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IMPLICATIONS FOR FORENSIC INVESTIGATIONS

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EFFECTS OF INSECTICIDES ON POSTMORTEM INTERVAL:
IMPLICATIONS FOR FORENSIC INVESTIGATIONS

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DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

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What children need most are the essentials that grandparents provide in abundance. They give unconditional love, kindness, patience, humor, comfort, lessons in life. And, most importantly, cookies. ~Rudolph Giuliani

Dedicated to two of the best grandparents in the world:
Ann and Willard Lyons

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Table of Contents

Chapter I: Introduction and Background	1
A. Brief History of Forensic Entomology.....	1
B. Stages of Decomposition of Bodies Following Death and Associated Gross Morphological and Biological Changes	4
C. Entomological Events Associated with Decomposition Processes.....	6
D. Stages of the Life Cycle in the Development of Flies.....	8
E. Qualitative and Quantitative Methods Employed in the Determination of the Post Mortem Interval (PMI) in Association with the Decay Process for a Dead Body	11
E. 1. Utilization of Livor Mortis in Assessing PMI.....	12
E. 2. Utilization of Algor Mortis in Assessing PMI	16
E. 3. Utilization of Rigor Mortis in Assessing PMI	20
E. 4. Utilization of Entomology in Assessing PMI	21
F. Effects of Perfused Chemicals in an Animal Model in the Alteration of the Life Cycles of Flies.....	27
G. Insecticides	34
Chapter II: Materials and Methods	44
A. Chemical Insecticides Used in Field and Laboratory Studies	44
B. Field Studies	45
B. 1. Solutions Used to Treat the Pig Carcasses.....	45
B. 2. Preparation of Pig Carcasses Prior to Placement in the Field.....	46
B. 3. Environmental Surroundings in the Field Studies.....	47
B. 4. Sampling of Various Stages of Flies in the Vicinity of the Carcasses.....	49
C. Lethal Concentration (LC₅₀) Determinations for Insecticides	53
C. 1. Preparation of Solutions and Vials for Subsequent Fly Exposure	53
D. Gas Chromatograph with Mass Spectrometer Detector	56
E. Laboratory Growth of Flies in the Presence of Insecticides and Metabolic Clearing of the Insecticides in Subsequent Growth	59
E. 1. Determining Chronic Effects of Insecticides on Larval Growth.....	60
E. 2. Determining Acute Effects of Insecticides on Larval Growth.....	61
E. 3. Determining Extended Acute Effects of Insecticides on Larval Growth ..	62
E. 4. Limitations on Chronic, Acute, and Extended Acute Studies Due to the Particular Insecticide Employed.....	62
F. Analysis of Insecticides Using Gas Chromatography and Selected Ion Monitoring with a Mass Spectrometer	64
F. 1. Preparation of Standard Solutions and Standard Curves.....	64
F. 1. a. Stock and Working Solutions of the Internal Standard	65
F. 1. b. Stock and Working Solutions of the Insecticide Malathion	66
F. 1. c. Stock and Working Solutions of the Insecticide Carbaryl.....	67
F. 1. d. Stock and Working Solutions of the Insecticide Permethrin	68
G. Extraction Efficiency for Insecticides in Larva	69
G. 1. Insecticide Solutions for Determination of Extraction Efficiency	69

G.2. Homogenization of Larvae.....	70
G.3. Preparation of Standards and Determination of Percent Extraction.....	72
Chapter III: Results and Discussion.....	73
A. Gas Chromatography with Mass Spectrometry (GC/SIM-MS) in the Analysis of Insecticides	73
A. 1. GC/SIM-MS Chromatograms and Spectra for Standards	73
A. 2. GC/SIM-MS Chromatograms and Spectra for Unknown Samples.....	89
A. 3. Typical Calibration Curves for Analysis of Unknowns	101
B. Determination of the Half-Lethal Concentration of Insecticide.....	104
B. 1. LC ₅₀ Determination for Malathion.....	105
B. 2. LC ₅₀ Determination for Carbaryl.....	106
B. 3. LC ₅₀ for Permethrin	106
B. 4. LC ₅₀ Summary	107
C. Extraction Efficiency	107
D. Laboratory Studies of Insecticides and Fly Larvae	108
E. Field Studies of Interruption of Normal Decay by Insecticides.....	111
E. 1. Prior Treatment of Carcasses with Insecticides.....	112
E. 2. Decay Processes of Carcasses in Field After Insecticide Treatment.....	113
E. 3. Monitoring of Adult Flies in the Vicinity of Pig Carcasses Used in Field Experiments 1 and 2	127
E. 4. Insecticide Levels at Various Life Stages of Flies Collected in the Vicinity of Treated and Control Pig Carcasses Undergoing Decay.....	131
F. Conclusions	136
Bibliography.....	139
Appendix A: Dichotomous Key for identifying Diptera	149
Appendix B: Dichotomous key for identifying Coleoptera	163
Appendix C: Temperature and Relative Humidity for Field Trials....	165

Abstract

Postmortem interval (PMI) is the length of time that occurs between death of a person and the subsequent discovery of the body. PMI can be determined in a variety of ways depending on how long the body has been exposed to the environment. Forensic entomologists can use the identity and growth stages of insects to determine the PMI. During the initial stages of decomposition, the primary insects to attack the body are blow flies and flesh flies. The abundance and age of the fly larvae are used to calculate the PMI according to established life cycle times for each species. Several factors can alter the apparent PMI via alterations in the insect activity. These factors include temperature, humidity, sunlight, and chemicals present in or on the body. This research focused on the alteration of PMI by insecticides applied to the body after death. Readily available insecticides were chosen from three classes of pesticides: malathion, an organophosphate; carbaryl, a carbamate; and permethrin, a pyrethroid. Pig carcasses were coated with a 0.5% solution of one of the three pesticides and placed in a remote site protected from predation. The pigs were monitored daily for the appearance of fly larvae. Larvae were collected and analyzed by GC/MS for the presence of the insecticides. The insecticides deterred the arrival of flies in the following decreasing potency: permethrin, carbaryl, and malathion.

Chapter I: Introduction and Background

A. Brief History of Forensic Entomology

Since the beginning of recorded time, individuals have demonstrated an ability to be less than kind to one other. Indeed, people have been killing other people throughout our documented history, beginning biblically when Cain murdered Abel.¹ Not surprisingly, others became interested in ascertaining exactly who had committed such heinous crimes against other(s). Eyewitness accounts were helpful in this regard.² Scientific evidence directly or indirectly implicating a perpetrator was also found to be helpful. Such evidence in the ancient past was certainly not as detailed and specific as it can and should be today. For example identification of a simple weapon with tissue and/or blood stains lying next to a corpse might implicate the owner of that instrument. If the instrument were very unique, this could help to solve the mystery and/or indicate the identity of the perpetrator. More recently, of course, crime scene investigations incorporate a vast array of scientifically useful information including the degree of development of algor mortis which involves the measurement of body temperatures, the degree of development of livor mortis which involves the pooling of blood, and the degree of development of rigor mortis which involves muscular rigidity. Additionally, simple presence of nearby relevant objects, tissue samples, blood samples, DNA samples, fibers,

unique fragments of various objects, cigarette butts, gum wrappers, and other things discovered in the immediate vicinity of the site can provide clues as to the presence and/or involvement of others.^{3,4}

An interesting area which has enjoyed much more broad-reaching application for the investigation of crime scenes, including homicide, is that of entomology.^{2,5-13} When used in the solution of a crime, this is known as forensic entomology. This field has gained a great deal of prominence in, particularly, the past two decades, and is now the preferred method for assessing the time of death from individuals who have been deceased between 36 hours and up to 2-4 months for modest temperature, humidity, and other environmental parameters associated with the crime scene.^{14,15}

The initial recorded report of the use of entomology for forensic purposes involved a murder investigated by a Mr. Sung Tz'u, a lawyer and death investigator in China during the 13th century. Mr. Tz'u was presented with a dead body and what appeared to be a cut that was most probably, as deduced from the size, shape, depth and location of the mortal wound, delivered by a scythe. He gathered all the nearby farmers and examined the individual scythe of each. None of these instruments had any obvious indication of having been used as the murder weapon, so Mr. Tz'u laid them all out on the ground and simply watched to see which, if any, would attract flies. Indeed, only one did attract flies, which indicated a trace of blood and/or tissue, invisible to the naked

eye. When he confronted the owner of the scythe with this evidence and his reasoning, the individual broke down and admitted to the crime. Surprisingly, further documented utilizations of forensic entomology were not reported until some 600 years later in France by Bergeret and Megnin.² In 1855, Bergeret was investigating the nature of insect infestation of dead bodies, particularly paying attention to fly pupae.² He incorrectly assumed that the life cycle of the fly from egg to adult was approximately one year. And, unfortunately, he testified to this in a court of law when asked to assess the time of death. Megnin in 1878 was called in to help with a case concerning a mummified infant located in an apartment wall.² He was attempting to determine if the infant had been placed in the wall by a current or a former tenant.² He investigated the larvae from butterflies of the aglossa genus to help address the problem. Based on the number of observed larvae, he testified that the body had been in the wall for 6-7 months. We also now know that this conclusion was incorrect. Nonetheless, these two separate applications of forensic entomology indicate that society and legal venues were beginning to accept such data to assess the actual time of death of a subsequently discovered body.

B. Stages of Decomposition of Bodies Following Death and Associated

Gross Morphological and Biological Changes

There are five commonly accepted stages of decomposition following the death of a mammal:^{14,16}

Fresh

Bloat

Decay

post-Decay

Skeletal

The duration of each of these stages is dependent on multiple different factors including the environmental temperature, the amounts and frequency and timing of precipitation events, the exposure or lack of same to bright sunlight, the geographic location of the sample (particularly if it has been moved post mortem), the altitude, the local flora and fauna, and the presence of any unusual chemicals or biological species in the nearby vicinity whether such was intended or not.

In the first, fresh, stage of decomposition, the body begins to undergo differential cooling in various regions while the muscles begin to experience stiffening manifested as increasing rigidity. Normal bacteria within the body begin to multiply far beyond their normal levels due to the lack of a functioning immune system to keep them in check.^{14,16,17} Death of the organism leads to the

loss of vitality of individual cells, which is of particular concern in regards to the white blood cells, phagocytes, and other protective cells. Upon loss of viability, these cells release their contents, including potent antibiological enzymes, such as lysozymes, and other chemical species into the surrounding tissue regime. This quickly leads to the destruction of the surrounding cells through a process commonly called autolysis. Simultaneously, the abnormally bolstered endogenous bacterial flora is involved in the active degradation of body proteins in a process called putrefaction.^{3,4,14-17} Autolysis and putrefaction produce substantial quantities of gases like ammonia and hydrogen sulfide, and these processes can proceed for a few hours under a given set of conducive conditions to a few days, months or even years in unfavorable conditions. If the whole organism contains no significant external wounds, the body might appear to simply be resting as one proceeds through the fresh stage of decomposition.

At some point, the gases produced by autolysis and putrefaction begin to become substantial in volume compared to the initial size of the organism. At this point, we enter the next stage of decomposition: bloat. The bloat stage is accepted to begin when the abdomen is observed to noticeably expand beyond its normal and starting condition.^{14,16-18} Bloat can occasionally cause a physical change in the position of the body. Investigation of livor mortis, or the pooling of the blood, could be affected by such a position shift, since the pooling typically occurs before, but can also overlap the time frame of bloating.

Bloating continues until the gases create sufficient pressure to physically rupture the skin of the organism. This skin rupture marks the beginning of the next stage of decomposition: decay.¹⁴

Upon skin rupture, the now liquefied internal contents, which were formerly tissue, spill onto the area immediately around the body, and the organism enters the decay phase of decomposition. The liquefied tissues have a very high viscosity and a substantial lipid content, forming an ideal growth medium for subsequent insect infestation. The gases escape, and shortly thereafter the body deflates. The liquefied tissues continue to be consumed biologically by either the previously discussed lysozymes and bacteria or novel outside agents like the fly larvae discussed below.

When consumption of all the liquefied tissue mass has been completed, the body is classified to be in the post-decay stage. In the post-decay stage, only skin, bone and some cartilage remain of the original biological organism.^{14,15}

After all the skin and cartilage have been consumed, typically by insects and other environmental scavengers, the final stage is reached, appropriately labeled the skeletal stage. At this stage only the bones of the animal remain.

C. Entomological Events Associated with Decomposition Processes

During each of the five individual stages of decomposition, different insects are interacting with the body.^{5,7-11,13-17,19-24} During the fresh, bloat and

decay stages, the primary insects exhibiting such interactions are blow flies (from the general order/family of Diptera: Calliphoridae) and flesh flies (from the general order/family of Diptera: Sarcophagidae). These two broad families of flies typically arrive within minutes of death and deposit eggs on the carcass in a process known as ovipositing (Calliphoridae) or deposit live larvae on the carcass through a process known as larvapositing (Sarcophagidae). Oviposition and larvapositing are quite similar processes. In both cases eggs are developed in the abdomen of the female fly following fertilization by a male fly. In oviposition, the female fly directly places these on the carcass, whereas in larvapositing the fertilized eggs have matured inside the female fly beyond the egg stage and are, thus, deposited as first instar larvae. The various instar stages of larvae are discussed below. After oviposition, the eggs hatch and the resultant larvae, sometimes called maggots, begin to feed on the body directly if the organism has entered the decay phase of decomposition. If the organism is still in either the fresh or the bloat stage, the larvae will attempt to enter the inner regions via any available openings. The openings are typically from wounds or, alternatively may simply be the natural openings of the body like the mouth, anus, ears, and so forth. If the larvae successfully enter the organism a reasonably long time prior to the skin rupture signaling the beginning of the decay stage, a substantial maggot mass, rather than simple liquefied tissue, spills out.

During this time, a second round of insects, composed of beetles from the order Coleoptera, begin to approach the body. These beetles not only feed on the liquefied tissues, but also use the blow fly larvae as a food source.

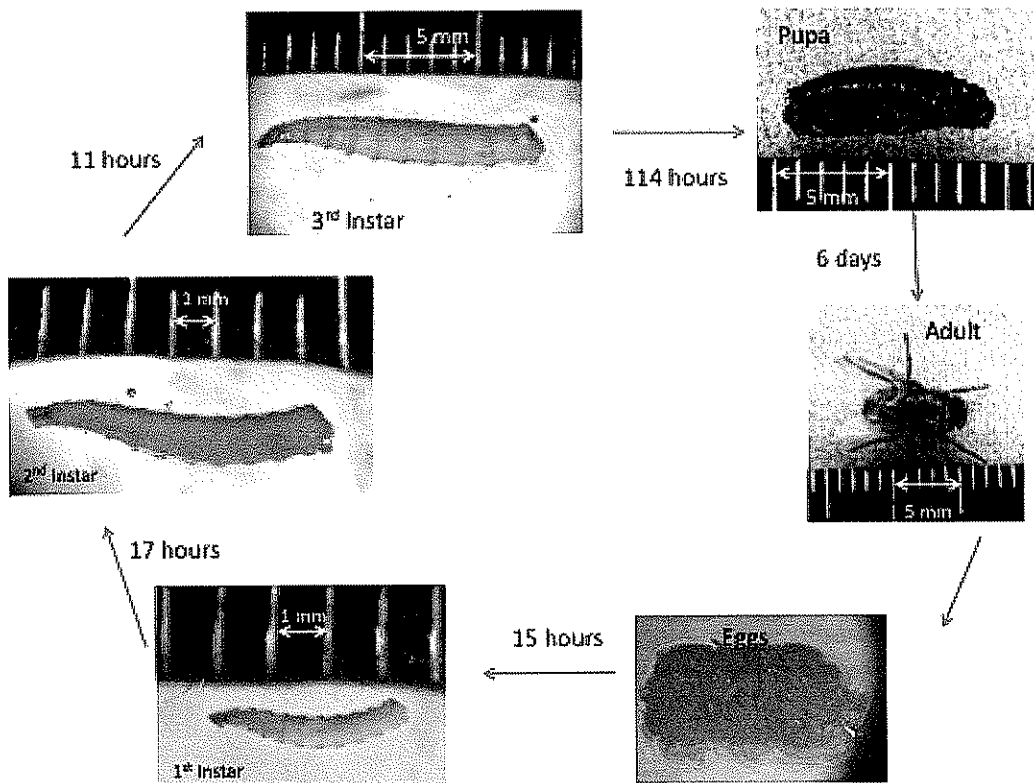
Typically, a third round of insects, composed of beetles from different families from the order Coleoptera, arrive at the corpse near the very end of the decay phase and at the beginning of the post-decay decomposition phase. These insects effectively consume the remaining, and relatively dry, skin of the carcass. At the beginning of the post-decay stage, the previously prevalent fly larvae of the families Calliphoridae and Sarcophagidae begin to migrate as a rather impressive mass away from the body in order to pupate and, thus, enter the immediate pre-adult stage of their life cycles. Coleoptera remain the predominant insects found on the body throughout the post-decay phase.

D. Stages of the Life Cycle in the Development of Flies

The life cycle of a fly begins, as mentioned above, when it is oviposited from the adult female or larvaposited. Flies which begin as eggs hatch to the first larval stage, which is the same stage at which those which were larvaposited began. The larvae exhibit 3 distinct stages in a life cycle. These three stages are all labeled as instars and are, in succession, the first, second and third instar stage of larval life and/or larval growth. The insect is identified as either a larva or a maggot at any or all of these three larval stages. The size of

the larvae successively increases as one moves from the first to the third instar. Similarly, the complexity of some specific body parts, like the mouth apparatus, increase throughout the stages and the number of spiracle slits increases from the first to the third instar. Following the third instar, the larva transforms into a pupa through the process known as pupation. In this state, the pupa appears to initially be a condensed and slightly modified version of the larva with a very hard outer shell. During the time spent in the pupa phase, the larva completely changes its shape, metamorphosing into a mature adult fly. The time required between each developmental stage for the fly is, of course, dependent upon the environmental surroundings and, particularly, the temperature. A typical example of the developmental progression timeline and the sizes of the maggots during each of the three larval instars are shown for *Protophormia terra-novae*, a blow fly known as a blue bottle fly, at 80°F in Figure 1-1 below.

