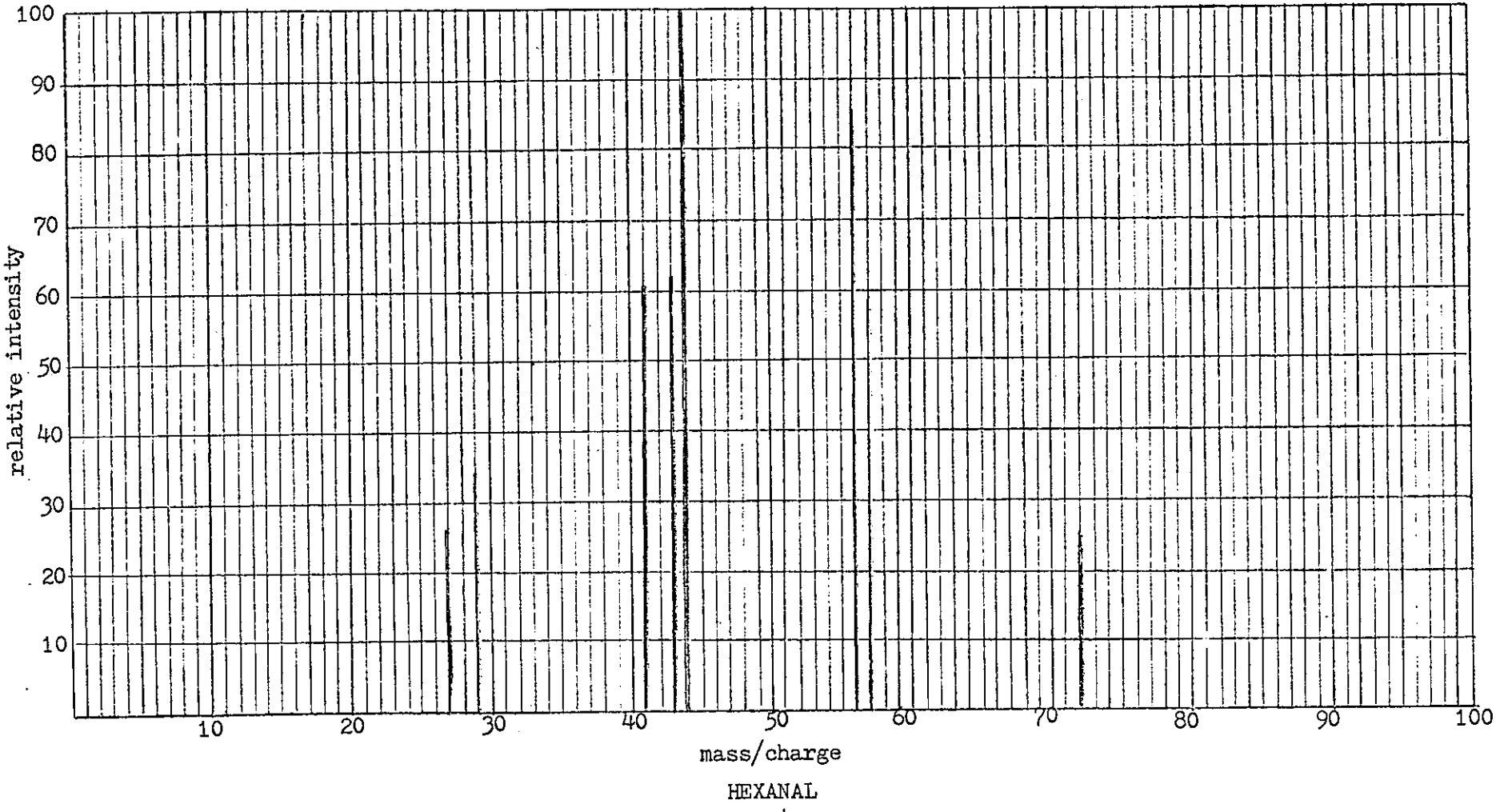
Figure 67



HEXANAL

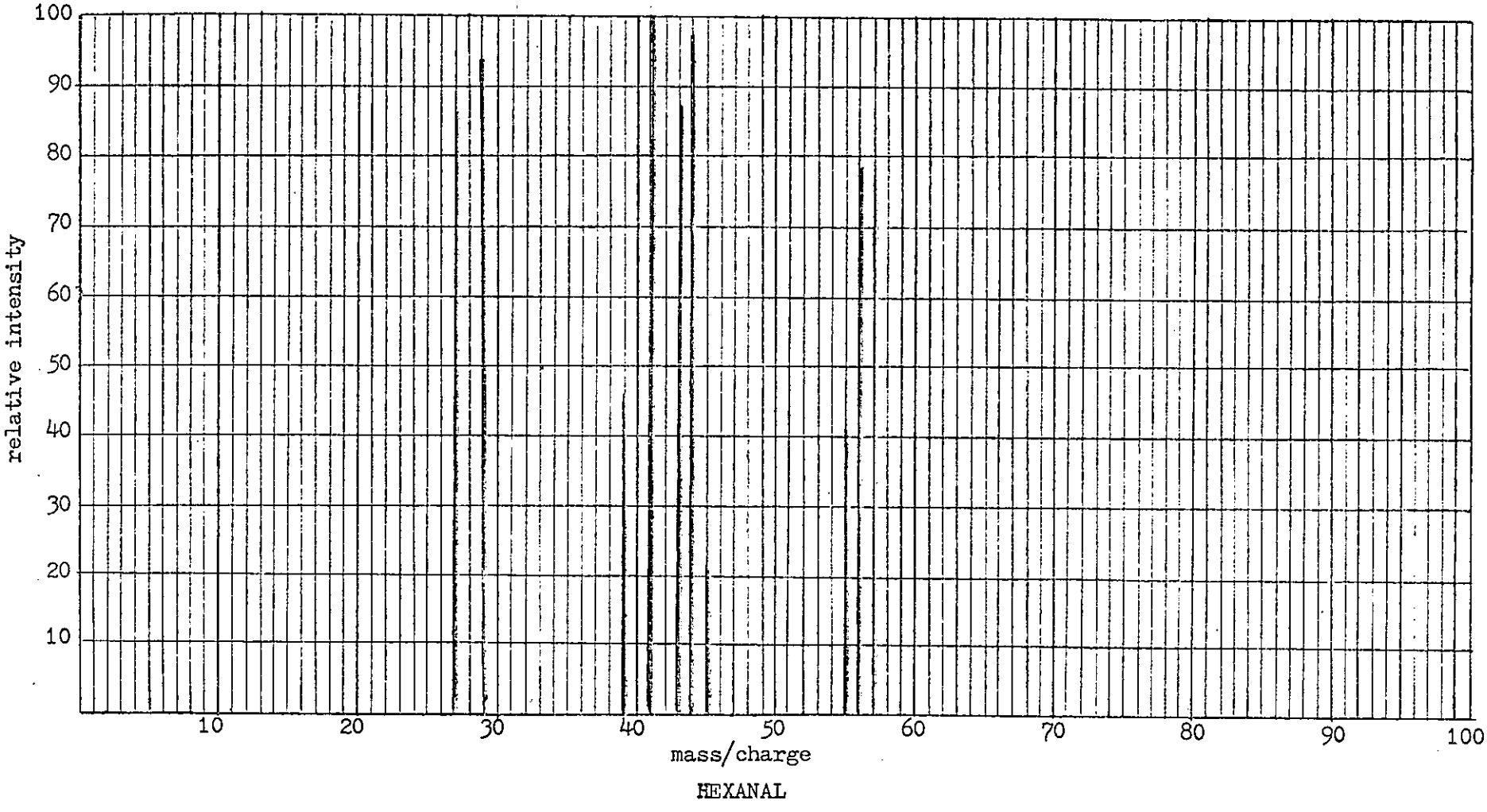
Source: Compilation of Mass Spectral Data, Vol.1, 2nd edition
A. Cornu & R. Massot, Heyden & Sons, Inc., 1975