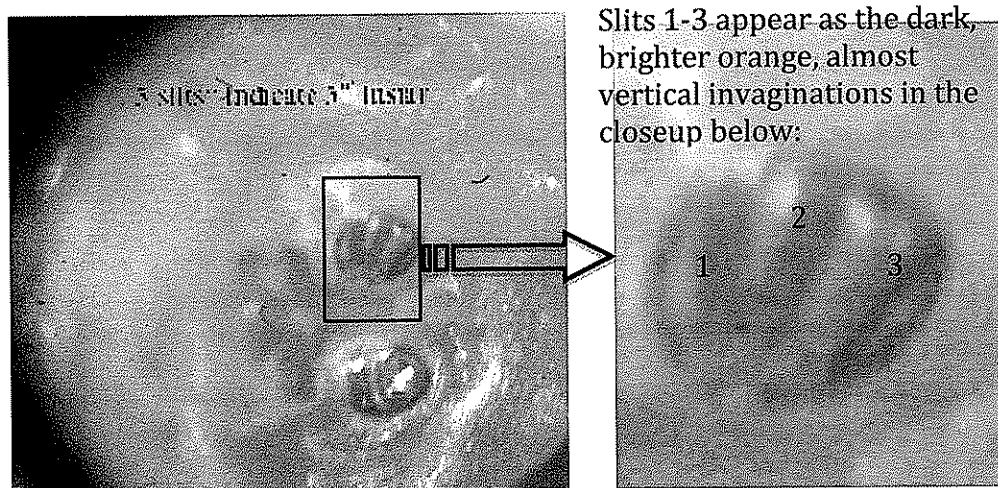
Figure 1-1: Distinctive Stages Observed in Life Cycle of
***Protophormia terra-novae* at 80 °F**



Larvae have a very hard exoskeleton made of chitin which must eventually be shed in order to allow growth to a larger size. The most commonly employed method to identify the age of the larva requires examination of the posterior end of the maggot. The larva exhibit two spiracles, which might initially appear to be eyes due to the spherical nature of these structures; but, the maggot has no eyes. Each spiracle contains one or more distinctive slits. The number of slits exactly reflects the particular instar of larval development and may be used to determine the age of the maggot. For a

first instar larva, there is one slit per spiracle, for a second instar, there are two and for a third instar there are three slits. An example of the slits in a third instar larva is shown in Figure 1-2 below.

Figure 1-2: Posterior Region of a Third Instar Larva of *Protophormia terra-novae* Showing the Characteristic Three Slits



E. Qualitative and Quantitative Methods Employed in the Determination of the Post Mortem Interval (PMI) in Association with the Decay Process for a Dead Body

The postmortem interval (PMI) is defined as the length of time between death and discovery of a body. PMI can be determined in a variety of ways depending on how long the body has been exposed to the environment and the nature of the specific environment.¹⁻³

depending on how long the body has been exposed to the environment and the nature of the specific environment.¹⁻³

Once death occurs, the body undergoes a reasonably predictable series of changes that allow extrapolation back to the time at which death occurred. Some of these changes employ quantitative measurements, such as algor mortis in which one measures the temperature of the body. Other changes are relatively qualitative, like the appearance or lack of some of livor mortis or rigor mortis, in which one judges the extent of pooling of the blood or the degree of rigidity of the muscles post mortem, respectively.¹

E. 1. Utilization of Livor Mortis in Assessing PMI

Livor mortis, also commonly called lividity, involves the “pooling” or “collection” of blood at the lowest non-pressurized point of the body due to its position and the simple effect of gravity. Lividity is commonly observed by direct visual inspection and appears as a kind of “bruise”, with the strongest blood collection occurring in the lowest point of the body which is not pressurized. For example, a modest sized person lying supine on his/her back at death would subsequently develop the clear appearance of lividity on his/her back (dorsal) side, with distinctive white patches for the buttocks and the shoulders region of the back, where the body was resting on the floor. The floor in this example exerted “pressure” against the body, blocking flow of blood to the outer (farthest downward) skin positions in the buttocks and shoulder region

of the back. Non-pressurized regions on the front (upper side) of such a body would not exhibit lividity; these regions would typically reveal a grayish color. At death, the heart eventually or immediately stops beating. When this occurs, blood ceases to circulate throughout the body. Gravity slowly but surely begins to move the blood toward the lowest point of the body that can be reached through the existing blood vessels. The blood vessels weaken post mortem due to the combination of the rapid bacterial proliferation and the lack of normal muscular pressure, facilitating blood flow through these vessels shortly after death. Typical development of livor mortis begins to be observed 1 hour after death and is considered “fully developed” at 3-4 hours, when the discoloration of the bruise-like area is maximally expressed. Livor mortis is said to be “fixed” at 12 hours.^{3,4} Although the visible collection of blood begins to appear after only an hour and maximizes after 3-4 hours, one can press on a relatively dark spot during or after this developmental time up to the “fixed” time of 12 hours and elicit a blanching or whitening of the area pressed. But, after the “fixed” time of 12 hours, such pressing will not elicit blanching, since the discoloration is no longer due to the presence of “liquid” or flowable components but rather “solid” components like clots or components that cannot escape their immediate vicinity. The onset of lividity prior to the “fixed” time of 12 hours would cause a partially or considerably discolored tissue to lighten due to movement of the

body to a new resting position assuming the area of concern was higher in the new position than it was in the previous position.

Recent research on livor mortis has attempted to more accurately assign the extent of progression of this process by spectrophotometric methods and, thereby, narrow the uncertainty in the estimate of the PMI derived from this livor mortis.²⁵ Usumoto et al. conducted examinations on 21 individuals, each known to be dead less than 72 hours at the time of the investigation. These investigators specifically attempted to relate the change in light reflection with a consideration of particular color effects to assign a PMI which was then compared to the known PMI.²⁵ They used a portion of skin with no lividity and compared it to a second portion skin with maximum visually observed lividity from the same individual. They derived two equations to assist in determining the experientially determined PMI. The first equation was given as:

$$\begin{aligned} \text{PMI}(\text{hr}) = & -20.47 - 3.79(\Delta L^*) - 0.52\left(\frac{a_1^*}{a_1}\right) \\ & + 10.70(b^*) - 7.89\left(\frac{b_1^*}{a_1}\right) \\ & - 57.42\left(\frac{\Delta a^*}{\Delta L^*}\right) - 38.78\left(\frac{\Delta b^*}{\Delta a^*}\right) \end{aligned} \quad (\text{eqn. 1-1})$$

In this expression, L^* represents the brightness, a^* is the red-green chromaticity or Chroma, and b^* is the blue-yellow chromaticity or Hue. This equation does not account for extraneous, unusual, or abnormal factors that could affect the color characteristics of the lividity such as CO exposure in/around death or

exposure to cold near/around death. Even without inclusion of these factors, the uncertainty (rms error) associated with the calculation was specified to be 6.95 hours. This is roughly comparable to the visual estimation one could make, which, if lividity is present, can really only accurately be stated to have a PMI between 1 and 12 hours.²⁵ Considering some of the autopsy derived data for the individuals in the study, these workers were able to create a more refined version of the above equation. This revised version was presented as:

$$\begin{aligned}
 \text{PMI (hr)} = & -30.75 - 1.01(\Delta L_c^*) + 5.30(a_c^*) + 0.99(a_l^*) \\
 & - 6.64(\text{sex}^t) - 0.35(\text{age, yrs}) + 11.52(\text{CO}^u) \\
 & - 8.42(\text{cold}^v) - 6.51(\text{blood clot } 1^w) \quad (\text{eqn. 1-2}) \\
 & - 1.35(\text{blood clot } 2^x) - 3.12(\text{msv hemrg}^y) \\
 & - 6.03(\text{resuscitation}^z)
 \end{aligned}$$

where the subscripts c and l refer, respectively to the control sample and the sample with lividity. The remaining terms in the above equation, indicated by the superscripted lower case letters t-y, have the following meanings and values:

t	sex of patient	1 = male	-1 = female
u	CO Exposure	1 = yes	-1 = no
v	cold exposure after death	1 = yes	-1 = no
w	blood clot observed - yes or no and with “chicken fat” or CF	1 = yes	0 = no and CF
x	blood clot observed - yes or no and with “chicken fat” or CF		

		1 = no	0 = yes and CF
y	massive hemorrhage	1 = yes	-1 = no
z	resuscitation used	1 = yes	-1 = no

With the additional factors in this second equation, the uncertainty (rms error) in the PMI estimation dropped to 4.76 hours for the same group of individuals as used previously with equation 1-1.²⁵ While this is an admitted improvement in the PMI estimation, many of these additional factors are simply not known and/or unavailable in the usual cases of missing persons or homicides. In spite of this generally known post mortem developmental effect on dead bodies, livor mortis is obviously not a useful technique to assess PMI under normal conditions beyond the first 12 hours following death. In addition, the precision with which one can qualitatively or quantitatively assess the particular time point a body occupies within the associated 12 hour window is severely limited as well.

E. 2. Utilization of Algor Mortis in Assessing PMI

Algor mortis, or body temperature, is another very important method employed in PMI estimation. In order to assess PMI using body temperature, a thermometer is inserted into the body, usually the liver or rectum, and the temperature is subsequently recorded as a function of time. The temperature of

the location where the body was found is also recorded since environmental temperature affects the rate at which the body cools. Different mathematical models have been derived to determine the PMI for the different body locations in which the temperature was monitored. The first reported quantitative assessment of the cooling rate was published by Rainy in 1868.²⁶ This investigation incorporated recordings of the temperature of the environment, presumed to be reasonably constant, in which the body was discovered as well as the actual body temperature at intervals of one hour following death. Based upon this work, he produced the following formula for the calculation of PMI using relatively simple temperature measurements:

$$\text{PMI (hr)} = \frac{\log T_{\text{ext}} - \log T_{\text{final}}}{\log R} = \frac{\log \left(\frac{T_{\text{ext}}}{T_{\text{final}}} \right)}{\log R} \quad (\text{eqn. 1-3})$$

In this equation, R is the ratio of the initially recorded temperature of the body, T_{initial} , to the temperature of the body recorded one hour later, T_{final} . The measured temperature of the surrounding environment in which the body is discovered is T_{ext} . All the temperatures are given in °C. This equation, as shown, was useful in providing an estimate of the minimum interval of time since death. To estimate the maximum time since death, one simply multiplies the result from the above equation 1-3 by a factor of 1.5. Rainy determined this factor using cases where time of death was known. He found that, in cases

where the rectal temperature was below 85 °F, all of the times fell below the time calculated by multiplying equation 1-3 by 1.5. The range of the derived minimum to maximum time since death is obviously more reliable than either one of these independently.

Practical utilization of algor mortis to assess PMI using equation 1-3 proved to be difficult by many law enforcement officials and others who were not very comfortable with mathematical calculations using logarithms. So, a simplified version of the equation was developed and presented as:

$$\text{PMI (hr)} = \frac{[98.6 - (T_m, ^\circ\text{F})]}{1.5} \quad (\text{eqn. 1-4})$$

where T_m is the measured temperature of the body in degrees Fahrenheit. This equation possesses considerable simplicity and has found remarkably widespread use.

Subsequent examinations of multiple cooling curves for various environmental and body conditions, including the amount of body covering or fat content of a victim²⁷⁻³³, have been utilized to more exquisitely and quantitatively model the observed decay of body temperature vs. time after death. As one might surmise, a priori, the body temperature decays in at least a single, if not multiple, exponential fashion.^{26,27,29,30} While such advancements in the mathematically predicted temperature decay in dead bodies have been presented and even accepted by the forensic community, equation 1-4 persists

today as the most widely used algor mortis tool for determination of PMI in the majority of death and homicide investigations.

Newer research in the field of algor mortis has brought into question the body temperature at the time of death. Previously, the temperature at the time of death was, with little question, assumed to be the “normal” body temperature (37°C or 98.6°F). Indeed, “normal” body temperatures of typical individuals under normal living circumstances would appear to vary less than a few degrees from this “normal” value. Thus, this should be a good estimator of the temperature at the time of death under “normal” circumstances for reasonably healthy individuals. However, al-Alousi examined a number of patients who died from advanced age, advanced disease states, or other conditions involving less than “normal” maintenance of “normal” body functions. For such persons, he determined that the liver temperature of individuals who were naked or covered, respectively, at the time of death was $27.5 \pm 3.1^{\circ}\text{C}$ and $32.7 \pm 2.9^{\circ}\text{C}$, while the rectal temperature of the same individuals was $36.6 \pm 2.0^{\circ}\text{C}$ and $32.2 \pm 4.8^{\circ}\text{C}$, respectively.^{27,28} Unfortunately, after a period of 18-30 hours, the body temperature can no longer be used to assist in the determination of PMI since the body essentially assumes the temperature of the surroundings thereafter; therefore, investigators must rely on techniques other than algor mortis as sources for assistance in PMI determinations after 18-30 hours following death.

E. 3. Utilization of Rigor Mortis in Assessing PMI

Rigor mortis is a stiffening of the body observed post mortem. Rigor mortis is a result of post mortem depletion of ATP in the muscles, along with associated glycogen degradation and lactic acid accumulation.^{16,24,34-38} The rigidity normally begins to initially appear in the smaller muscles of the face and progresses down the body in a predictable and well-known pattern. Rigor mortis typically starts somewhere between two and six hours following death and exhibits maximal stiffening around twelve hours after death. Therefore, rigor mortis is qualitatively and semi-quantitatively useful for PMI estimations in the 2-12 hours following death under normal circumstances.^{3,4} After two to four days, the muscles begin to relax, and rigor is no longer useful to approximate PMI.^{3,4} Of course, an observed complete relaxation could be used to indicate that the body is *at least* four days post mortem assuming normal conditions. Rigor mortis can notably be “broken” or overcome by external forces. With pressure, the limbs can be forcibly manipulated, for example, to alternate positions, including what we consider to be a more natural position. This is obviously helpful in preparing a body for viewing under circumstances like an open casket funeral. Forensically, the application of external forces to alter a position during the process of rigor mortis will cause subsequent rigor mortis to develop in regards to the new position. However, depending on the elapsed time between the onset of rigor and the breaking of it, rigor mortis in the

new position could be roughly the same as or much weaker than the unaltered rigor mortis would have been. Several factors can influence the development of rigor mortis that make the use of this observational tool as an accurate indicator of PMI less reliable. Cold environmental temperatures, for example, can substantially slow, or even halt, the development of rigor. Such interference by cold or extremely cold temperatures would cause the PMI estimate to be unquestionably shorter than it was in actuality.^{24,35-39} On the other hand, a person involved in strenuous activity at or near the time of death, like participation in a physically demanding athletic event, will experience substantially reduced and/or essentially missing levels of ATP in the active muscles. The low to missing ATP content in the muscles would elicit much more rapid onset of rigor than normal circumstance with a concomitant incorrectly high estimate for PMI.

E. 4. Utilization of Entomology in Assessing PMI

Insect evidence has recently gained prominence as an alternative or supplemental method to determine PMI. When insect evidence is collected, one looks for the largest fly larvae, particularly blow flies, to increase the probability that samples from the third instar will be present, enabling the identification of the precise species. Forensic entomological investigations typically begin with a sampling of the various insects present on and in the body. Visual inspection is then commonly used to initially identify the species and stage of the insects

present. For some species, this information may not be very helpful due to a lack of specificity. However, the various stages of flies can usually be readily assessed including the egg, the various larval instars, the pupa, and the adult fly. And, flies infest virtually all dead bodies in virtually all regions of the world. Thus, flies have become a particularly useful insect for forensic investigations. In the latter stages of decay, the flies leave the body to pupate, and beetles briefly become the major insects of concern.

Identification of the particular stage of development of the fly, as shown above, is quite distinctive in general. However, the particular genus and species of the fly observed may not be quite as straightforward, but also an important item to ascertain. The third instar larvae of flies, along with a detailed dichotomous key, can be extremely helpful in identifying the precise genus and species of a fly. An example of a dichotomous key is given in Appendix A. The third instar stage of development is usually the minimal one at which simple physical observation and the associated dichotomous key can be reasonably accurate in identifying the particular genus and species. The knowledge of the particular species is pertinent if one is to use tables of growth curves published by others to determine the developmental time that has elapsed for the fly observed.

The length of the longest observed larva and/or the weight of the most developed examples of each larval species found must be measured. Since larval

development is temperature dependent, the temperature at the scene must also be determined. Area temperatures surrounding the time of discovery are obtained from local meteorological offices. The temperature at the scene is, if possible, recorded for three to five days following discovery of the body. The temperature at the scene in the three to five days post discovery is then correlated to the meteorological data. Extrapolation of the meteorological data, corrected for the correlation to the temperature at the scene, is then used to assign temperatures at the scene for a reasonable period of time, hopefully extending to the time of death, prior to discovery. If time constraints and appropriate sampling at the crime scene allows, the species found would be subjected to a single growth cycle under laboratory controlled environmental conditions, providing a growth curve for the particular species of concern. However, when such sampling and/or adequate time are not available, one can use standard tables of growth curves for the same or a similar fly species.

Growth curves for fly larvae are obviously an essential tool in the determination of PMI. These are plots of time versus larval length, weight, or morphological stage of a specific species of blow fly.⁴⁰⁻⁴⁵ These are specific to the species of fly and are constructed using laboratory data at a controlled temperature. Ongoing research is currently being conducted to construct curves showing development at a variety of different ambient temperatures.^{22,44,46} Another method commonly employed in determining PMI using insect data

involves calculation of the accumulated degree days (ADD) and subsequent back extrapolation to the actual time of death. One could also calculate and use the accumulated degree hours by simply using hours instead of days for all pertinent time parameters. In order to proceed with this calculation, one additional, but highly pertinent piece of information is required. This additional piece of information is the minimum temperature, also called the baseline temperature, above which development will occur for the species in question. Stated another way, each species has a lower temperature limit, known as the baseline temperature, below which growth will not occur. Hopefully, the baseline temperature will be accessible from the literature. Unfortunately, not all species have been examined to determine their respective baseline temperatures. If the baseline temperature is not available in the literature, one will have to determine it experimentally before being able to proceed. Once the baseline temperature is known or determined, the investigator can calculate the total number of days, ADD_{total} , required to reach the maximal larval or other growth stage observed in the body sample presuming the species of concern were raised in a controlled environment at the fixed temperature used to produce the growth curve, T_{growth} . The controlled environments typically involve a fixed temperature in the range of 20-30°C. A typical ADD_{total} , for example, would then be calculated using the approach shown below as equation 1-5.

$$ADD_{\text{total}} (\text{days}) = (t_{\text{dev}}) [T_{\text{graph}} - T_{\text{baseline}}] \quad (\text{eqn. 1-5})$$

where the parameters employed are defined as

t_{dev} the time in days required for the particular species of fly to reach the desired larval length (or other stage).

T_{graph} the temperature ($^{\circ}\text{C}$) at which the growth curve was established.

T_{baseline} the temperature ($^{\circ}\text{C}$) below which the species will not grow.

However, the total number of accumulated degree days, , is also the sum of the individual degree days since death that the body has been lying at the scene, or

$$ADD_{\text{total}} = \sum_{i=1}^n (\text{degree day})_i \quad (\text{eqn. 1-6})$$

And, while days each come in 24 hour units, the degree day unit must be corrected for the average degree reading on each given day. This is done for a given day considering the temperature at the scene:

$$ADD_{\text{total}} = \sum_{i=1}^n (1 \text{ day}) [T_{\text{ave, day}_i} - T_{\text{baseline}}]_i \quad (\text{eqn. 1-7})$$

where the parameters employed are defined as

T_{ave, day_i} the average temperature ($^{\circ}C$) for day i , where average is defined as one-half of the difference between the recorded high and the recorded low temperature for that day.

$T_{baseline}$ the temperature ($^{\circ}C$) below which the species will not grow.

In actual practice, one does not determine the PMI by using equation 1-7, but rather works backwards one day at a time from the time of discovery, subtracting the ADD for a particular day from the ADD_{total} to obtain a “revised” ADD_{total} . This is done successively until the remaining “revised” ADD_{total} becomes zero. ADD for the final iteration, typically, will be less than a whole unit. In this case, one can simply estimate the fraction of the day and convert it to hours. The PMI is then the number of whole and fractional iterations required to produce a “revised” ADD_{total} of zero. Utilization of ADH to determine PMI is done in the same fashion as ADD; one simply uses hours instead of days as outlined above.

There are several factors that are known to affect the abundance of flies on a body post mortem, and these can obviously adversely interfere with the estimate of the PMI outlined above. In the above approach, one assumes the growth rate is roughly linear with temperature in the modest temperature range

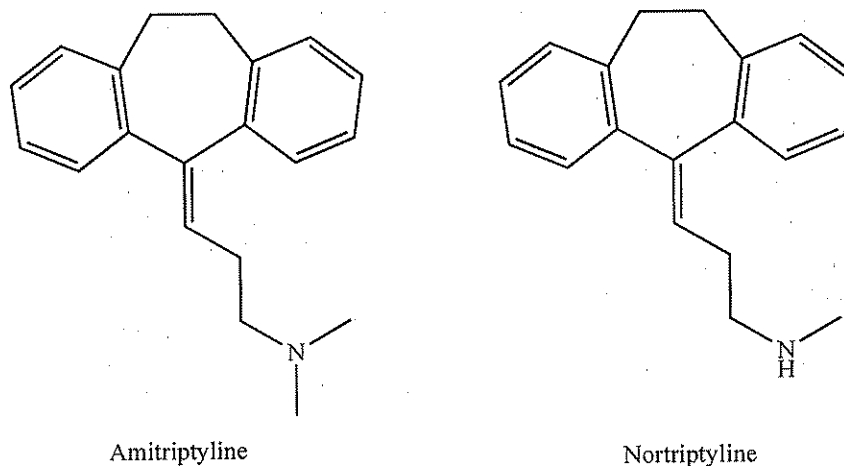
one expects to encounter. However, if it is extremely hot or extremely cold, flies will not as frequently approach the body to lay eggs as they would at the optimal growth temperature. This optimal growth temperature is typically near 80°F for many species of blow flies and is the temperature at which optimal growth is observed for all stages of development.¹⁴ If the temperature is substantially above or below 80°F, the number and, sometimes, even the species of flies will be altered. Exposure of the body to the sun, exposure of the body to the shade, covering the body with clothing, and/or leaving the body uncovered all can and do affect the numbers and species of flies that are attracted to the body. Ingestion of chemicals in pertinent quantities prior to death or topical application of chemicals to the body, not surprisingly, can and do alter the life cycles of the flies and/or affect the attraction of the flies to be, at most, of nominal concern. The possible effects of chemicals, on the other hand, are the primary subject of this dissertation and, as seen from prior and current data, can and do produce very large differences between the “apparent” and the actual PMI.

F. Effects of Perfused Chemicals in an Animal Model in the Alteration of the Life Cycles of Flies

Previous studies have reported the effects of decay of animal carcasses which had ingested or had been infused with various chemicals prior to death.

There have also been selected human case studies where the victim was known to have ingested significant quantities of a specific chemical prior to death. Goff, for example, examined the effect of amitriptyline and MDMA in the rabbit.^{47,48} The chemical structures of these two compounds along with their primary in vivo metabolites, respectively, are shown below in Figures 1-3 and 1-4. In the amitriptyline study, he

Figure 1-3: Chemical Structures of Amitriptyline and Its Primary Metabolite Nortriptyline



infused the rabbits in the ear vein with three different concentrations of the drug: 300 mg, 600 mg, and 1000 mg. These values notably represent a lethal dose (LD), two times the LD and 3.3 times the LD respectively. Only one rabbit was used at each dose level. The rabbit receiving the LD appeared moribund at one minute post administration and was dead at three minutes post administration.

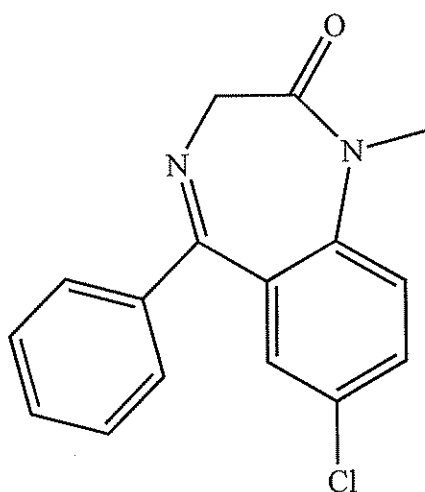
The rabbit receiving twice the LD was dead immediately post administration. The rabbit receiving the highest dose died during administration of the dose. Following expiration, the livers of the treated rabbits were excised and exposed for 15 minutes to necrophagous flies (*Parasarcophaga ruficornis*, commonly called flesh flies). Samples of the blood and liver were taken for subsequent chemical analysis (see below) immediately after death. The livers were incubated at 26°C in the presence of a 12 hour on/12 hour off daily photoperiod. He collected larvae for size analysis every 6 hours throughout the entire larval growth period. At 48 hours into the growth cycle following hatching, he took samples of the larvae for chemical analysis (see below). The recorded length of the larvae were used to determine the rate of development. Significant differences ($P > 0.05$) in the entire larval development time, taken as the time between hatching of the egg and transformation of the third instar larva to a pupa, were found for samples taken from all the treated animals when compared to the control values. The resulting mean larval lifetimes were: 159.9 hr (ctrl), 173.4 hr (300 mg), 190.1 hr (600 mg), and 172.7 hr (1000 mg). The rabbit blood, rabbit liver, and larvae samples collected from each treated animal and the control animal were submitted for analysis by HPLC to determine the levels of amitriptyline and its primary metabolite nortriptyline. Based primarily upon his observations of the increased lifetime of the fly in the rabbit model through the larval and puparial stages for the chemically treated animals, Goff estimated

treatments. The measured concentrations of the MDMA in the liver samples taken from the lowest to highest dose, respectively, were 0.4, 1.6, and 7.9 ng/mg. The concentrations of MDMA in the larvae at 72 hours for the three doses, respectively, were 0.6, 1.5, and 1.7 ng/mg. The corresponding concentrations of MDMA in the pupae were 1.1, 6.6, and 22.8 ng/mg. Measurements of 3,4-methylenedioxyamphetamine (MDA), the primary metabolite of MDMA, were only reported for the pupae; however, these values followed the same trends observed for the MDMA results for the pupae. Perhaps surprisingly, only the very highest dose significantly ($P < 0.05$) altered the lifetime of the larvae compared to controls. The larval lifetime at the largest dose was 190.9 hours compared to a lifetime of 214.3 for the controls. In this single instance, MDMA actually increased the rate of growth for the larvae. No significant difference was observed at any of the doses examined for the duration of puparial stage. As a result of these observations, Goff projected that MDMA would not substantially effect the PMI estimation in humans.⁴⁸

Carvalho et al. studied the effects of diazepam, shown below in Figure 1-5, in a manner similar to that employed by Goff for MDMA and amitriptyline.⁴⁷⁻⁴⁹ They administered a 50 mg dose of diazepam in the ear vein to the rabbits, which were sacrificed immediately after the administration. The livers of the animals were excised and exposed to two separate species of flies: *Chrysomya putoria* and *Chrysomya albiceps*. Both of these species are from the order

Diptera and the family Calliphoridae, and they are commonly known as the latrine fly and hairy maggot blow fly, respectively.

Figure 1-5: Chemical Structure of Diazepam



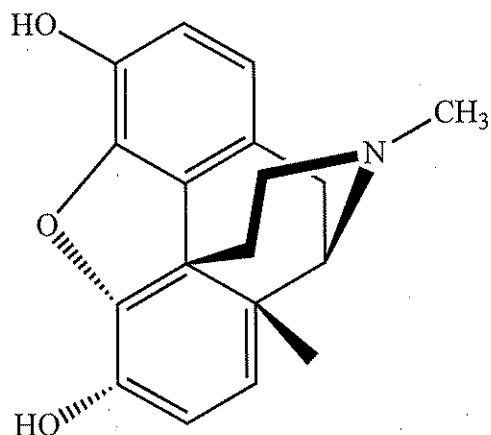
Diazepam

Larvae were sampled, weighed and measured at six hour intervals following exposure. The larvae taken at 54 hours post mortem were subjected to analysis by GC/SIM-MS for diazepam content. For both species, the growth rate of the flies exposed to diazepam was observed to be significantly ($P < 0.05$) greater than the controls at all times greater than 18 hours into the larval development stage; however, no significant differences were observed for times less than 18 hours.

They also found that diazepam significantly shortened the time required for pupation for both species. The concentration of diazepam found in the larvae of each species was found, respectively, to be 0.342 µg/mg and 0.479 µg/mg. These values were notably not significantly different ($P>0.05$) from each other.

Bourel (1999), also using the identical rabbit model, investigated the effects of morphine, shown below in Figure 1-6, on fly development.⁵⁰ Using *Lucilia sericata*, which are generally identified as blow flies from the order Diptera and the family Calliphoridae, he finally reached the conclusion that morphine caused slower than normal larval development and that this compound would, thus, cause a estimation of PMI for humans so treated to be up to 24 hours too low.⁵⁰ In a second study involving morphine, Bourel raised the flies on a simple substrate of minced beef which was infused with 100 mg/kg or 1000 mg/kg of the drug.⁵¹ Analysis of the subsequently sampled flies at various stages of development by RIA revealed morphine concentrations of 1.19 ± 0.69 ng/mL and 214.09 ± 21.25 ng/mL, respectively, in puparial cases. He also found morphine levels at concentrations of 0.42 ± 0.43 ng/mL and 36.14 ± 3.76 ng/mL in the desiccated adult flies.

Figure 1-6: Chemical Structure of Morphine



Morphine

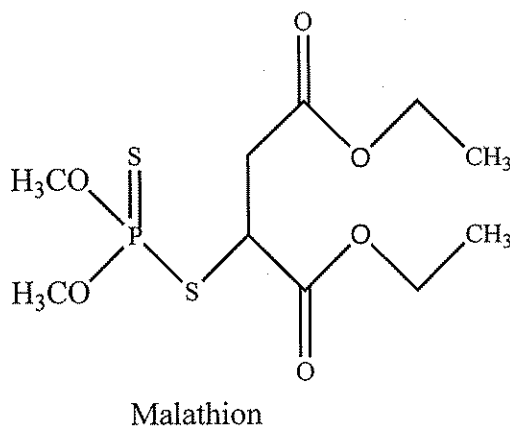
G. Insecticides

Malathion [O,O-dimethyl-S-(1,2-dicarbethoxy)ethylphosphorodithioate], shown below in Figure 1-7, is a phosphodithioate insecticide, a subclass of the organophosphate insecticides that contains two sulfur atoms in the phosphoric acid portion of the molecule.⁵² It was first registered with the EPA for use in 1956 against various agricultural, garden and house pests.⁵³ The mode of action for malathion is primarily via inhibition of acetylcholine esterase (AChE). AChE inhibitors work by preventing the breakdown of acetylcholine (ACh) into acetic acid and choline. Thus, this results in an accumulation of ACh in the

extraneuronal space and the enhancement of cholinergic transmission in general.

When this

Figure 1-7: Chemical Structure of Malathion

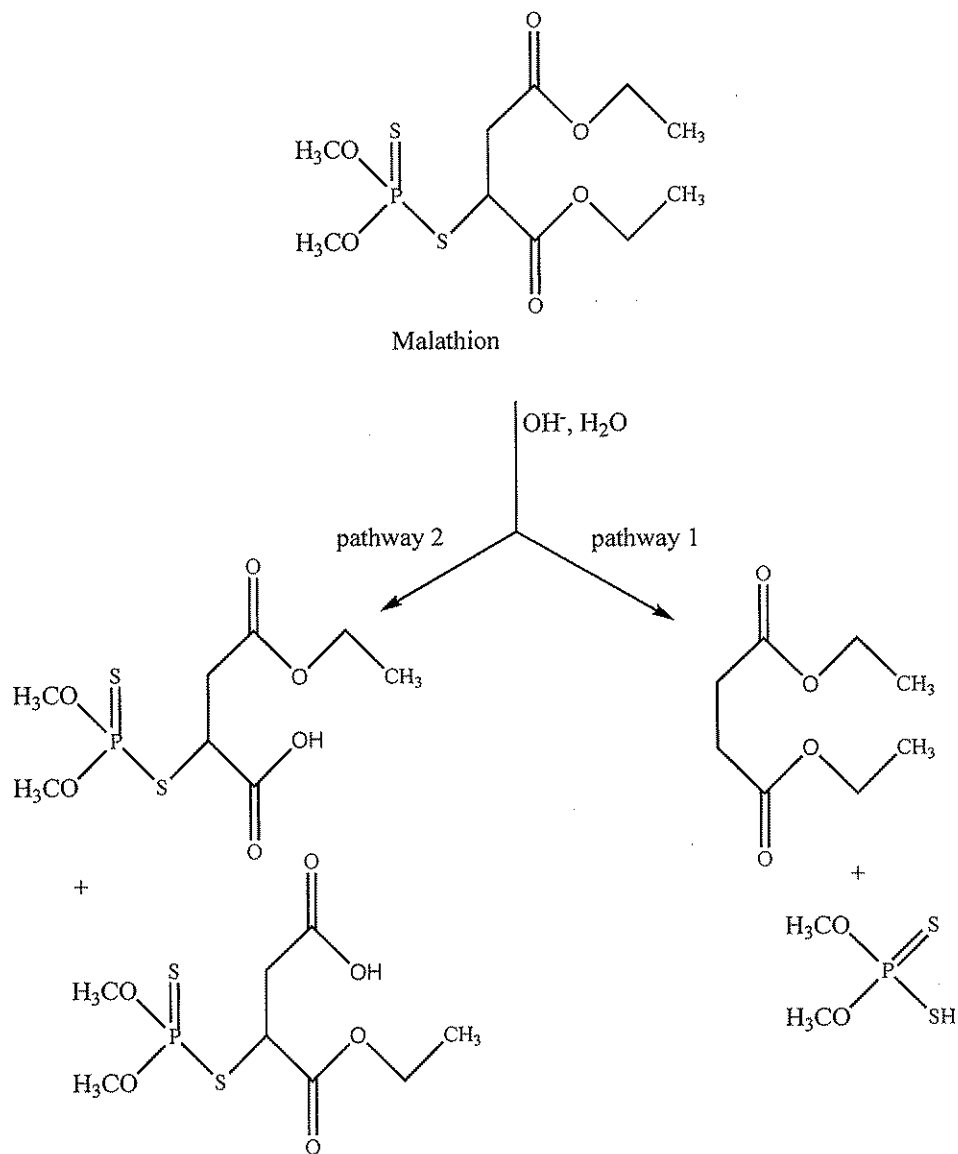


accumulation builds up in the vicinity of a cholinergic receptor, the resultant outcome may become rapid and constant firing of the neurons, which if they happen to be motor neurons can lead to tremors, paralysis, and perhaps even death. All of these observed behavioral phenomena are generally accepted to be symptoms of exposure to AChE inhibitors. In the case of malathion, this process is slow and, unfortunately, irreversible. There have been at least 289,000 pounds of malathion manufactured and used in the United States annually from 1990 to 2006.⁵⁴

Malathion undergoes degradation by basic hydrolysis, by photolysis and/or by microorganisms leading to a considerable number of by-products which could be involved in the action of malathion.⁵⁵⁻⁶⁹ Wolfe et al., examining

the aqueous chemical degradation of malathion, found that hydrolysis in acid, hydrolysis under neutral conditions, and the potential oxidation by molecular oxygen were all unlikely under normal circumstances.⁶⁹ However, the basic hydrolysis of malathion was found to be considerably more rapid, leading to an overall second order rate constants of $k_1=3.9\pm0.2 \text{ M}^{-1}\text{s}^{-1}$ and $k_2=1.4\pm0.1 \text{ M}^{-1}\text{s}^{-1}$, respectively, for the pathways indicated as 1 and 2, respectively in Figure 1-8 below.⁶⁹ The temperature was fixed at 27°C. To give one a feeling for how effective these processes could be in degrading malathion, we note that these rate constants at 27°C would yield half-lives for malathion of 21 and 57 days, respectively, in solutions having a pH of 7.00. Combining degradation by the two pathways and fixing the solution pH to a value of 8.00, the half-life for malathion becomes ca. 36 hours. Using photolysis with $\lambda > 290 \text{ nm}$ at pH 6.0, these same authors measured a $t_{1/2}$ of 990 hours (41 days).