Figure 68



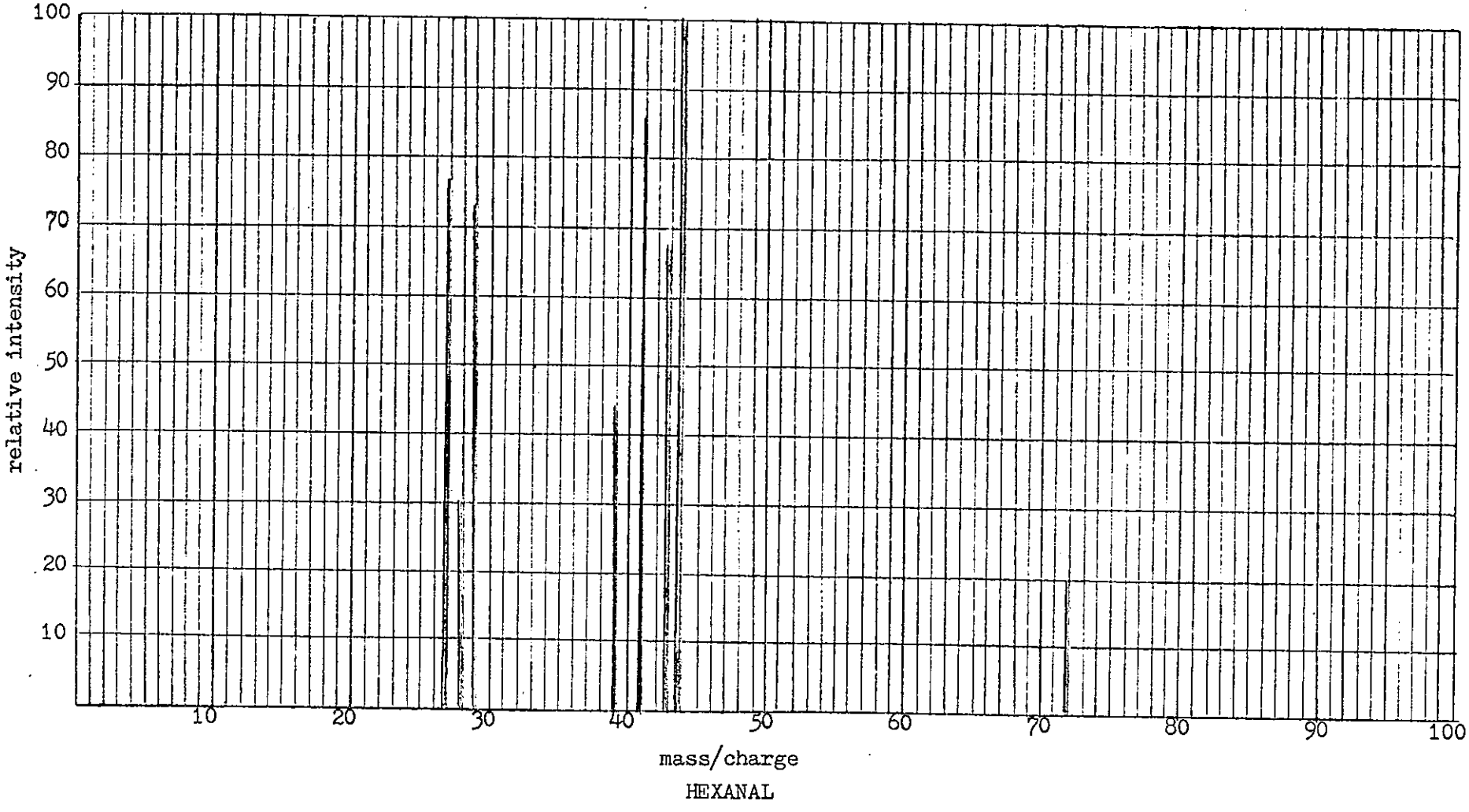
Source: Eight Peak Index of Mass Spectra, Vol.1,1st edition
Mass Spectrometry Data Center, AWRE
Aldermaston, Reading, U.K.

Figure 69



Source: Compilation of Mass Spectral Data, Vol.1, 2nd edition
A. Cornu & R. Massot, Heyden & Son, Inc., 1975

Figure 70



Source: D. Luke
Unilever Research Lab
Port Sunlight, Chesire, U.K.

Figure 71

pheromone: Hexanal and Mesityl Oxide. The first test of these two chemicals involved checking their retention times on the OV-17 column. If the times did not match that of peak A, the active peak, the chances were that these chemicals were not the sex pheromone. Mesityl Oxide was tried first. One drop of the Mesityl Oxide was mixed in one ml of methylene chloride. Ten μl of it were injected into the G.C. fitted with an OV-17 column. The temperature program rate was 4°/min, the setting used in collecting peak A. The Mesityl Oxide came off the column a little before 50° (see Figure 72) - which was nearly identical to the large peak of cut A (see Figure 29). Hexanal also came off the column at 50° (see Figure 73), although there is an unidentified impurity coming off at 125°C.

A sample of Mesityl Oxide was subjected to N.M.R. Spectroscopy to check its structure. The N.M.R. Spectra (Figure 74) shows three peaks: at 6, 2.2, and 1.8 ppm. All the peaks were singlets and in the ratio 1:3:6 for peaks 6, 2.2, and 1.8 ppm respectively. The Spectra was the one expected for Mesityl Oxide: the singlet at 6ppm corresponded to the vinylic H, the singlet at 2.2 ppm was from the primary Hydrogen atoms nearest the carbonyl group, and the last peak at 1.8 ppm came from the six equivalent primary Hydrogen atoms on the other end of the molecule.

The Hexanal was tried next. The N.M.R. Spectra (Figure 75) was a bit more complex: a triplet at 9.8 ppm, a sextet at 2.5 ppm, a multiplet centered at 1.7 ppm, and a triplet at .8 ppm with peak ratios 1:2:6:3 respectively. The aldehydic Hydrogen of Hexanal was responsible for the peak at 9.8 ppm, and it was split into three peaks by the two Hydrogen atoms on the adjacent carbon atom. The