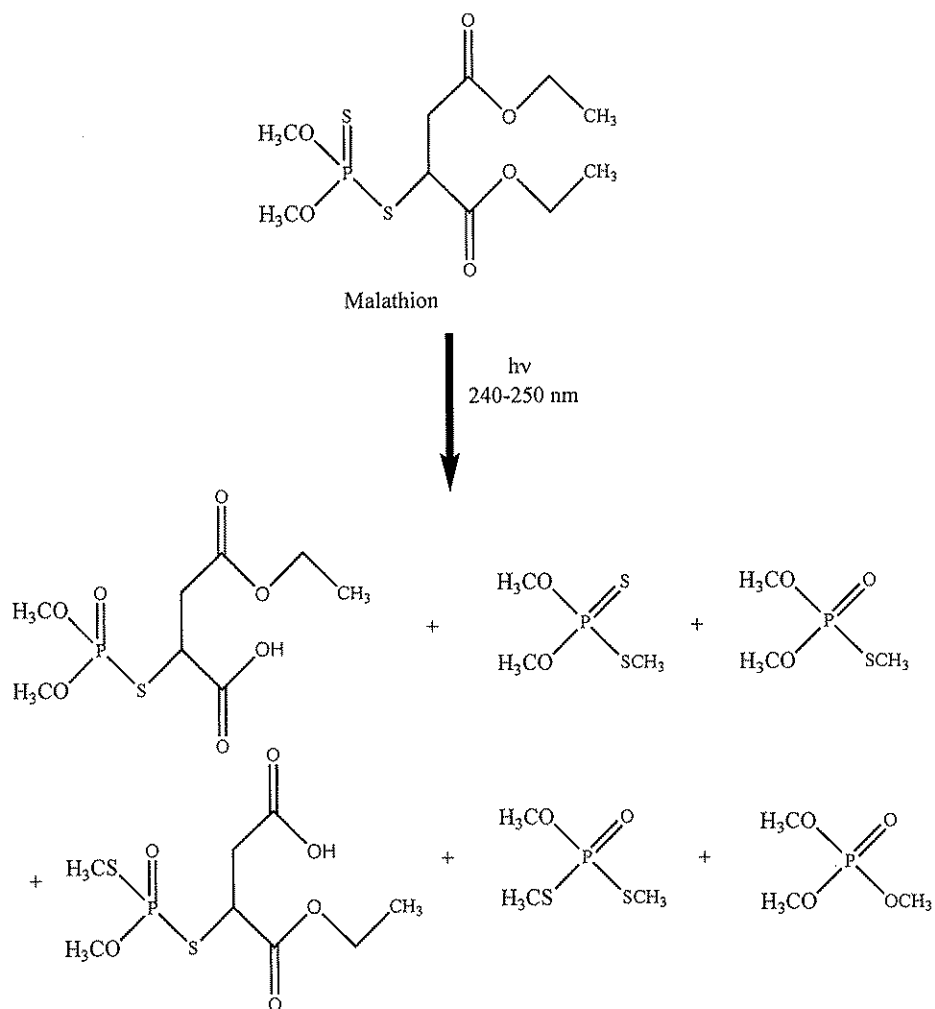
Figure 1-8: Aqueous Degradation of Malathion Under Basic Conditions



Aires et al. later examined the photodegradation of malathion in aqueous solution using a mercury lamp as a light source.⁵⁵ Given the nature of the

mercury lamp and the malathion absorption spectrum, the primary degradation was afforded by light in the wavelength range of 240-250 nm. The products at 25°C are shown below in Figure 1-9.

Figure 1-9: Photodegradation of Malathion in Aqueous Solution



These investigators examined five different pH values in the range of 4.17 to 10.23 with irradiation times of 60 to 200 min, with the latter progressively

increased with an increase in the former. The percent degradation varied from 88 to 100% under these conditions.⁵⁵

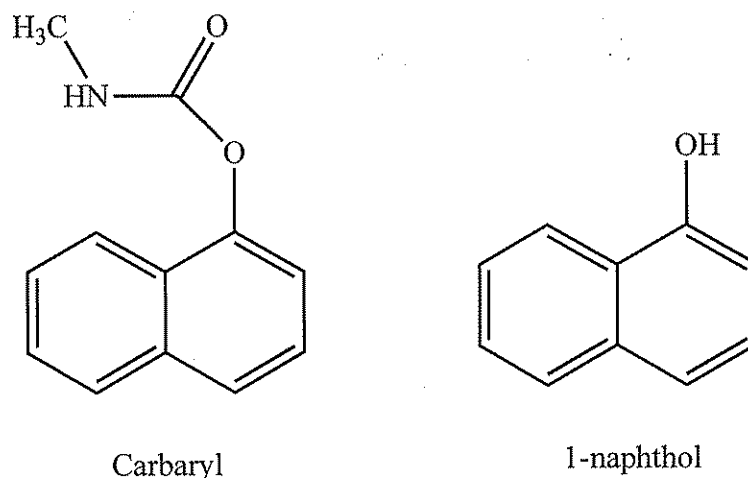
Bacterial degradation of malathion has also been shown to occur in aqueous solutions and in soil under normal conditions via the carboxylesterase enzyme.^{57,59,67} Singh et al., for example, exposed malathion to a strain of *Pseudomonas* and found that the major products were the two malathion monocarboxylic acids, which are sometimes identified as the malathion monoacids.⁶⁷ These monoacids are shown as products on the left hand side of the above Figure 1-8. After 32 hours, TLC measurements showed an initial 200 ppm solution of malathion had decreased to a level of 150 ppm when the temperature was maintained at 30°C and the aqueous solvent was modified to contain 1% ethanol. Even though this strain of bacteria was able to degrade the malathion at this level, it is worth noting that concentrations above 200 ppm revealed a readily discernible decrease in bacterial growth from which the authors inferred a toxicity of malathion toward the bacteria.⁶⁷

Carbaryl [1-naphthyl-N-methylcarbamate], as shown below in Figure 1-10 along with its primary in vivo metabolite, 1-naphthol, is widely sold under the trade name of Sevin®. The intent to utilize this compound as an insecticide was originally registered with the Environmental Protection Agency, or EPA, in 1959.⁷⁰ Well over 100 different species of pests have been targeted for control and/or eradication by carbaryl when used in either agricultural settings or even

directly on domesticated animals and pets. It is a member of the general category of carbamate insecticides and, like malathion, finds its primary mode of action to be its ability to act as an AChE inhibitor. As with malathion, the blockade of AChE by carbaryl is irreversible. However, with the case of carbaryl, the blockade is much more rapid acting. During the years 1990 to 2006, an annual average of 364,000 pounds of carbaryl were manufactured and, for the most part, used in the United States.⁵⁴ The only degradation product typically found with carbaryl is 1-naphthol, which is more commonly called α -naphthol.

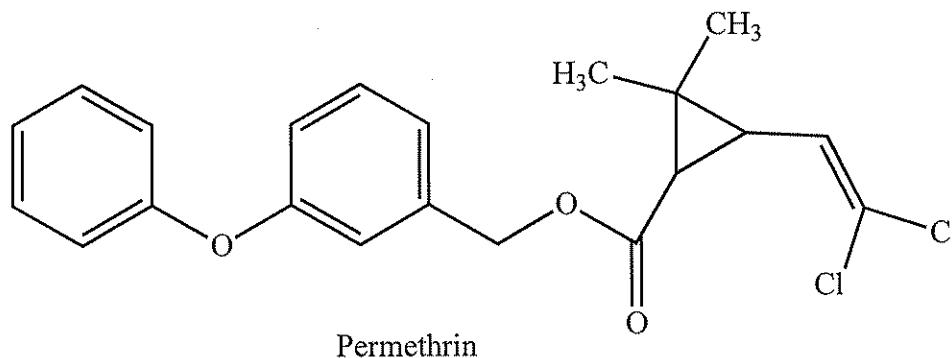
Bondarenko et al. determined the half-life of carbaryl in drained San Diego Creek (a fresh water creek) sediment under both aerobic and anaerobic conditions at room temperature ($21\pm 2^{\circ}\text{C}$) to be 1.8 and 125 days, respectively.⁵⁶ Samples of sediment taken from Bonita Creek (a salt water creek) provided half-lives of carbaryl in aerobic and anaerobic conditions at room temperature ($21\pm 2^{\circ}\text{C}$) of 4.9 and 746 days, respectively.⁵⁶ Starner et al. examined carbaryl in aqueous systems at two different times of the year, April and August, affording primarily a difference in the nature of the bacteria.⁷¹ The April samples produced half-lives of carbaryl of 22 and 2 days, respectively, at incubation temperatures of 10 and 25°C .⁷¹ The August samples incubated at the same temperatures, respectively, produced half-lives of 16 and 1 days.⁷¹

**Figure 1-10: Chemical Structures of 1-Naphthyl-N-methylcarbamate
(Carbaryl) and Its Primary Metabolite, 1-Naphthol (or,
 α -Naphthol)**



Permethrin is a pyrethroid insecticide which has enjoyed agricultural use for many years. Pyrethroids are synthetic products which are derived from the chrysanthemum flower.⁵² Permethrin, shown in Figure 1-11, was initially registered with the EPA in 1979.⁷² It is used in households, in agriculture and in the field in military applications to control a variety of pests including body lice. The mode of action of permethrin is quite different from either malathion or carbaryl; it is primarily a sodium channel blocker. Permethrin is manifested as hyperactivity, tremors and paralysis. Between 1990 and 2006, 129,000 pounds were manufactured on average annually, and the majority of this was utilized in the United States.⁵⁴

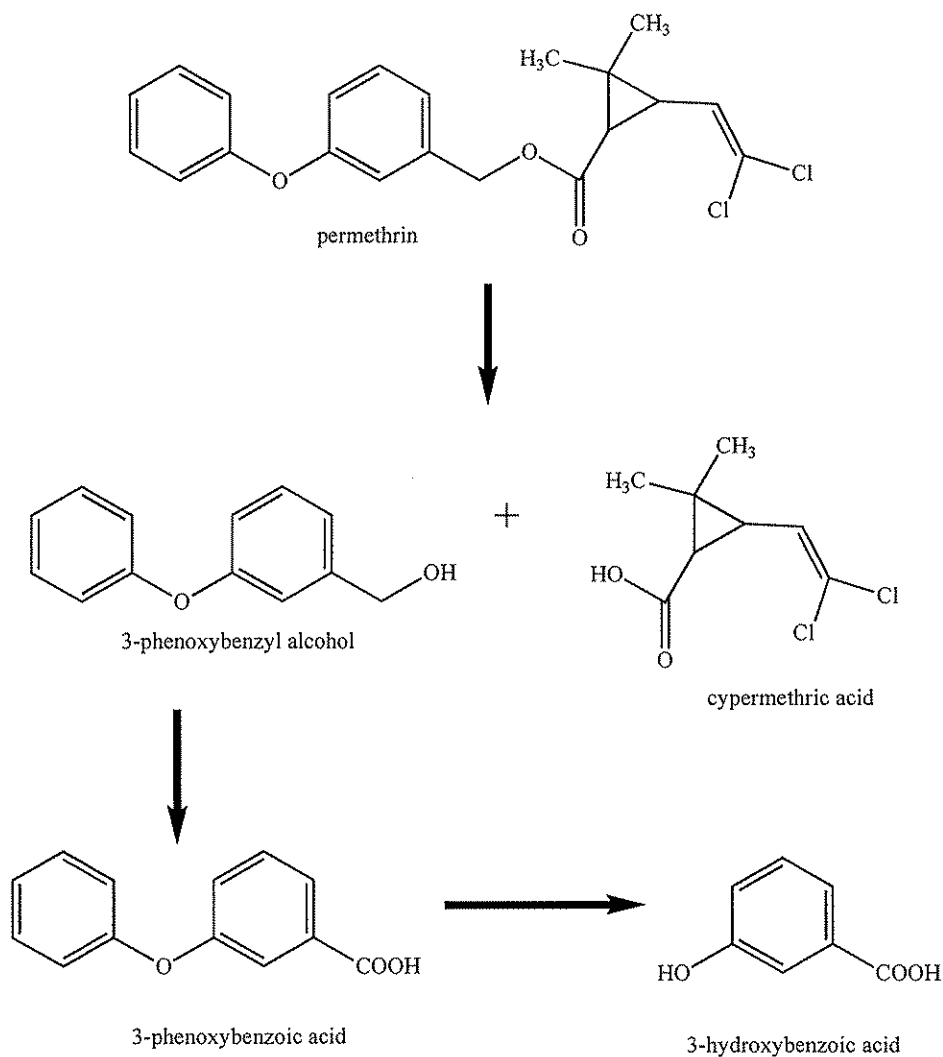
Figure 1-11: Chemical Structure of Permethrin



Commercial preparations of permethrin are typically 50:50 mixtures of the cis- and trans-isomers with respect to the terminal double bond. Studies have shown that permethrin is degraded in soil via hydrolysis into four major products: cypermethric acid, 3-phenoxybenzyl alcohol, 3-hydroxybenzoic acid, and 3-phenoxybenzoic acid.⁷³⁻⁷⁹ This degradation pathway is shown below in Figure 1-12. In 2006, Qin and Gan found that these products were only transitory in nature and were subsequently quite rapidly converted into carbon dioxide.⁷⁸ They also noted that the trans-permethrin was less persistent than the cis-permethrin.⁷⁸ The half-life of both isomers in soil has been studied, and a rather wide range of values, from three weeks to twenty-one months, have been reported.^{75,76,80-83} Maloney et al. found that thermophilic bacteria acting on permethrin produced a half-life of four weeks, with degradation products similar

to those shown in Figure 1-11.⁷⁶ Again, the trans-isomer was removed more rapidly by the

Figure 1-12: Degradation of Permethrin



bacteria than the cis-isomer, and they postulated that this was due to it being “less insecticidally active”.⁷⁶

Chapter II: Materials and Methods

A. Chemical Insecticides Used in Field and Laboratory Studies

Standards for the individual insecticides were purchased from Sigma-Aldrich (St. Louis, MO), Fluka Analytical (St. Louis, MO) or Supelco (St. Louis, MO) and used without further purification; specifications for these standard materials are given below in Table 2-1.

Table 2-1: Sources and Purity of Chemical Standards

Chemical	Source	Purity
Malathion	Supelco	99.1%
Carbaryl	Fluka Analytical	99.8%
Permethrin ^a	Fluka Analytical	98.0%
Phenanthrene	Sigma-Aldrich	98%

^aPermethrin is a mixture of cis- and trans- isomers.

For the field studies, commercial preparations of the insecticides were purchased from local home improvement stores (Home Depot, Norman, OK and ACE Hardware Store, Norman, OK). These commercial preparations were selected to be as close as possible to the form of the compounds that would be

accessed by the public at large if ever used in an attempt to interfere with the subsequent determination of the post mortem interval, PMI, following the commission of a homicide, for example. The active ingredients listed for each of the commercial preparations was carefully checked to ensure that no synergists or interfering substances were present. The following commercial brands of the individual insecticides were used: Spectracide® Malathion (50% wt/vol solution of malathion in a xylene range aromatic solvent, St. Louis, MO), Sevin® (22.5% wt/wt suspension of carbaryl in an aqueous solvent, Atlanta, GA) and ACE Soil & Turf Insect Control® (2.5% wt/vol suspension of permethrin in low molecular weight organic solvents, Oak Brook, IL).

B. Field Studies

B. 1. Solutions Used to Treat the Pig Carcasses

The commercial insecticides were diluted to 0.50% (0.50 g per 100 mL) with respect to the active ingredient for the field studies. For the Spectracide® Malathion, 20.0 mL of the concentrated solution was transferred to a 2.00 L volumetric flask, diluted to the mark with singly distilled water (dH₂O) and mixed thoroughly. For the Sevin® sample of carbaryl, 44.4 mL of the concentrated solution was transferred to a 2.00 L volumetric flask, diluted to the

mark with dH₂O and mixed thoroughly. For the ACE Soil & Turf Insect Control®, 200.0 mL of the concentrated permethrin solution was transferred to a 2.00 L volumetric flask, diluted to the mark with dH₂O and mixed thoroughly.

B. 2. Preparation of Pig Carcasses Prior to Placement in the Field

Male and female domestic pig carcasses, *Sus scrofa*, were purchased from a local swine farm (Dreomm Smith farm, Newalla, OK). The weight of each pig was measured using a digital spring based scale with a hook (Rapala Fish Scale model RGSDS-50 with range of zero to fifty pounds). The pigs were treated in groups of four with one pig for each of the (1) control, (2) malathion treated, (3) carbaryl treated, and (4) permethrin treated. Each of the treated pigs were placed in thirty gallon polyethylene tubs and washed with one of the 0.50% commercial insecticide solutions for ca. five minutes. Movement of the solution by hand, using rubber gloves, of course, ensured uniform and comprehensive coverage of all surfaces with the insecticidal solution.

The treated pigs were all placed in a remote area of the Fred G. Oliver Wildlife Preserve located just southeast of the corner of Chautauqua and Oklahoma State Highway 9 in Norman, OK. Each pig was placed in a cage to protect it from consumption by carnivorous animals in the surroundings or any detritivores. The frame of the cages were constructed from pieces of 2x4 lumber,

and the entire structure which was roughly a cube measuring ca. 3 ft on a side, was then covered with steel mesh chicken wire with openings typically less than one inch in size. The bottom or floor of the cage was covered completely with chicken wire, intended to prevent even moles from reaching the carcass via burrowing from the ground below. The four pigs, each in individual cages, were located approximately at the corners of a square measuring ca. 10 meters on a side.