Chart speed: 1 in/min

Mesityl Oxide in Methylene Chloride

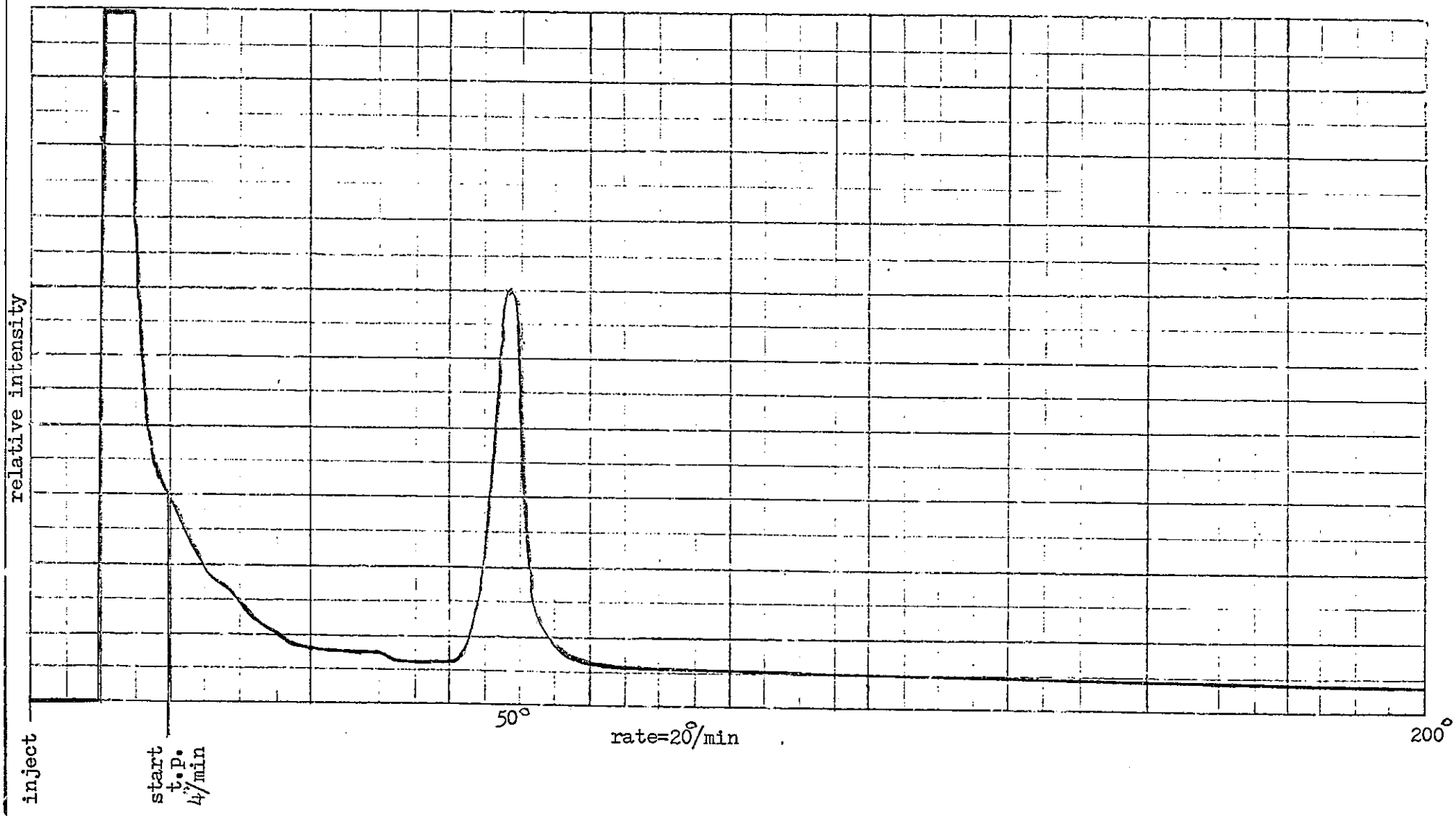


Figure 72

Chart speed - 72 in/min

Hexanal (New Bottle) in Methylene Chloride

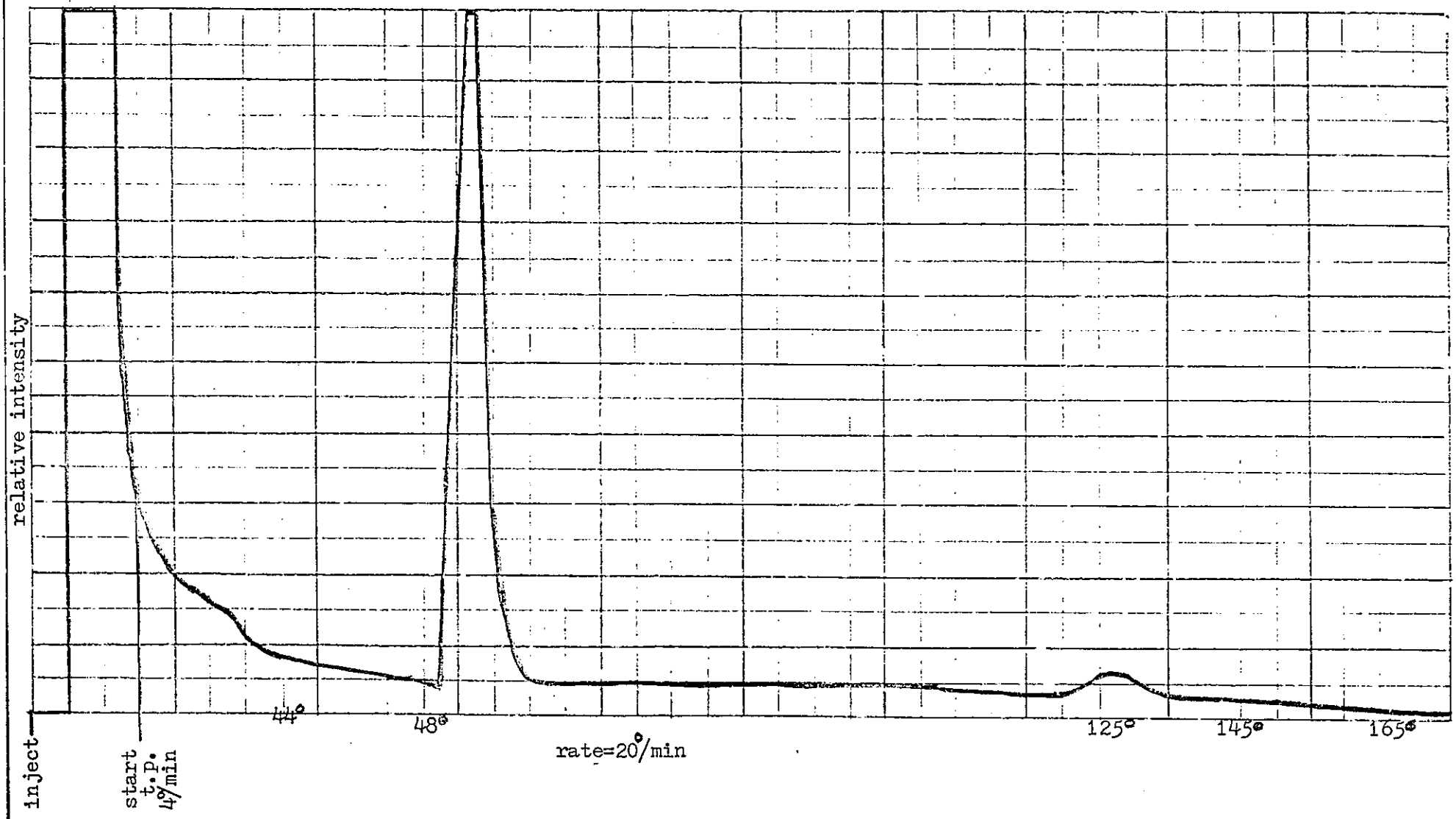
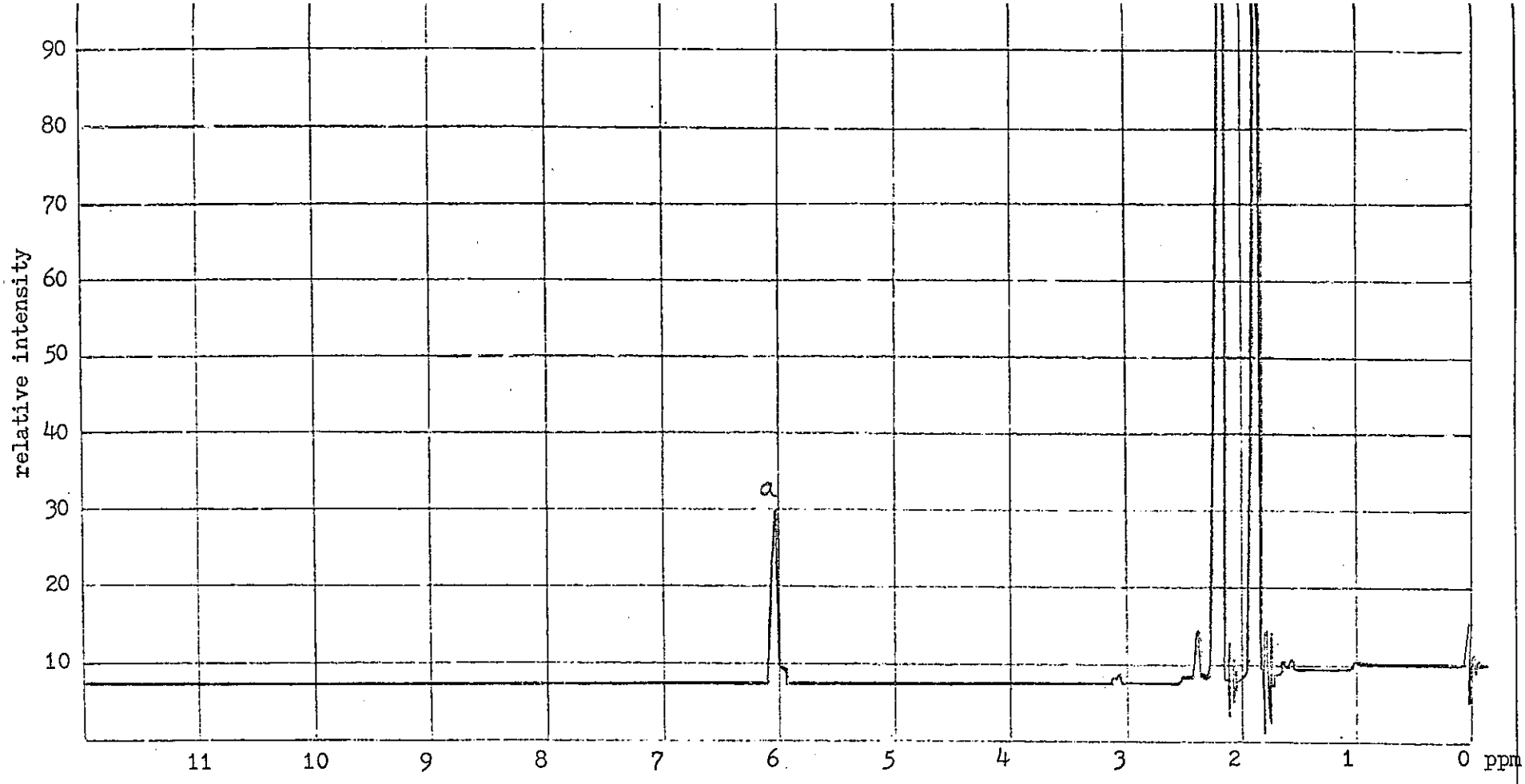