In order to guarantee that the insects reasonably contacted and/or experienced the insecticide located on the external surfaces of the pig carcass, we carefully plugged all the normally accessible orifices (mouth, nostrils, ears, and anus) with sterile gauze.

B. 3. Environmental Surroundings in the Field Studies

The temperature and relative humidity data was recorded using a centralized HOBO® Temperature Logger (Onset® Computer Corporation, Cape Cod, MA) with five separate probes. Four of the probes were capable of temperature measurement, while the fifth was capable of measuring both temperature and relative humidity. Each of the four temperature probes was placed in each of the four cages, respectively. Each was attached to the center of the roof of the structure and allowed to hang down into the cage so that the

actual probe was ca. one foot above the upper region of the pig carcass. The probe that had the ability to simultaneously measure the temperature and the relative humidity was placed in the center of the square arrangement of the pig carcasses.

Measurements of temperature and relative humidity taken from the five sensors, as noted above, were recorded every hour that the carcasses were left in the field. There were a total of three different experiments performed using, thus, a total of 12 different pig carcasses. The three field experiments were conducted in the following time periods:

1. Summer Exp't # 1 -- July 27, 2010 to August 7, 2010
2. Summer Exp't # 2 -- August 8, 2010 to August 18, 2010
3. Winter Exp't # 1 -- January 22, 2010 to April 30, 2010

Unfortunately, the data collected in the third experiment was subsequently not useful due to the environmental conditions. In the initial portion of the time period of the third experiment, the temperature of the carcasses was constantly below the baseline temperature for the local fly population. By the time the environmental temperature rose above the baseline temperature, the carcasses were mummified and no longer in a phase of decomposition that would attract blow flies. Thus, no flies laid any eggs on the carcasses; no eggs, larvae, pupae, or adult flies were observed on the carcasses. When the pigs were finally removed from the remote location, degradation had progressed to the post-decay

stage via bacterial action and autolysis, but no insect involvement. The tabulated temperature and relative humidity vs. time data for the first two experiments are presented in Appendix C.

Rainfall was another important environmental parameter that was of concern at the outset of this investigation. Thus, rain gauges were uniformly attached to the northern-most side of the individual cages to measure rainfall during the field tests. The directional location of the gauges was not important; however, the uniformity of position was judged prior to the experiments to be potentially important. But, the inclusion of the rain gauges to the experiments turned out to be of no consequence since no rain was observed during either of the two summer field tests conducted in the current study.

B. 4. Sampling of Various Stages of Flies in the Vicinity of the Carcasses

Following placement of the carcasses in their field locations, we commenced daily monitoring to detect the initial appearance of flies. Flies in the immediate vicinity of each of the carcasses in the field studies were detected and sampled for taxonomic identification using Catchmaster™ glue boards (AP&G, Brooklyn, NY) placed ca. one foot from the carcass, as shown in Figure 2-1 below. One glue board per carcass was initially placed in the cage on the same day as the pigs were positioned in the field. The glue board for each carcass was

replaced by a second, fresh one, 24 hours later. The date, time of placement, time of removal, and carcass identification for each glue board was recorded, typically on the back of the board while in the field. A total of two glue boards per carcass were deemed necessary since the majority of larval growth had already begun by the end of the second day. Glue boards were routinely stored in a freezer at -4 °C until species identification and abundance was determined from each of the glue boards.

Figure 2-1: Field Setup for Typical Carcass Showing Glue Board



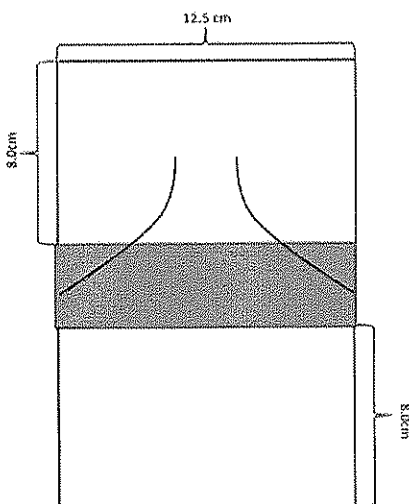
Upon placement of the carcasses in their field locations, we also commenced daily visual monitoring of the individual carcasses to detect the appearance of any ovipositing. The initial occurrence and location of ovipositing on the carcass was documented. Ova were examined macroscopically to determine viability. If viable, ova remained on the carcass; however, non-viable were taken and placed in a glass vial (15x45 mm, Kimble Chase, Vineland, NJ) for subsequent insecticide analysis by GC/SIM-MS as described below. The combination of a gas chromatograph and a mass spectrometer operated in the selected ion mode is commonly abbreviated as simply GC/SIM-MS. When first instar larvae were found, a sample containing typically 50 larvae was transferred to a glass vial, and the location of the larvae on the carcass as well as the date were documented. Pupae were not collected for analysis, since they were not easily located and/or could not certainly be identified to be associated with the carcass of concern. Similarly, no adults collected in the vicinity were subjected to analysis since the association with the carcass degradation process could not be verified.

A second sample of first instar larvae, when initially discovered on the carcass of each treated and each control pig carcass, was transferred to a separate, plastic rearing chamber (BioQuip® Products, Rancho Dominguez, CA), as shown below in Figure 2-2, for the isolated growth of the insects in an insecticide-free environment. The rearing chamber was covered loosely with an

opened plastic zip-lock bag inverted over the top to prevent rain entry through the ventilated screen located in the center of the top. It was then placed in the nearby vicinity to the carcass so it would subsequently experience the same temperature variations thereafter as did the carcass. The rearing chambers contain an upper and a lower chamber. The lower chamber was filled with a small layer of Vermiculite®, on top of which was placed a small container of a very particular type of minced cat food (Purina® Fancy Feast® Chicken and Liver, Gray Summit, MO). The maggots taken from the carcass were placed on the cat food directly, and the upper and lower portions of the rearing chamber were then re-assembled for subsequent incubation. When larval feeding ceased, the larvae migrated into the Vermiculite®, which provided a dry place for the larvae to pupate. Upon adult emergence, the flies flew into the upper chamber.

Samples of the various stages of larvae, pupae and adult flies were collected from the rearing chambers and recorded along with the dates of collection and the associated carcass for each sample. The samples were subjected to subsequent GC/SIM-MS analysis as described below.

Figure 2-2: BioQuip® Rearing Chamber



C. Lethal Concentration (LC₅₀) Determinations for Insecticides

In order to get an assessment of the toxicity of the various insecticides employed in regards to the flies, we performed a series of experiments with adults. These studies were aimed at assessing the amount (ng/cm²) of the insecticide which, when placed on the inside of a liquid scintillation vial containing the adult flies, caused death in 50% of the flies after 24 hours.

C. 1. Preparation of Solutions and Vials for Subsequent Fly Exposure

For the malathion studies, 0.0010% (w/v) solutions of malathion were prepared by adding 2.03 µL of the 99.1% standard malathion to a 250.0 mL

volumetric flask, diluting to the mark with dH₂O and mixing thoroughly. Due to solubility constraints, the final carbaryl solutions were only 0.00050% (w/v). In a 500.0 mL volumetric flask, 2.5 mg of carbaryl was added to an initial aliquot of 10 mL of ethanol (200 proof alcohol from Aaper Alcohol, Shelbyville, KY) with the latter placed in the 500.0 mL flask to solubilize the carbaryl. After complete dissolution of the carbaryl in the ethanol, the mixture was diluted to the mark with dH₂O and mixed thoroughly. Previous studies by Joanna Buley⁸⁴ have already provided comparable numbers for permethrin, so no LC₅₀ studies were performed on this latter insecticide.

Scintillation vials (20 mL, internal surface area of 29.46 cm²) were cleaned and sterilized in an autoclave prior to treatment with an insecticide solution. The volume of insecticide solution described below in Table 2-3 for the particular compound was pipetted into the bottom of a scintillation vial. The

Table 2-3: Volumes of Insecticides* in Each Vial for LC₅₀ Assays

Malathion Volume (μL)	Carbaryl Volume (μL)	Final Surface Dose (ng/cm ²)
2.0	4.0	0.68
4.0	8.0	1.4
8.0	16.0	2.7
10.0	20.0	3.39
12.0	24.0	4.07

* Concentrations employed were: malathion – 0.0010%
carbaryl – 0.00050%

vial was rolled on a hotdog roller (NostalgiaTM Electronics, Helman Group Ltd., USA), without heat application, to facilitate drying of the solvent and even distribution of the insecticide on the internal glass surface. One adult fly, one to five days following emergence from the pupa stage, was placed in each vial. The vial was covered with single layer of sterile gauze, attached with a rubber band around the upper neck of the vial, to block escape of the fly. The vial was kept in the lab near a natural light source, an unshaded window, for 24 hours. After 24 hours, the fly was observed for mortality. Mortality was determined to be either actual death or exhibition of ataxia, since all flies exhibiting the latter behavior expired within two hours of the termination of the experiment. Ataxia in blow flies is determined as “wobbly” flying, loss of the righting ability when placed upside down, and/or shakiness when walking. For each insecticide

examined, there were three sets of experiments using 30 flies at each dose listed in Table 2-2. That was a total of 900 individual flies used for the complete investigation.

D. Gas Chromatograph with Mass Spectrometer Detector

An Agilent model 6980 gas chromatograph coupled with a HP-5973 mass selective detector (Agilent Technologies, Santa Clara, CA) was used for the analysis of all insecticide samples. The column was a 30 m x 0.25 mm, model number HP-5, coated on the internal surface with 5% phenylmethylsiloxane to obtain a 0.25 μ m film thickness. We employed splitless injection of 1.00 μ L samples into a injector maintained at a constant temperature of 220°C.

Due to longer retention time for permethrin and problems with carryover, a higher final temperature was required for the analysis of permethrin. We slightly altered the remaining chromatographic temperature programming parameters from those initially employed for malathion and carbaryl. For both malathion and carbaryl, the column oven temperature program was held constant at 160°C for 5 minutes, followed by a linear temperature ramp at 20°C/minute up to 260°C, and, then, held constant at 260°C for 5 minutes. For the permethrin, the oven temperature program was held constant at 160°C for 5 minutes, followed by a linear temperature ramp at

20°C/minute up to 300°C, and, then, held constant at 300°C for 5 minutes. The higher final temperature for permethrin was found necessary to obtain elution of this insecticide in a reasonable time frame.

For all insecticide analyses, the transfer line temperature was maintained at 280°C. The mass spectrometer was operated in the electron ionization mode (70 eV ionization potential) and set to perform selected ion monitoring for the individual insecticides and the internal standard. In the selected ion monitoring mode for this instrument, one can simultaneously monitor up to four different single mass windows. This practically means one can see four individual ions at the same time. However, the particular four ions monitored may be altered during the elution up to 50 times, with switching times for one set of four to another set of four being accomplished in less than 100 seconds. Thus, if a number of masses beyond four is desired to be monitored during a single injection, one can program the switching to occur at the appropriate times following injection if sufficient separation can be achieved between the corresponding peaks one desires to monitor. The individual retention times and ions monitored for each of the insecticides and the internal standard are listed below in Table 2-4.

Table 2-4: Mass Spectrometer Ions Monitored for Individual Chromatographic Peaks

Analyte	Retention Time (min)	Ions Monitored
Carbaryl	9.3	201, 115, 127
Malathion	9.0	173, 158, 143, 330
Permethrin	15.4, 15.5 ^a	183, 163, 184,
Phenanthrene	7.6 ^b	178, 152, 176
	6.8 ^c	

^a Permethrin is a mixture of cis- and trans- isomers. It is not known if the initially shown time is for the cis- or the trans- isomer.

^b Retention time shown for the internal standard under elution conditions employed for malathion and carbaryl determinations.

^c Retention time shown for the internal standard under elution conditions employed for permethrin determinations.

Between each sample injection the syringe was rinsed thoroughly with the hexanes solvent followed by a thorough rinse with the appropriate solvent to prevent any carryover. The appropriate solvent used for the rinse was the same as that used for dissolution and homogenization of the individual insecticides of concern, which was dichloromethane for malathion and carbaryl, and ethyl acetate for permethrin. Prior to each injection, the syringe was carefully rinsed with the solution being analyzed to ensure that no dilution occurred due to the previous rinsing. Individual samples were routinely analyzed in triplicate.

Between sample solutions, an injection of the appropriate solvent, either dichloromethane or ethyl acetate, was analyzed to ensure that there was no undesirable carryover from the previous solution which may have been retained on the column or elsewhere in the analytical system.

E. Laboratory Growth of Flies in the Presence of Insecticides and Metabolic Clearing of the Insecticides in Subsequent Growth

We were initially concerned about the general localization and/or subsequent normal metabolic expulsion of any insecticide to which the fly larvae were exposed in the field studies. As such, we proceeded to conduct a controlled experiment in the laboratory in which flies were hatched and raised throughout the larval stages on an insecticide treated food source. This chronic experiment provided information about altered developmental times in these stages as well as the levels of insecticide incorporated into the larvae at various times following hatching. After the initial hatching of the eggs and the appearance of the first instar stage of larvae in the chronically treated group just described, a sample of the larvae were transferred into a new growth chamber that contained liver with no insecticide. This second group provided information concerning both developmental time and insecticide concentrations throughout growth following acute exposure to the insecticide. After the chronically treated group initially expressed the second instar of growth, another sample of larvae

were transferred to another new growth chamber containing liver with no insecticide. This third group provided information on developmental time and insecticide concentrations in the larva throughout the subsequent growth following what we have labeled as extended acute exposure to the insecticide. Thus, we created chronic, acute and extended acute groups for observation and data collection.

E. 1. Determining Chronic Effects of Insecticides on Larval Growth

Approximately 50 pupae from blow flies commonly known as blue bottle flies i.e., *Protophormia terra-novae*, were placed in BioQuip® rearing chambers as previously described, with a bed of Vermiculite® and ca. 10 grams of raw chicken liver in a Petri dish sitting on top of the Vermiculite®. The chicken livers were purchased from a local grocery store (Homeland, Norman, OK), divided into ca. 10 gram portions in weigh boats, wrapped in cellophane and frozen at -4°C. Approximately 24 hours prior to utilization, the desired number of 10 gram portions of liver were placed in the refrigerator at 4°C and allowed to thaw. Individual portions of raw liver placed in the rearing chambers were always replaced with fresh pieces of liver every 24 hours. The rearing chambers were kept at a constant temperature of 25°C. Dried milk and granular sucrose were placed in a separate dish on top of the Vermiculite® to provide

sustenance as a carbohydrate source for the adult flies when these initially emerged from the pupae. The fresh chicken liver served as a source of protein for the adult flies as well as an ovipositing site. Each of the 50 flies, incidentally, would typically produce ca. 30 eggs, and ca. 70% of the eggs hatched. The hatching from all 50 flies typically occurred within a 3-5 hour time frame. After oviposition, the eggs were transferred to fresh rearing chambers containing livers treated, by surface exposure, to 1.00 mL of 0.010% insecticide (malathion, carbaryl, or permethrin) placed in the bottom of the Petri dish. The insecticides were spread over the surface of the meat quite evenly via multiple manual rotations, using rubber gloves, of the liver in the liquid. A fresh sample of liver, as mentioned above, was added each 24 hours that the experiment continued. Simultaneous to the addition of the fresh liver, a fresh 1.00 mL aliquot of the insecticide was added each 24 hours as well. Once every 24 hours, a small sample of ca. 10 larvae were removed from the rearing chamber and subjected to insecticide analysis by GC/SIM-MS.

E. 2. Determining Acute Effects of Insecticides on Larval Growth

A sample containing approximately one-fourth of the first instar larvae from the chronic group for a particular insecticide, as described above, was transferred to a new rearing chamber with a fresh 10 grams of untreated chicken liver. The larvae in this sample were subsequently identified as having received

the acute insecticide treatment. A fresh sample of liver was added each 24 hours that the experiment continued thereafter. Also, once every 24 hours, a small sample of ca. 10 larvae were removed from the rearing chamber and subjected to insecticide analysis by GC/SIM-MS.

E. 3. Determining Extended Acute Effects of Insecticides on Larval Growth

A sample containing approximately one-fourth of the initially appearing second instar larvae from the chronic group for a particular insecticide, as described above in section D.1, was transferred to another new rearing chamber with a fresh 10 grams of untreated chicken liver. The larvae in this sample were subsequently identified as having received the extended acute insecticide treatment. A fresh sample of liver was added each 24 hours that the experiment continued thereafter. Also, once every 24 hours, a small sample of ca. 10 larvae were removed from the rearing chamber and subjected to insecticide analysis by GC/SIM-MS.

E. 4. Limitations on Chronic, Acute, and Extended Acute Studies Due to the Particular Insecticide Employed

The controlled laboratory experiments described above for chronic, acute, and extended acute exposure of the larvae to insecticides was initially intended to be performed on each of three insecticides: malathion, carbaryl, and

permethrin. In actual execution of the experiments, we found that the experimental design only worked for malathion.

In the cases of both carbaryl and permethrin, the eggs laid by the adult flies on the insecticide treated liver never hatched. Examination of these eggs under a dissecting microscope (model Nikon 80X, Nikon® Instruments, Inc., Melville, NY) revealed no unusual characteristics. Thus, we created a modified treatment plan for the carbaryl and permethrin treated groups. In the adjusted experimental paradigm for these two insecticides, we allowed the eggs to be laid by the adult flies on an untreated liver substrate. Upon hatching into the first instar larval stage, the larvae were transferred into a new rearing chamber containing insecticide treated liver, with the liver and insecticide solution being changed daily as described above. Larval samples taken daily thereafter formed what we subsequently labeled as a “modified chronic” group. When the modified chronic group initially expressed the second instar larvae, we again split a portion of these larvae and began to grow them in a new rearing chamber with an untreated liver source. This new collection, subsequently grown on only untreated liver, was labeled as the “modified extended acute” group. The livers, treated or untreated, respectively, were changed daily as described previously for the chronic and extended acute groups. Larval samples were taken daily from all groups during the entire larval growth period for GC/SIM-MS analysis.