Figure 73



Reference - TMS
Sweep Width: 600 Hz
Sweep Time: 250 sec
Time Constant: .01
Shift: 0 ppm
R.F. Field: 5×10^3 μV
Sensitivity: 5 x 1

Mesityl Oxide

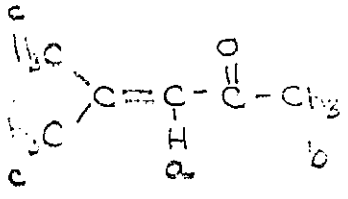
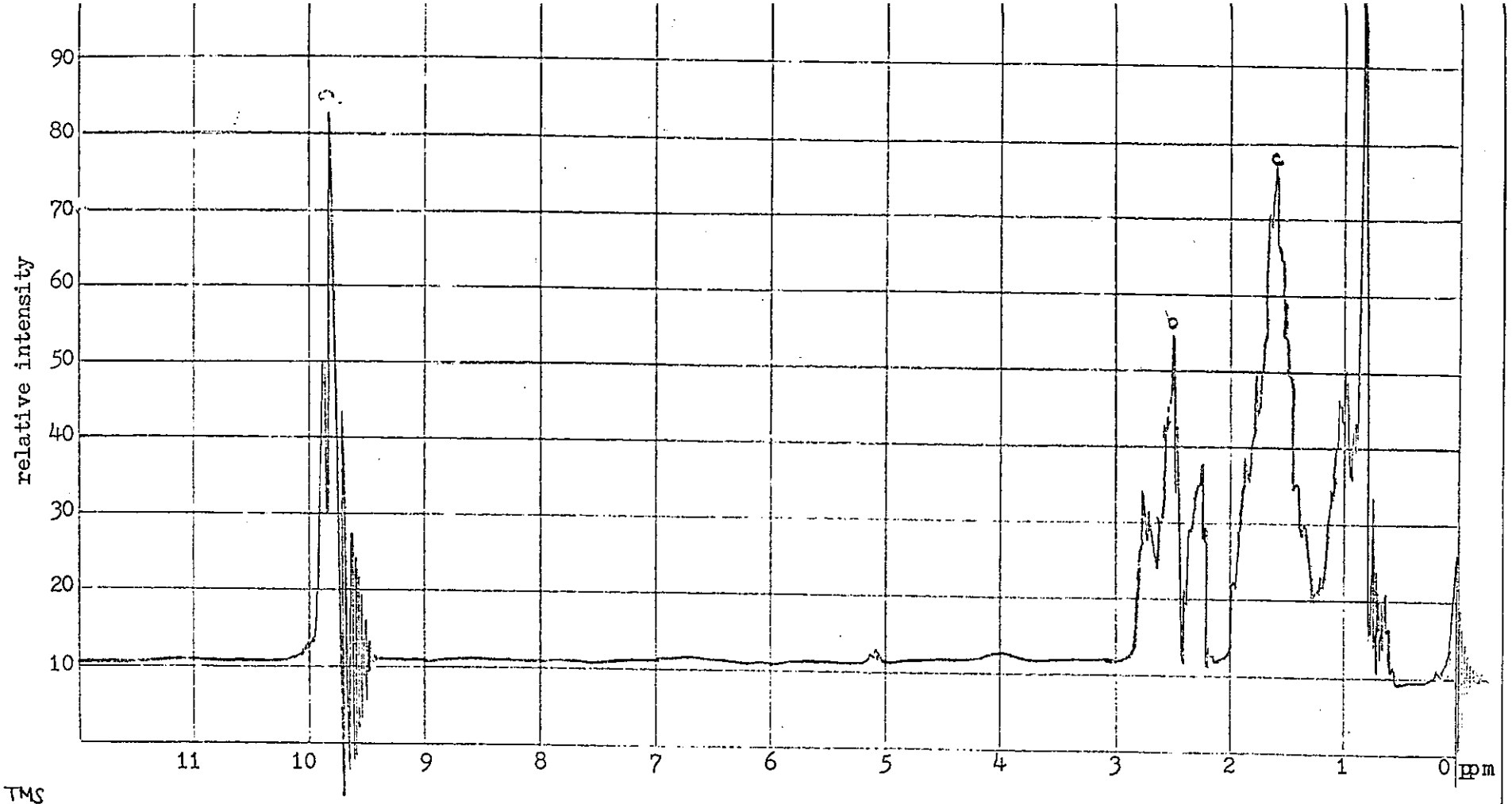