Unfortunately, the eggs transferred to the permethrin treated livers to form the modified chronic group for permethrin never hatched. Thus, in the end we had no controlled laboratory data for the permethrin treated samples.

F. Analysis of Insecticides Using Gas Chromatography and Selected Ion Monitoring with a Mass Spectrometer

Quantitative determinations of malathion, carbaryl, and permethrin in prepared and larval derived samples were performed using a commercial gas chromatograph which employed a mass spectrometer as a detector (Model No. HP-5985B from Hewlett-Packard, Van Nuys, CA). The mass spectrometer was programmed to perform selected ion monitoring for the pertinent ions of the compound(s) of concern. The collected combination of these analytical units is commonly abbreviated as simply GC/SIM-MS.

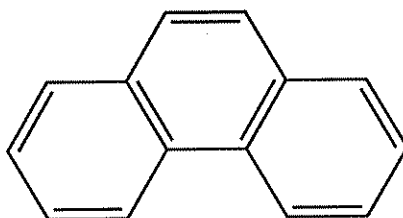
F. 1. Preparation of Standard Solutions and Standard Curves

Quantitative determinations of malathion, carbaryl, and permethrin in various samples employed the use of standard curves. These were established using serial dilution to produce known concentrations of the analyte. Simultaneously, we employed an internal standard to minimize errors due to sample loss and/or variable momentary analytical procedural alterations.

F. 1. a. Stock and Working Solutions of the Internal Standard

Phenanthrene (Sigma-Aldrich, St. Louis, MO) was used as the internal standard for all GC/MS analyses. Two 0.050% stock solutions were prepared for use with the different insecticide standards. The first stock solution was prepared

Figure 2-3: Chemical Structure of Phenanthrene



Phenanthrene

by weighing 5.0 mg of phenanthrene, transferring to a 10.0 mL volumetric flask, diluting to the mark with dichloromethane (ACS grade, Mallinckrodt Baker, Phillipsburg, NJ), and mixing thoroughly. The second phenanthrene stock solution was prepared by weighing 5.0 mg of phenanthrene, transferring to a 10.0 mL volumetric flask, diluting to the mark with ethyl acetate (ACS grade, Mallinckrodt Baker, Phillipsburg, NJ), and mixing thoroughly. Two 0.0010% working standard phenanthrene solutions were prepared by, in each case, transferring 200.0 μ L of a stock solution into another 10.0 mL volumetric flask, diluting to the mark with the appropriate solvent, and mixing thoroughly. We refer to these stock and working internal standard solutions as phenanthrene in

dichloromethane and phenanthrene in ethyl acetate, respectively. Working standard solutions were prepared fresh on the day of analysis when needed.

F. 1. b. Stock and Working Solutions of the Insecticide Malathion

A stock solution of 0.010% malathion was prepared by adding 2.03 μL of the standard, having a density of 1.23 g/mL, to 25.00 mL volumetric flask, diluting to the mark with dichloromethane and mixing thoroughly. This stock solution was used to make the diluted standards for the calibration curve. Seven working concentrations containing 0.00, 0.200, 0.400, 4.00, 8.00, 20.0, and 40.0 ppm malathion, respectively, were prepared in seven separate 1.50 mL eppendorf tubes. The first of these, of course, is a blank. For each of the seven working concentration tubes, we began preparation by the addition of a fixed 100 μL of the working 0.0010% internal standard solution of phenanthrene in dichloromethane. For the seven individual working concentrations listed above, we next added the 0.010% malathion stock standard in volumes (μL), respectively, of 0.00, 2.00, 4.00, 40.0, 80.0, 200.0 and 400.0. Finally, we wanted to have the final total volume be 1.00 mL. Considering the insecticide solution and the internal standard solutions already employed, we thus finally added, respectively, the following amounts (μL) of dichloromethane: 900, 898, 896, 860, 820, 700, and 500.

Analysis of larvae from our samples revealed concentrations of malathion to be consistently below 8.0 ppm. We then decided to employ the working standards only from 0.0 to 8.0 ppm to establish our standard curve. Working standard solutions were prepared fresh on the day of analysis when needed.

F. 1. c. Stock and Working Solutions of the Insecticide Carbaryl

A stock solution of 0.010% carbaryl was prepared by (1) adding 5.0 mg of the standard to 10.00 mL volumetric flask, diluting to the mark with dichloromethane, and mixing thoroughly and, then, (2) delivering 2.00 mL of the initial solution to 10.00 mL volumetric flask, diluting to the mark with dichloromethane, and mixing thoroughly. This second, stock solution was used to make the working standards for the calibration curve. Five working concentrations containing 0.00, 5.00, 10.0, 25.0, and 50.0 ppm carbaryl, respectively, were prepared in five separate 1.50 mL eppendorf tubes. The first of these, of course, is a blank. For each of the five working concentration tubes, we began preparation by the addition of a fixed 100 μ L of the working 0.0010% internal standard solution of phenanthrene in dichloromethane. For the five individual working concentrations listed above, we next added the 0.020% carbaryl stock standard in volumes (μ L), respectively, of 0.00, 50.0, 100, 250, and 500. Again, we wanted to have the final total volume be 1.00 mL. Considering the insecticide solution and the internal standard solutions already

employed, we thus finally added, respectively, the following amounts (μL) of dichloromethane: 900, 850, 800, 750, and 400. Working standard solutions were prepared fresh on the day of analysis when needed.

F. 1. d. Stock and Working Solutions of the Insecticide Permethrin

A preliminary stock solution of 0.020% permethrin was prepared by adding 5.0 mg of the standard to 25.00 mL volumetric flask, diluting to the mark with ethyl acetate, and mixing thoroughly. A 500 μL aliquot of this preliminary stock solution was transferred to a 10.00 mL volumetric flask, filled to the mark with ethyl acetate, and mixed thoroughly. The resultant mixture contained 0.0010% permethrin and was labeled the stock solution. The stock solution was used to make the working standards for the calibration curve. Seven working concentrations containing 0.00, 0.10, 0.25, 0.50, 0.75, 1.00, and 2.50 ppm permethrin, respectively, were prepared in seven separate 1.50 mL eppendorf tubes. The first of these, of course, is a blank. For each of the seven working concentration tubes, we began preparation by the addition of a fixed 100 μL of the 0.0010% internal standard solution of phenanthrene in ethyl acetate. For the seven individual working concentrations listed above, we next added the 0.0010% permethrin stock standard in volumes (μL), respectively, of 0.00, 10.0, 25.0, 50.0, 75.0, 100, and 250. Again, we wanted to have the final total volume be 1.00 mL. Considering the insecticide solution and the internal standard

solutions already employed, we thus finally added, respectively, the following amounts (μL) of ethyl acetate: 900, 890, 875, 850, 825, 800, and 650. Working standard solutions were prepared fresh on the day of analysis when needed.

G. Extraction Efficiency for Insecticides in Larva

The extraction efficiency for each of the three insecticides in the larval-homogenizing solvent mixture was measured to see that we were not losing too much of the analyte to the procedure. Initially thinking that the high molecular weight components, lipids, and chitin might serve as major inhibitors to the dissolution of an insecticide during homogenization, we decided to employ filtration followed by multiple washings with solvent alone following homogenization. This approach, unfortunately, led to substantially lowered percent recoveries for each of the insecticides. Thus, we decided to see if this procedure might work with a more direct utilization of the homogenate. Indeed, exclusion of the filtering provided a better recovery, and that is what we used for the subsequent determinations.

G. 1. Insecticide Solutions for Determination of Extraction Efficiency

A stock solution containing 0.50% malathion was prepared from the 99.1% pure compound by transferring 40.7 μL , having a density of 1.23 g/mL,

to a 10.0 mL volumetric flask, diluting to the mark with dichloromethane, and mixing thoroughly. This stock solution was then diluted to a 0.0050% working solution by transferring 10.0 μ L of the 0.50% solution to an eppendorf microcentrifuge tube and adding 990 μ L of dichloromethane.

A stock solution of 0.050% carbaryl was prepared from the 99.8% pure standard by transferring 5.0 mg to a 10.0 mL volumetric flask, diluting to the mark with dichloromethane, and mixing thoroughly. This stock solution was then diluted to 0.0050% working solution by transferring 100.0 μ L of the 0.050% solution to an eppendorf microcentrifuge tube and adding 900 μ L of dichloromethane.

A stock solution of 0.020% permethrin was prepared from the standard by transferring 5.0 mg of the standard to a 25.0 mL volumetric flask, diluting to the mark with ethyl acetate, and mixing thoroughly. This stock solution was then diluted to a 0.0020% working solution by transferring 100 μ L of the 0.020% solution to an eppendorf microcentrifuge tube and adding 900 μ L of ethyl acetate.

G.2. Homogenization of Larvae

Third instar blue bottle fly larvae, *Protophormia terra-novae*, were used to determine the interference of protein, fats, chitin, and other components of the

larvae on the extraction efficiency of each of the insecticides. The larvae were starved for 24 hours prior to sacrifice to minimize interference from the wide variety of possible food-derived products in the gut. After being food-free for 24 hours, the larvae were quickly sacrificed by placement on dry ice. They were subsequently stored at -4°C until analysis by GC/SIM-MS.

A single larva, weighing typically between 50 mg and 100 mg, was allowed to briefly reach a temperature above freezing. It was then weighed and the weight was recorded. The larva was injected with a known volume of one of the working standard solutions (0.0050% for carbaryl and malathion; 0.0020% for permethrin). The volume of the working standard of a particular insecticide solution was varied to obtain multiple total amounts of insecticide with which to assess the extraction efficiency. The following volumes were used on individual larva: 2.0, 4.0, and 6.0 μL . The injected larva was placed in a test tube with the solvent and homogenized for 60 seconds or until the solution was milky in appearance with a mechanical homogenizer (Kinematica Polytron model PT 1200, Zurich, Switzerland). The homogenate was subjected to centrifugation in an Fisher table-top centrifuge model (Fisher Scientific, Pittsburgh, PA) for 60 seconds to pelletize the solids. The homogenate was evaporated to dryness by exposure to blowing air over the surface of the microcentrifuge tube. Reconstitution of the sample to a final volume of 1.00 mL was achieved by adding 900 μL of the appropriate (dichloromethane for malathion and carbaryl;

ethyl acetate for permethrin) solvent after addition of 100 μ L of the appropriate working internal standard solution described immediately above. The reconstituted solution was stirred by vortexing, and, then, was transferred to an eppendorf microcentrifuge tube and stored in the freezer until analysis by GC/SIM-MS.

G.3. Preparation of Standards and Determination of Percent Extraction

For comparison to the larvae treated standards, solutions of pure insecticides were prepared by appropriate dilutions of the working standard insecticide. Aliquots of 2.0, 4.0, 6.0, 8.0 and 10.0 μ L of the working standard insecticide were placed in each of five separate eppendorf microcentrifuge tubes. We then added 100 μ L of the working internal standard solution and sufficient solvent (dichloromethane for malathion and carbaryl; ethyl acetate for permethrin) to make a total volume of 1.00 mL. The solution was briefly mixed on a vortex stirrer, and it was stored in the freezer until analysis by GC/SIM-MS, which occurred on the same day as analysis of the related larvae extracts.

The peak areas of the insecticides to that of the internal standard determined from the GC/SIM-MS determinations for the larvae extracts were compared to the same ratio from the pure standard samples to determine the extraction efficiency.

Chapter III: Results and Discussion

A. Gas Chromatography with Mass Spectrometry (GC/SIM-MS) in the Analysis of Insecticides

Gas chromatography with selected ion monitoring mass spectrometry was employed to measure the insecticides and the internal standard.

A. 1. GC/SIM-MS Chromatograms and Spectra for Standards

GC/MS total ion current, TIC, chromatograms for standard samples for each insecticide were recorded. The retention times for the insecticide and the internal standard, phenanthrene, were noted for future reference and use. Three or four selected ions were chosen for the selected ion monitoring of each insecticide and the internal standard.

As shown in Figure 3-1 for a standard sample containing 8.0 ppm malathion and 1.0 ppm phenanthrene, baseline chromatographic separation was easily achieved between the phenanthrene at 7.63 minutes and the malathion at 9.08 minutes. The chromatographic run was extended well past the malathion peak to ensure that no carry over would occur from injection to injection. We independently ran a chromatogram of a sample containing a substantially larger concentration of the malathion and a corresponding high levels of the internal

standard under a continuous scan mode to identify the pertinent three or four ions for utilization in the selected ion monitoring of each of the corresponding peaks for all future GC/SIM-MS analyses. The continuous scanning mode performed cyclic, continuous scanning of unit m/z values in the range of 50 to 800. The resultant spectrum indicated that the three pertinent SIM ions to monitor for phenanthrene were 152, 176, and 178 (base). The three pertinent ions so determined for malathion were 158, 173 (base), and 330. Examination of these SIM ions for phenanthrene and malathion in the sample used above for Figure 3-1 yielded Figure 3-2, showing the SIM ions for a phenanthrene peak and Figure 3-3, showing the SIM ions for the malathion peak.