Figure 74



Reference: TMS
Freq Width: 600 Hz
Freq Time: 250 sec
Time Constant: .01
Shift: 0ppm
R.F. field: 5×10^3 μV
Sensitivity: 8 x 1

Hexanal (New Bottle)

$$H_3C-\overset{d}{C}H_2-\overset{c}{C}H_2-\overset{c}{C}H_2-\overset{b}{C}H_2-\overset{a}{C}HO$$

Figure 75

next peak, at 2.5 ppm, came from the two Hydrogen atoms on the carbon atom next to the carbonyl group. It was split into six peaks by the one Hydrogen atom on the one adjacent carbon atom and the two Hydrogen atoms on the other adjacent carbon atom. The multiplet at 1.7 ppm was a result of an overlap of the peaks of the remaining secondary Hydrogen atoms in the molecule. The last peak at .8 ppm came from the three primary Hydrogen atoms on the carbon atom furthest removed from the carbonyl group. It was split into three parts by the two Hydrogen atoms on the adjacent carbon atom.

The crucial tests were the bioassays - would the female flesh flies be attracted to either of the two compounds? One ml of Mesityl Oxide was dissolved in 100 ml of methylene chloride and stirred for an hour. The Y-tube was set up for the bioassay. Ten µl of the Mesityl Oxide-methylene chloride solution (about 100 µg of Mesityl Oxide) were shot onto a piece of filter paper and placed in one of the flasks (+1). Ten µl of pure methylene chloride on a piece of filter paper in the other flask (-1) served as a control. The 30 minute bioassay gave the following:

	Mesityl Oxide				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	2	1	0	7	-1.0
15 min	8	1	0	0	6	+2.5
30 min	3	1	1	3	7	-5.0

Although the concentration may have been either too high or too low, the female flesh flies were not attracted to the Mesityl Oxide.

Two solutions of Hexanal were prepared: one ml of Hexanal dissolved in 100 ml of methylene chloride and one ml of Hexanal in 50 ml of methylene chloride. Both were stirred for an hour.

A 10 μl shot of the less concentrated solution (containing about 100 μg of Hexanal) was used in the next bioassay along with a 10 μl blank of methylene chloride. The results after the first 15 minutes were:

	Hexanal				Blank	
	+1	+½	0	-½	-1	Total
15 min	9	2	1	0	2	+8.0

The test was repeated and gave the following:

	Hexanal				Blank	
	+1	+½	0	-½	-1	Total
5 min	5	2	2	0	6	0
15 min	7	0	2	0	6	+1.0
35 min	11	0	0	0	4	+7.0

The more concentrated Hexanal solution was tried in the next two bioassays. Only four μl were used in the tests (about 80 μg of Hexanal). The results of the first assay were:

	Hexanal				Blank	
	+1	+½	0	-½	-1	Total
15 min	10	2	4	0	2	+9.0

The second bioassay gave the following:

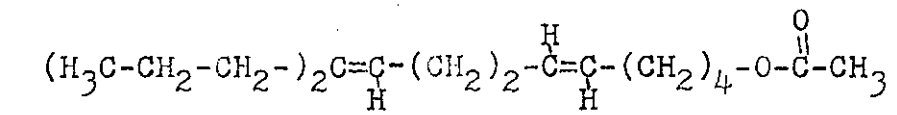
	Hexanal				Blank	
	+1	+½	0	-½	-1	Total
15 min	7	2	3	0	3	+5.0

Preliminary bioassays with Hexanal and male flesh flies seem to indicate that they are not attracted to it, implying that we have found at least one component of the sex pheromone in Sarcophaga bullata.

Discussion of Results and Conclusions

The evidence strongly suggests that Hexanal is a sex attractant for female flesh flies. We must consider, however, the work of John Budris: no activity was found in any cut made from the G.C. except the 80°-86° one. Why did he not find activity in the 50° region? No definitive answer can be given but it seems likely that he never collected the Hexanal with his dry ice cold trap. Even with the liquid nitrogen cold trap, which was much, much colder than the dry ice one, we had difficulty collecting the Hexanal peak. It took three capillary tubes of cut A to give less than a tenth of a microgram of Hexanal. It would hardly be surprising if Budris failed to collect the Hexanal. Since he did not re-inject a sample of his collections into the G.C. to verify that he actually did collect something, there is no way of knowing whether he was able to collect the Hexanal or not.

He did obtain, however, significant activity between 80°-86° C. from his G.C. work. Time did not permit us to find the compound responsible for activity in this region, but it certainly did not contradict our findings. In the literature, there are many reports of multi-component pheromone systems. For example, the sex pheromone of the pink bollworm moth, Pectinophora gossypiella, was found to be 10-propyl-trans-5-trideca-5,9-diene acetate:[37]



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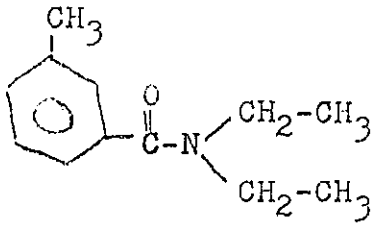
[37] Beroza, M., Chemicals Controlling Insect Behavior, New York: Academic Press, 1970.

Although it attracted male moths in the laboratory, the addition of N,N-diethyl-m-toluamide greatly enhanced the activity of the pheromone:



So it is entirely conceivable that Hexanal is only one of the components of the sex pheromone of the flesh fly Sarcophaga bullata and that the compound responsible for the peak between 80°-86° on the G.C. may be another. But Hexanal is an aldehyde and aldehydes oxidize fairly easily to form carboxylic acids. And carboxylic acids give very broad peaks on the OV-17 column. Futhermore, the tests of Mike Gottschalk indicated that the active peak which Budris had found was probably from a carboxylic acid. Could Hexanoic Acid, the oxidation product of Hexanal, be another component of the pheromone system?