Figure 3-1: Malathion Standard Chromatogram

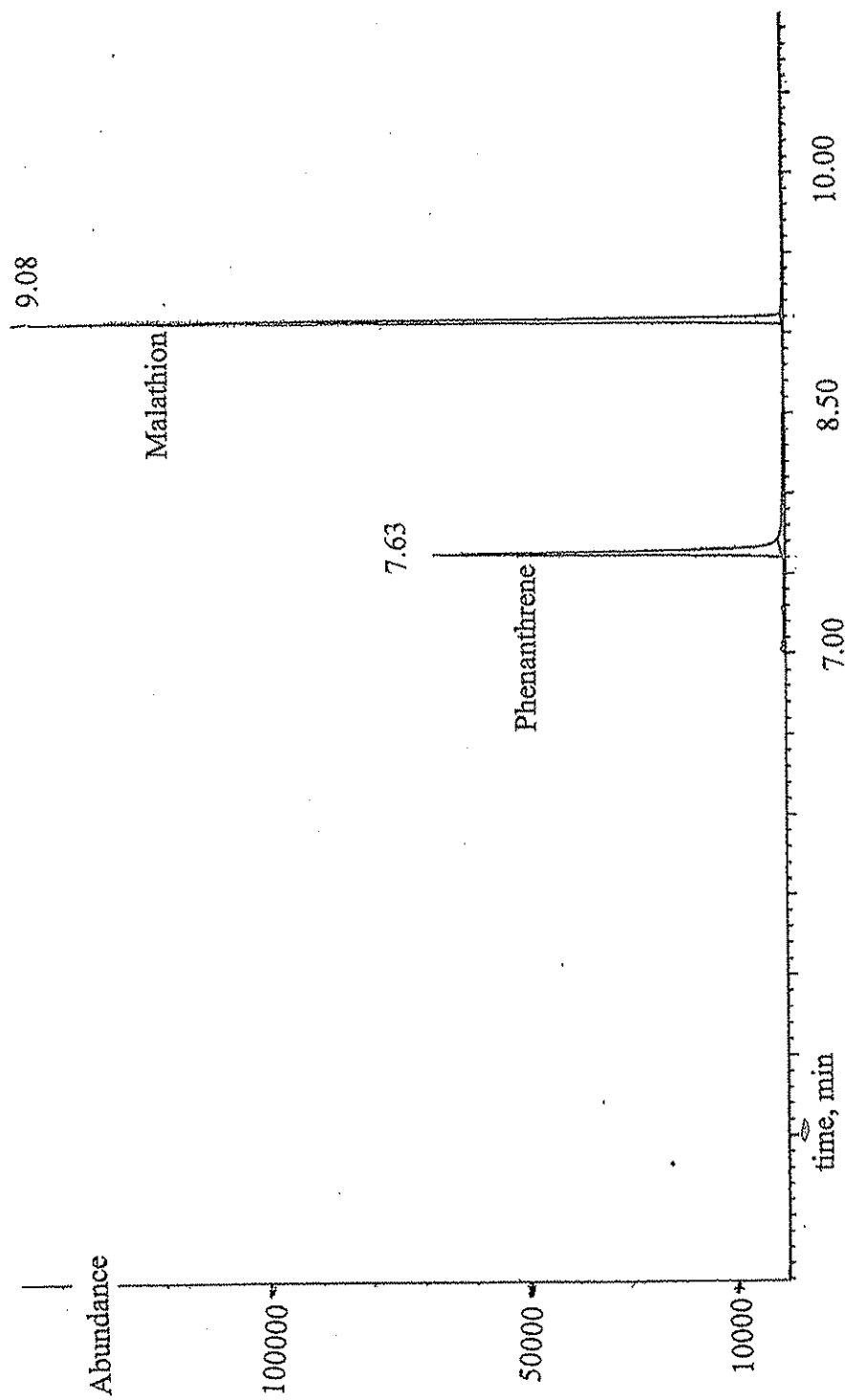


Figure 3-2: Phenanthrene SIM Spectrum

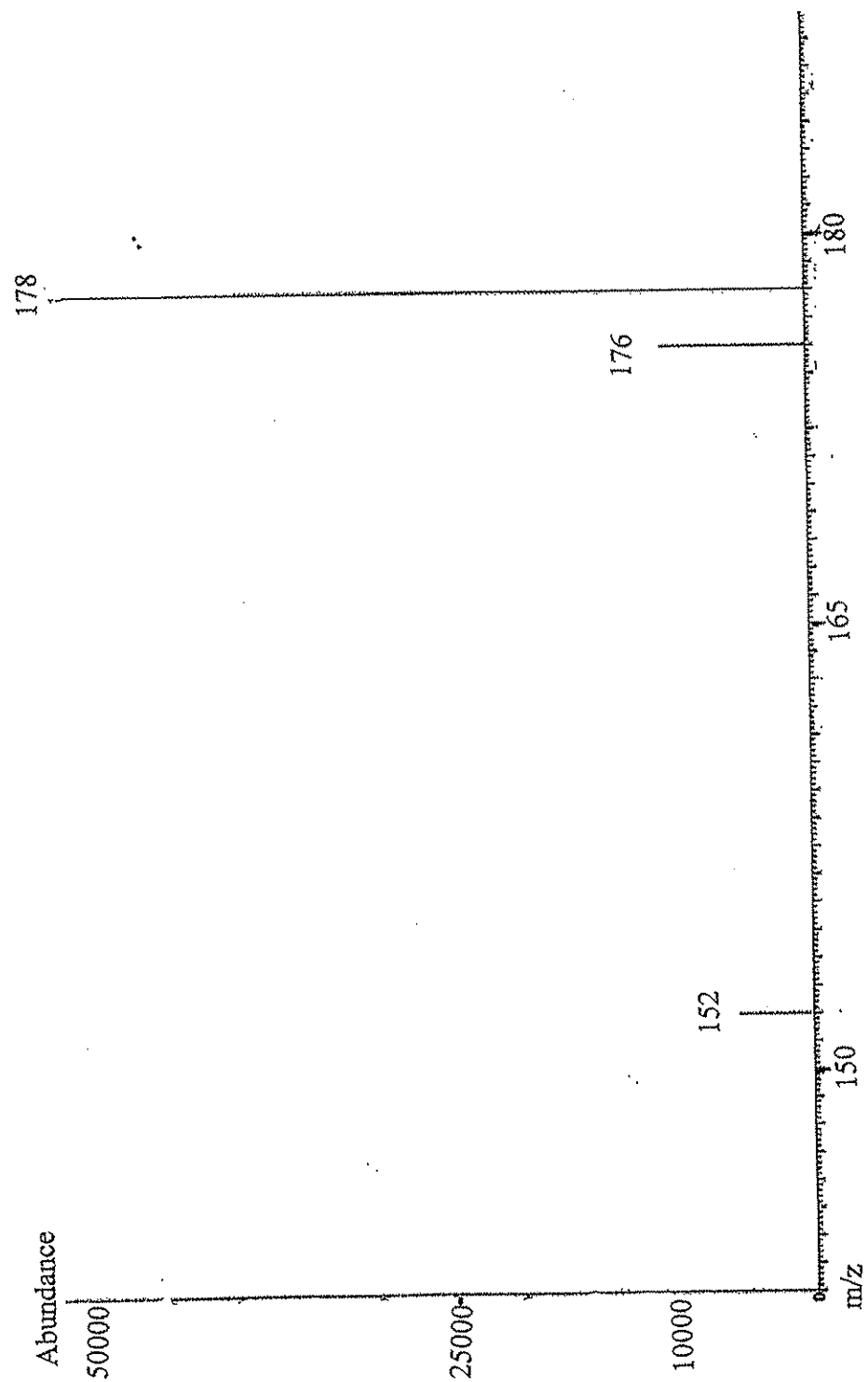
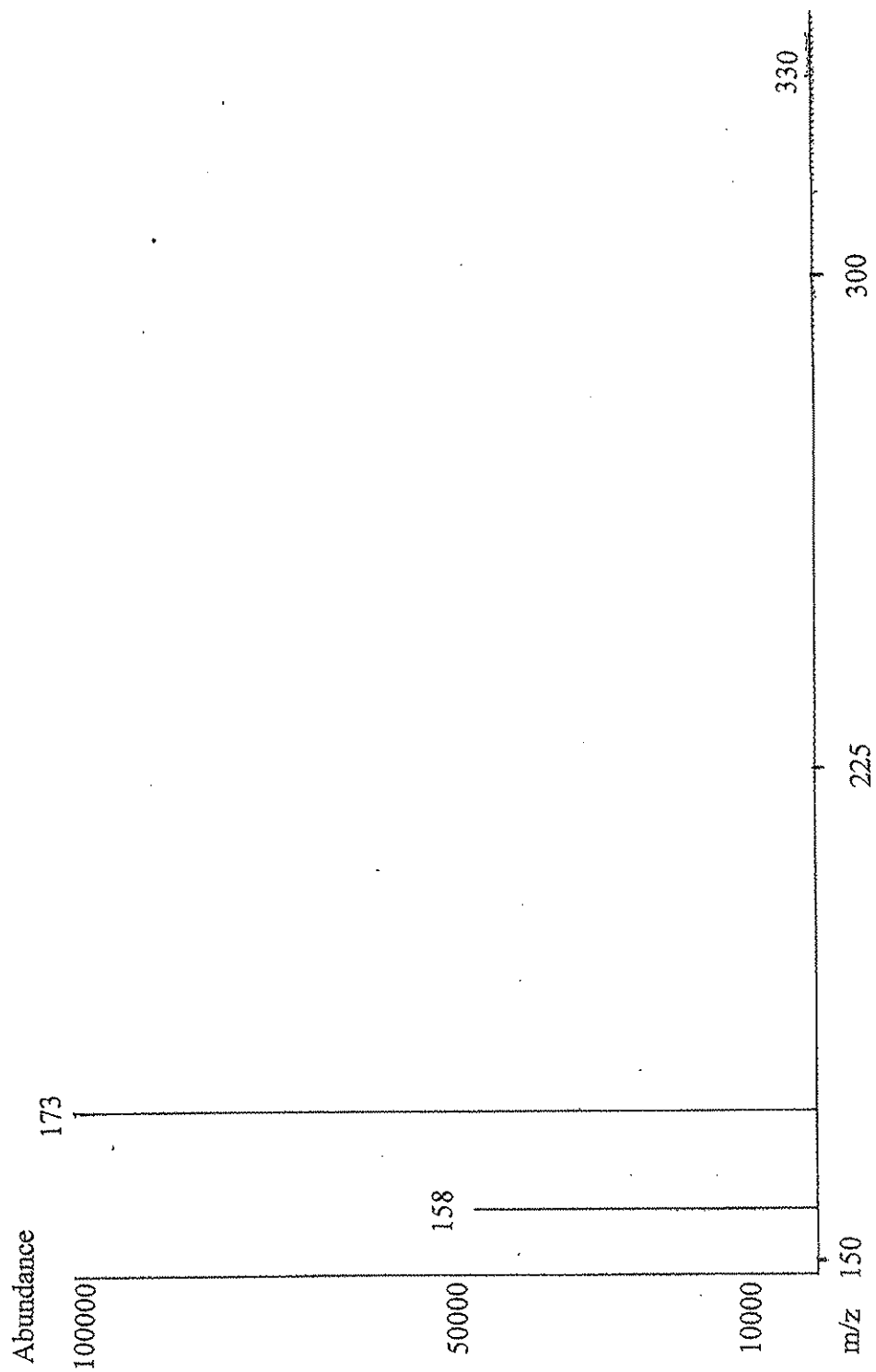


Figure 3-3: Malathion SIM Spectrum



A standard sample originally containing 2.0 ppm carbaryl and 1.0 ppm phenanthrene was allowed to age for a day so that it would have the opportunity to produce a significant concentration of the primary metabolite, α -naphthol. The result is shown in Figure 3-4. When injected into the GC/MS, baseline chromatographic separation was easily achieved between the α -naphthol at 4.55 minutes, phenanthrene at 7.64 minutes and the carbaryl at 8.64 minutes. The chromatographic run was extended well past the carbaryl peak to ensure that no carryover would occur from injection to injection. We independently ran a chromatogram of a sample containing a substantially larger concentration of the α -naphthol, carbaryl and a corresponding high level of the internal standard under a continuous scan mode to identify the pertinent three or four ions for utilization in the selected ion monitoring of each of the corresponding peaks for all future GC/SIM-MS analyses. The continuous scanning mode performed cyclic, continuous scanning of unit m/z values in the range of 50 to 800. The resultant spectrum indicated that the three pertinent SIM ions to monitor for α -naphthol were 89, 115, and 144 (base). The three pertinent ions for phenanthrene were, as found previously, 152, 176, and 178 (base). The three pertinent ions so determined for carbaryl were 115 (base), 127, and 201. Examination of these SIM ions for α -naphthol and carbaryl in the sample used above for Figure 3-4 yielded Figure 3-5, showing the SIM ions for a carbaryl peak and Figure 3-6, showing the SIM ions for a α -naphthol peak.

Figure 3-4: Carbaryl Standard Chromatogram

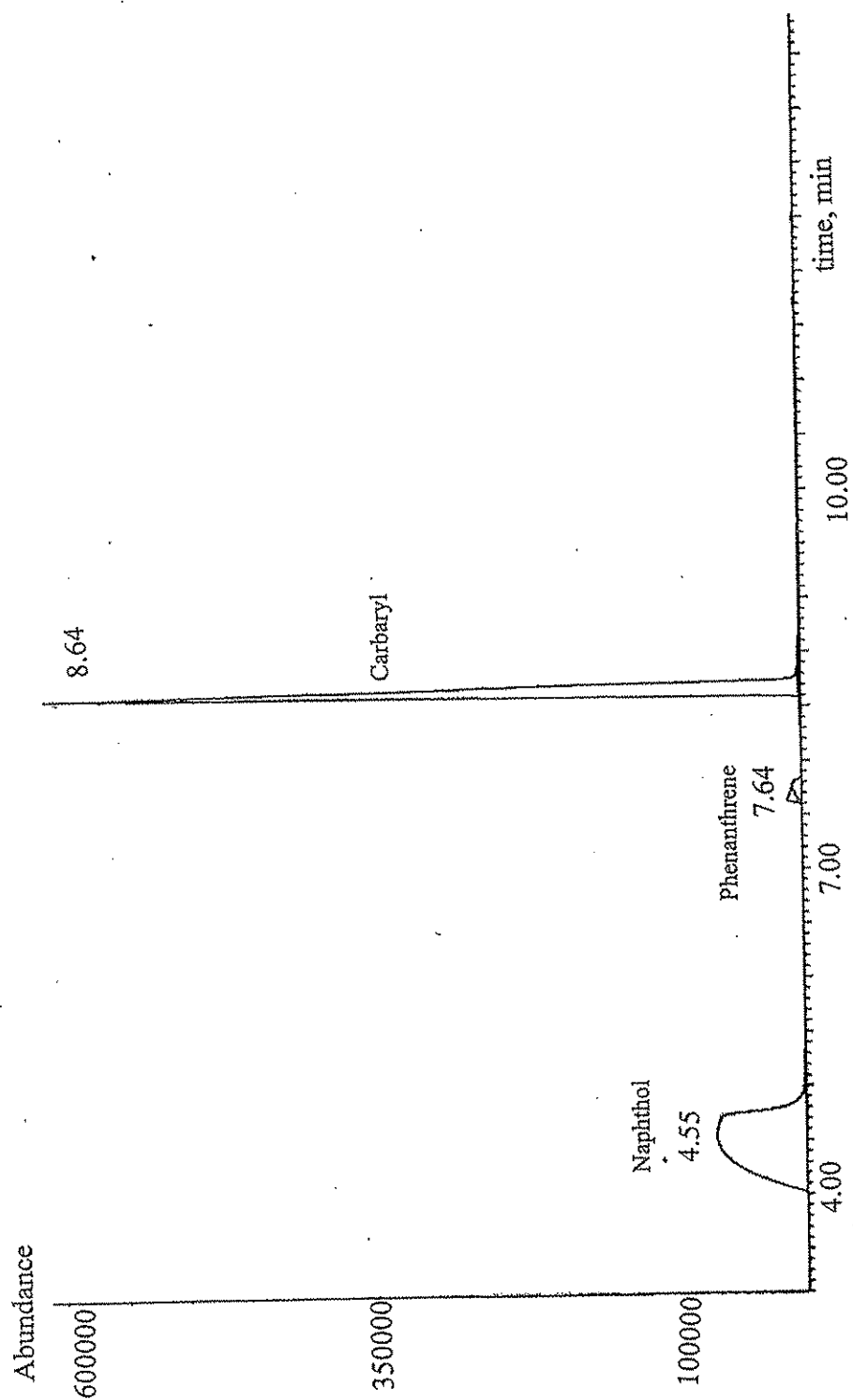


Figure 3-5: Carbaryl SIM Spectrum

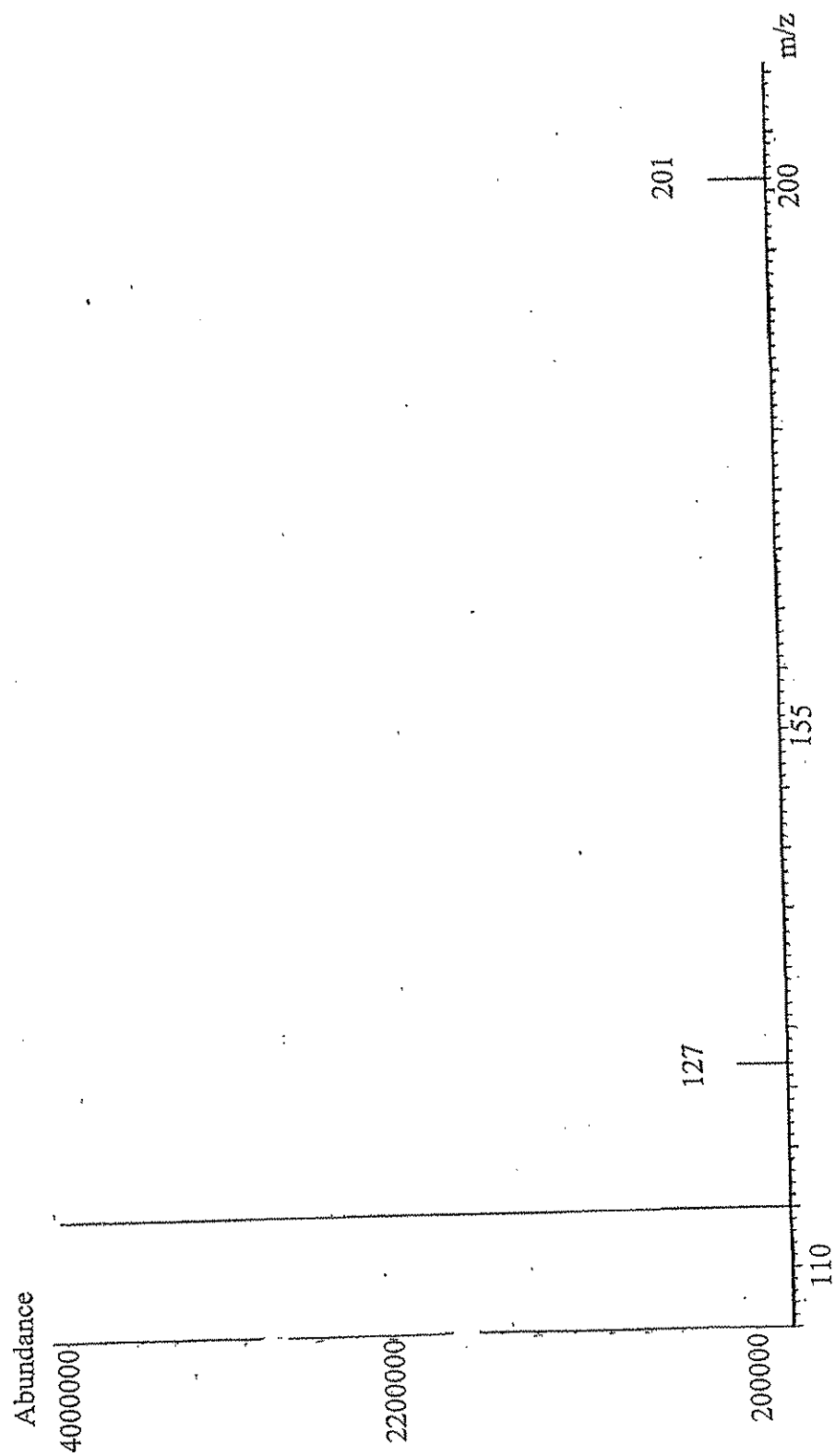
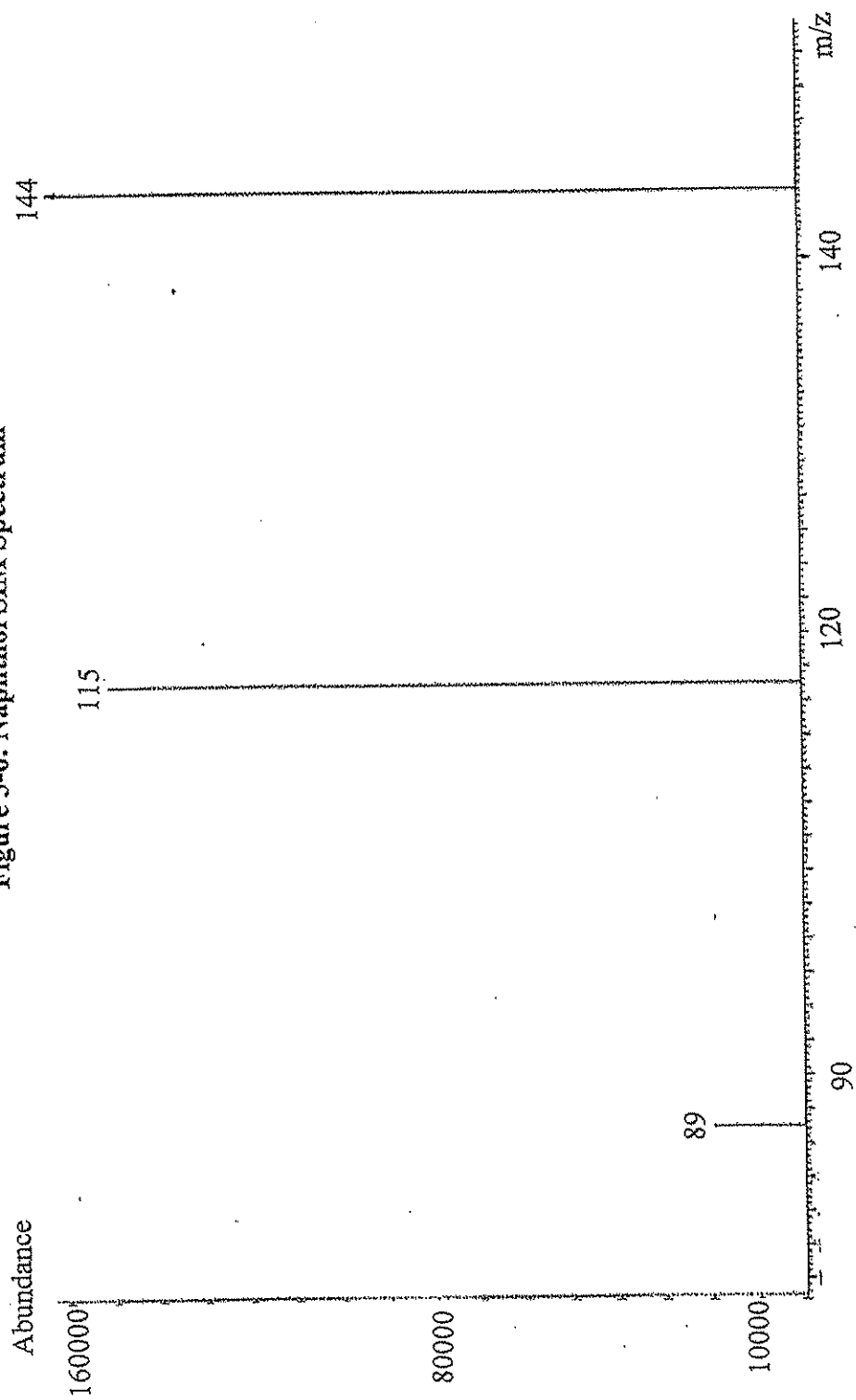


Figure 3-6: Naphthol SIM Spectrum



As shown in Figure 3-7 for a standard sample containing 2.5 ppm permethrin and 1.0 ppm phenanthrene, baseline chromatographic separation was easily achieved between the phenanthrene at 6.79 minutes and the two permethrin peaks at 11.90 and 11.96 minutes, respectively. These two peaks, as noted previously, represent the cis- and trans- isomers of the alkene functionality in permethrin. An expansion of the chromatogram in the area of the two peaks is shown in Figure 3-8. The chromatographic run was extended well past the permethrin peaks to ensure that no carryover would occur from injection to injection. We independently ran a chromatogram of a sample containing a substantially larger concentration of the permethrin and a corresponding high level of the internal standard under a continuous scan mode to identify the pertinent three or four ions for utilization in the selected ion monitoring of each of the corresponding peaks for all future GC/SIM-MS analyses. The continuous scanning mode performed cyclic, continuous scanning of unit m/z values in the range of 50 to 800. The resultant spectrum indicated that the three pertinent SIM ions to monitor for phenanthrene were, as found previously, 152, 176, and 178 (base). The four pertinent ions so determined for each of the individual permethrin peaks were 163, 183 (base), 184, and 391. SIM examination of the two permethrin peaks (either at 11.90 or 11.96 minutes) in the sample used above for Figure 3-7 yielded Figure 3-9, showing the expected SIM ions in the expected ratios.