A drop of Hexanoic Acid was added to one ml of methylene chloride and nine microliters of this was injected into the G.C. The Hexanoic Acid yielded a very broad peak - starting at about 81° and continuing until about 92°, after which it quickly dropped back to the baseline (see Figure 76). It must be mentioned that Budris used a temperature program rate of 2°/min when he collected the 80°-86° cut while I used a rate of 4°/min. It is very likely that the Hexanoic Acid would come off the OV-17 column at a slightly lower temperature if the slower programming rate were used, perhaps between 80°-86°. Unfortunately, preliminary bioassays with Hexanoic Acid have shown it to be inactive so the active peak found by John Budris may not be this compound. Perhaps it is some compound very similar to Hexanoic Acid - such as

Chart speed: 1 in/min

Hexanoic Acid in Methylene Chloride

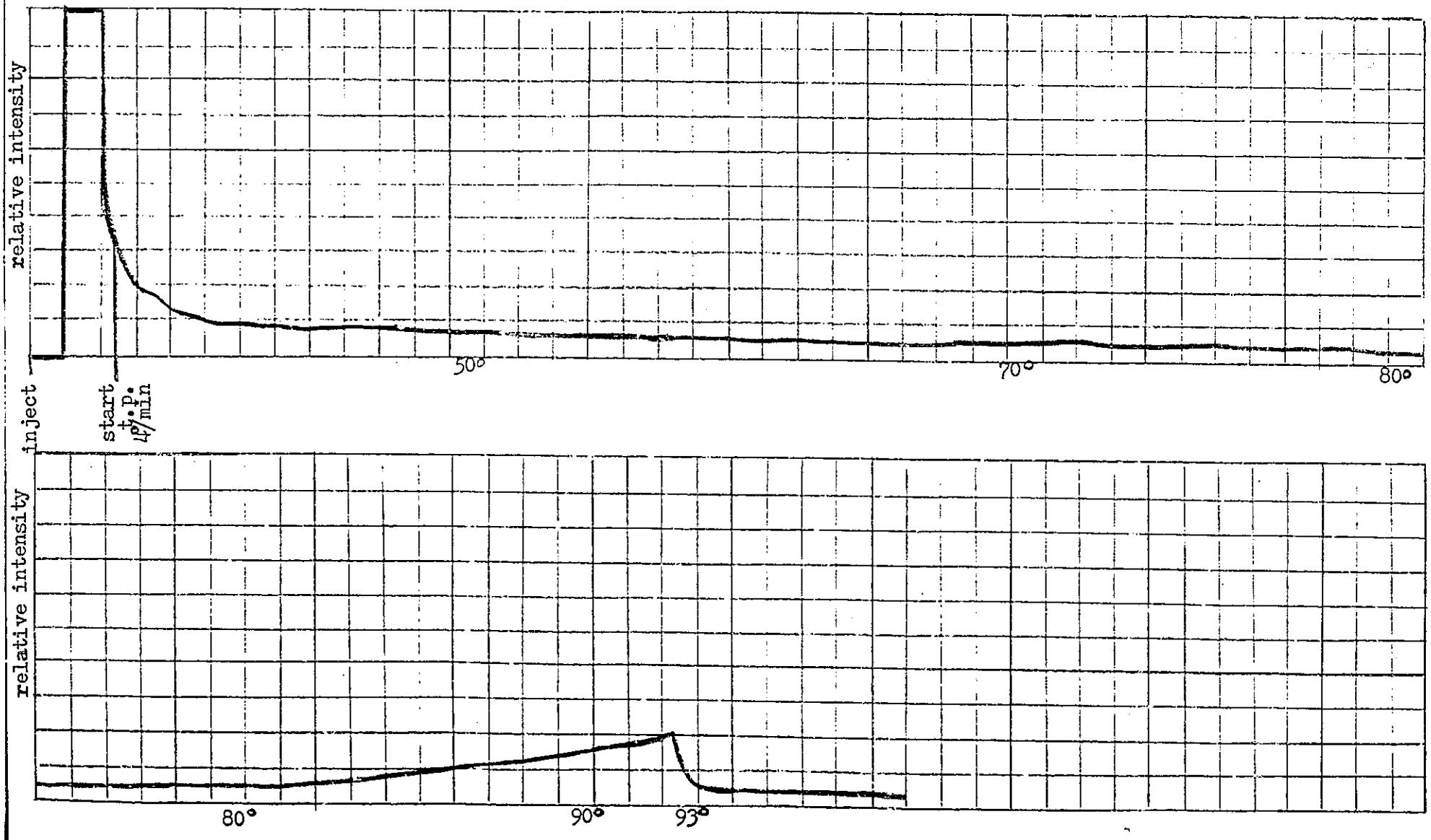


Figure 76

Hexenoic Acid.

The only other thing left to explain is the absence of a double bond in Hexenal: Gottschalk's work suggested that the pheromone contained a double bond. One possible explanation is that the compound giving rise to the peak at 80°-86° on the G.C. contains a double bond. Perhaps, too, the tests gave misleading results: the chemicals which he employed for the tests may have deactivated the Hexanal.

In conclusion, we believe that we have found at least one of the components of the sex pheromone of the flesh fly Sarcophaga bullata and that it is Hexanal. Hexanal has been found to attract female flesh flies. Furthermore, the Mass Spectra of one of the compounds in the active region (40°-57°) on the G.C. trace closely resembles that of Hexanal. None of the other identified compounds in that region have been found to be active. Also, the retention time of Hexanal on an OV-17 column falls within the region of activity. It is also quite volatile (B.P. =120°C.) and this could account for the observation that samples of the active extract 183-6 lost activity when left out in the air at room temperature. It seems likely that this compound would come off a Porasil column with 100% hexane, as found by Robin Vita.

If Hexanal does prove to be a good attractant for the female flesh fly - and only field tests will tell - it would probably be quite useful in helping to control the flesh fly. Many pheromones are so costly to synthesize that they are not practical alternatives to insecticides. Hexanal, on the other hand, is relatively easy to

synthesize and therefore inexpensive. Furthermore, its odor is not particularly offensive to humans. Once field studies have been completed, Hexanal may be used in flypaper and other types of traps to help control the flesh fly populations.

Bibliography

Beroza, M., Chemicals Controlling Insect Behavior, New York: Academic Press, 1970.

Beroza, M., Pest Management with Insect Sex Attractants, Washington, D.C.: American Chemical Society Publication, 1976.

Birch, M., Pheromones, New York: American Elsevier Publishing Company, Inc., 1974.

Blakeslee, D., Chinn, W., Introductory Statistics and Probability, Boston: Houghton Mifflin Company, 1975.

Brady, U., Pheromones, II: Current Research, New York: MSS Information Corporation, 1974.

"The Bugs Are Coming," Time, July 12, 1976, pp. 38-46.

Carlson, D., Beroza, M., "Field Evaluations of (Z)-9-Tricosene, A Sex Pheromone of the Housefly," Environmental Entomology, v. 2, August, 1973, pp.555-559.

Carlson, D., Mayer, M., Silhacek, D., James, J., Beroza, M., Bierl, B., "Sex Attractant Pheromone of the Housefly: Isolation, Identification and Synthesis," Science, v. 174, pp. 76-78.

Hardyck, C., Petrinovich, L., Introduction to Statistics for the Behavioral Sciences, Philadelphia: W.B. Saunders Co., 1969.

Hewitt, C., Houseflies and How They Spread Diseases, New York: Cambridge University Press, 1912.

Hughes, P., "Pheromones of Dendroctonus: Origin of α-Pinene Oxidation Products Present in Emergent Adults," J. Insect Physiol., v. 21, pp. 687-691.

Jacobsen, M., Insect Sex Pheromones, New York: Academic Press, 1972.

Jacobsen, M., Beroza, M., "Insect Attractants," Scientific American, v. 211, pp. 20-27.

Kafka, Wolf, "Physicochemical Aspects of Odor Reception in Insects," Ann. N.Y. Acad. Sci., v. 237, pp. 115-128.

Kaib, M., "Die Fleisch- und Blumenduftrezeptoren auf der Antenne der Schmeißfliege Calliphora vicina," J. Comp. Physiol., v. 95, pp. 105-121.

Leyrer, R., Monroe, R., "Isolation and Identification of the Scent of the Moth, Galleria mellonella, and a Revaluation of its Sex Pheromone," J. Insect Physiol., v. 19, pp. 2267-2271.

Bibliography - Continued

Libbey, L., Morgan, M., Putnam, T., Rubinsky, J., "Pheromones Released During Inter- and Intra-Sex Response of the Scolytid Beetle Dendroctonus brevicomis," J. Insect Physiol., v. 20, pp. 1667-1671.

Mayer, M., Thaggard, C., "Investigations of an Olfactory Attractant Specific for Males of the Housefly, Musca domestica, J. Insect Physiol., v. 12, pp. 891-897.

Meresz, O., Mansingh, A., Steele, R., Smallman, B., Mozogai, C., "Pheromone Effects of Cis-9-Long Chain Alkenes on the Common Housefly - An Improved Sex Attractant Combination," Can. Ent., v. 104, pp. 1963-1965.

Mitchell, E., "Disruption of Pheromonal Communication Among Coexistent Pest Insects with Multichemical Formulations," BioScience, v. 25, August 1975, pp. 493-499.

Muhammed, S., Butler, J., Carlson, D., "Stable Fly Sex Attractant and Mating Pheromones Found in Female Body Hydrocarbons," J. Chem. Ecol., v. 1, pp. 387-398.

Murvosh, C., Fye, R., Labrecque, G., "Studies on the Mating Behavior of the Housefly, Musca domestica," Ohio Journal of Science, v. 64, July 1964, pp. 264-271.

Murvosh, C., Labrecque, G., Smith, C., "Sex Attraction in the Housefly, Musca domestica," Ohio Journal of Science, v. 65, March 1965, pp.68-71.

Mustaparta, H, "Response of the Pine Weevil, Hylobius abietis, to Bark Beetle Pheromones," J. Comp. Physiol., v. 88, pp. 395-398.

O'Connell, R., "Olfactory Receptor Responses to Sex Pheromone Components in the Redbanded Leafroller Moth," Journal of General Physiol. v. 65, pp. 179-205.

Renwick, J., Hughes, P., Tanletin, D., "Oxidation Products of Pinene in the Bark Beetle, Dendroctonus frontalis," J. Insect Physiol v. 19, pp. 1735-1740.

Richter, I., "Sex Attraction of the Housefly by Moving Dummies Is Not Increased by Pheromones," Naturwissenschaften, v. 61, p. 365.

Rogoff, W., Gretz, G., Jacobsen, M., Beroza, M., "Confirmation of (Z)-9-Tricosene as a Sex Pheromone of the Housefly," Ann. Ent. Soc. Am., v. 66, pp.739-741.

Schneider, D., Boppré, M., Schneider, H., Thompson, W., Boriack, C., Petty, R., Meinwald, J., "A Pheromone Precursor and Its Uptake in Male Danaus Butterflies," J. Comp. Physiol., v. 97, pp. 245-256.

Sharma, R., Saxena, K., "Orientation and Developmental Inhibition in the Housefly by Certain Terpenoids," J. Med. Ent., v. 11, November 25, 1974, pp. 617-621.

Shorey, H., Animal Communication by Pheromones, New York: Academic Press, 1976.

Sláma, K., Romaňuk, M., Šorm, F., Insect Hormones and Bioanalogues, New York: Springer-Verlag, 1974.

Tamaki, Y., Yushima, T., "Sex Pheromone of the Cotton Leafworm, Spodoptera littoralis," J. Insect Physiol., v. 20, pp. 1005-1014.

Uebel, E., Sonnet, P., Bierl, B., Miller, R., "Sex Pheromone of the Stable Fly: Isolation and Preliminary Identification of Compounds that Induce Mating Strike Behavior," J. Chem. Ecol., v. 1, pp. 377-385.

Uebel, C., Sonnet, P., Miller, R., Beroza, M., "Sex Pheromone of the Face Fly, Musca autumnalis," J. Chem. Ecol., v. 1, pp. 195-202.