Figure 3-7: Permethrin Standard Chromatogram

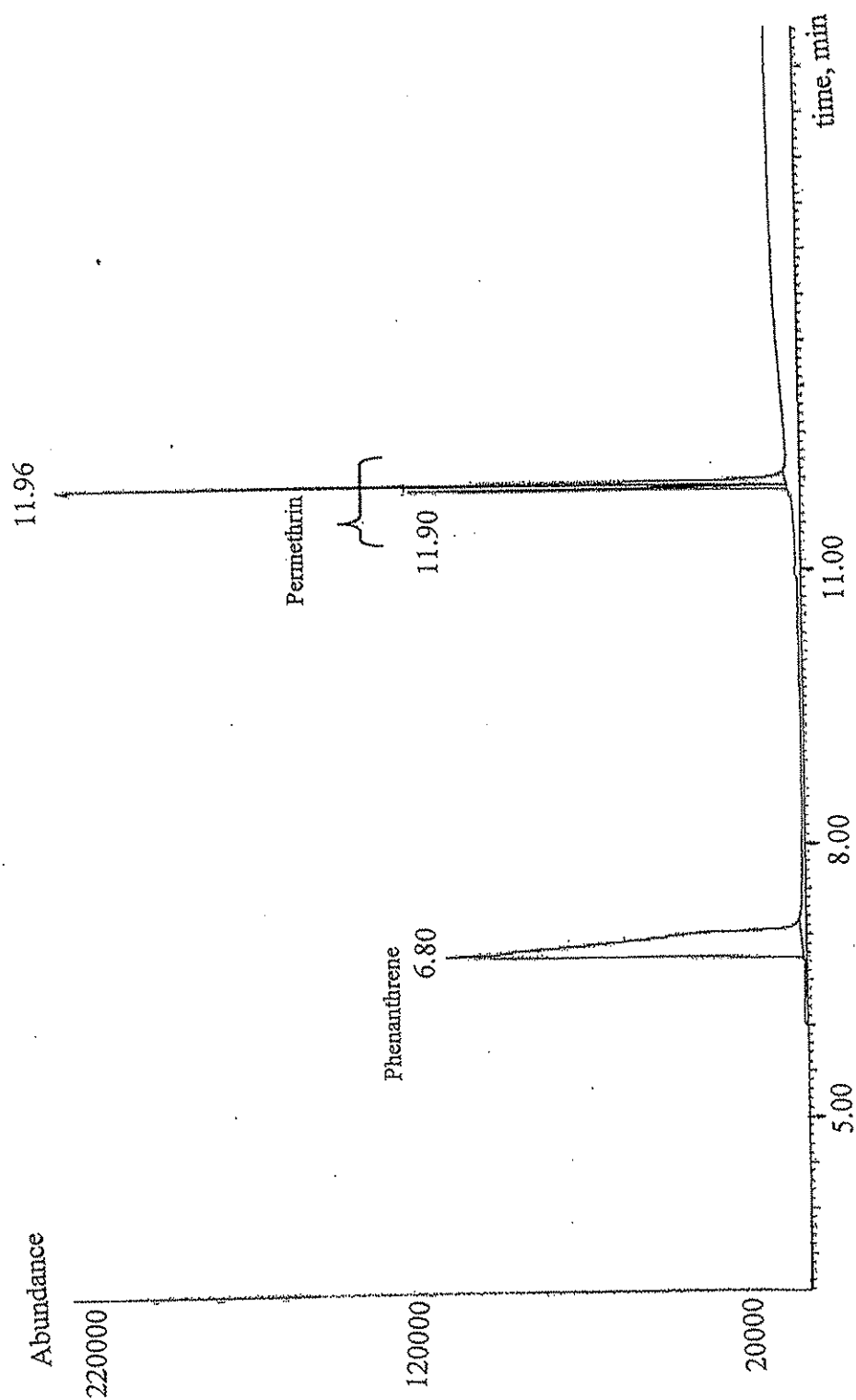


Figure 3-8: Permethrin Peaks from Standard Chromatogram

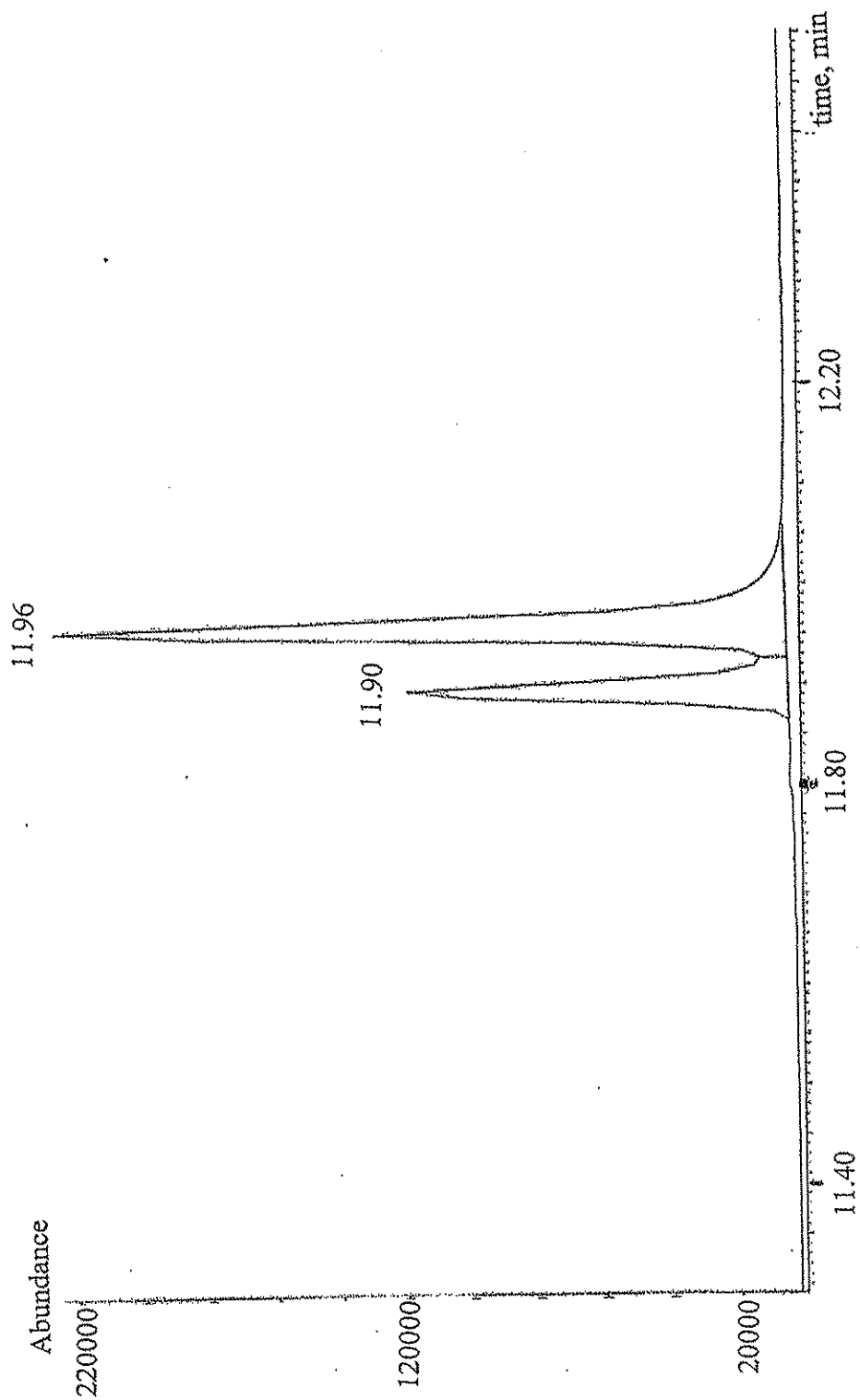
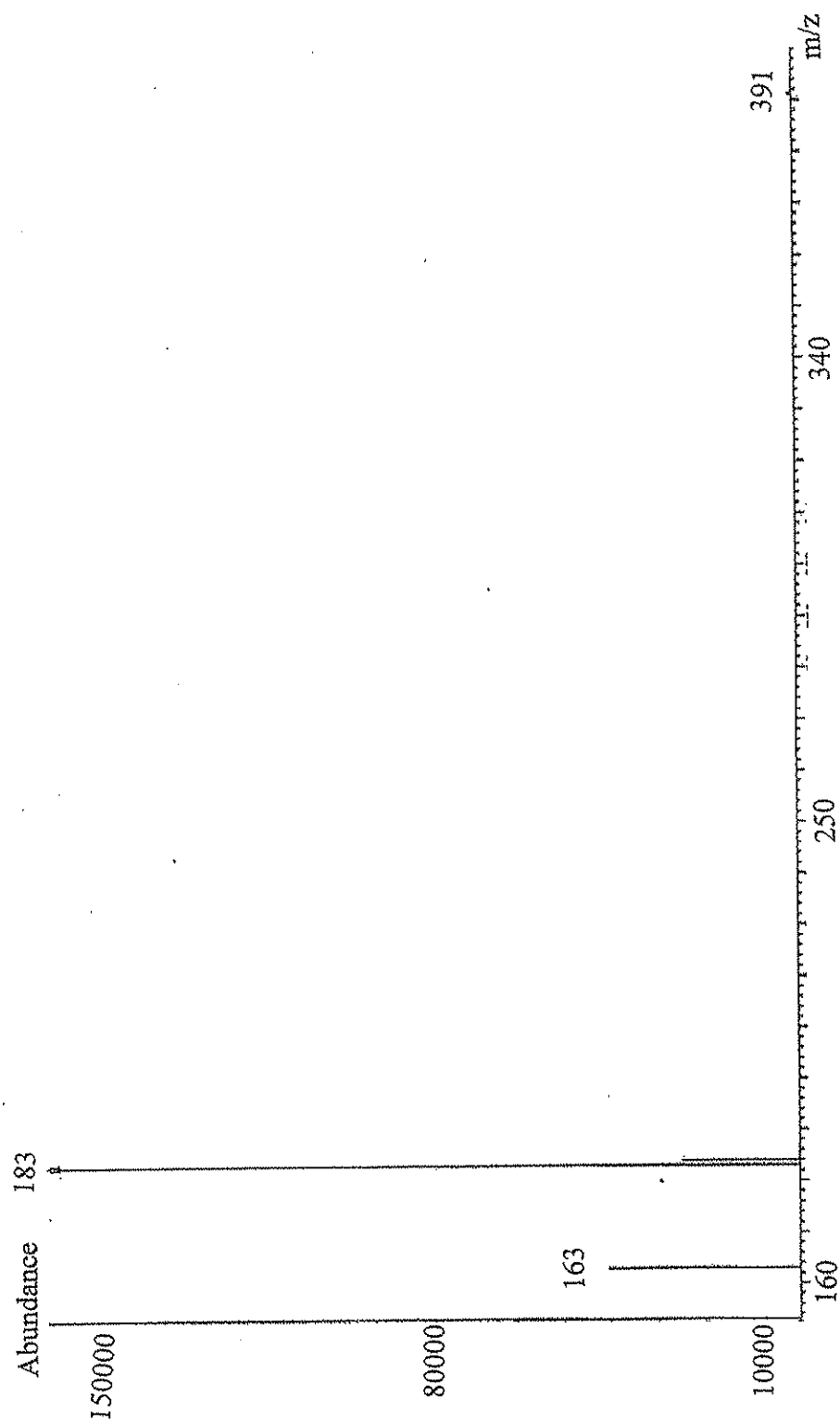


Figure 3-9: Permethrin SIM Spectrum



Careful analysis of all the standard samples provided the essential retention times and SIM ion ratios necessary to verify the identification of any peak observed at the proper retention time in an unknown sample as positively being one of the three known insecticides or the internal standard, phenanthrene. The retention times and the associated criteria for identification are shown in Table 3-1 below. The pertinent ions, their ratios, and the associated criteria for identification of each of the four compounds are presented in Tables 3-2 through 3-5, respectively, below.

**Table 3-1: Retention Times for Compounds Investigated
and Criteria for Ratios to Use in Subsequent
Positive Identification of Unknowns**

Analyte	Avg. Retention Time (min) ±s.d.	Range	Criteria for Substantiation of Peak Identification
Malathion	9.081±0.002	9.078–9.084	9.081 ± 0.005
Carbaryl	8.603±0.015	8.592–8.638	8.603 ± 0.030
Permethrin	11.895±0.002	11.891–11.898	11.895 ± 0.004
	11.959±0.002	11.954–11.960	11.959 ± 0.004
Phenanthrene	7.620±0.020	7.590–7.640	7.620 ± 0.040
	6.792±0.006	6.783–6.803	6.792 ± 0.01

**Table 3-2: Observed Ratios of Chosen SIM Peaks to Base Peak for
Phenanthrene for Standards and Criteria for Ratios to Use
in Subsequent Positive Identification in Unknowns**

Ion	Percent of Base Peak (178), Avg \pm s.d. (n)	Range of Percentages Observed	Criteria for Substantiation of Peak Identification
152	9.9 \pm 0.8 (12)	8.0 – 11.1	9.9 \pm 1.6
176	18.9 \pm 0.9 (12)	17.1 – 20.4	18.9 \pm 1.8

**Table 3-3: Observed Ratios of Chosen SIM Peaks to Base Peak for
Malathion for Standards and Criteria for Ratios to Use
in Subsequent Positive Identification in Unknowns**

Ion	Percent of Base Peak (173), Avg \pm s.d. (n)	Range of Percentages Observed	Criteria for Substantiation of Peak Identification
158	45 \pm 5 (8)	38 – 52	45 \pm 10
330	1.3 \pm 1.6 (8)	0.04 – 3.9	1.3 \pm 2

**Table 3-4: Observed Ratios of Chosen SIM Peaks to Base Peak for
Carbaryl for Standards and Criteria for Ratios to Use
in Subsequent Positive Identification in Unknowns**

Ion	Percent of Base Peak (115), Avg \pm s.d. (n)	Range of Percentages Observed	Criteria for Substantiation of Peak Identification
201	12 \pm 3 (8)	6.6 – 15	12 \pm 6
127	8 \pm 3 (8)	0.7 – 10	8 \pm 6

**Table 3-5: Observed Ratios of Chosen SIM Peaks to Base Peak for
Permethrin for Standards and Criteria for Ratios to Use
in Subsequent Positive Identification in Unknowns**

Ion	Percent of Base Peak (183), Avg \pm s.d. (n)	Range of Percentages Observed	Criteria for Substantiation of Peak Identification
163	32 \pm 12 (10)	19.6 – 54.5	32 \pm 24
184	17 \pm 1 (10)	15.4 – 19.9	17 \pm 2
390	2.3 \pm 1.9 (10)	0.37 – 5.6	2.3 \pm 3.8

A. 2. GC/SIM-MS Chromatograms and Spectra for Unknown Samples

Figures 3-10 through 3-18 represent GC/MS TIC chromatograms and SIM spectra for actual analyzed unknown insecticide samples for malathion, carbaryl, and permethrin, respectively.

Figure 3-10 exhibits a TIC chromatogram a larval homogenate from a malathion treated pig carcass, i.e. the carcass shown later as belonging to Experiment 2, Day 1. Figures 3-11 and 3-12, in order, examine the SIM output for the peaks observed in the chromatogram at 8.92 and 9.08 minutes. The observed ratios of the 158:173 and 330:173 SIM peaks in these two SIM spectra clearly indicate that the peak at 9.08 belongs to malathion, whereas the peak at 8.92, by comparison of the same ratios to the standard malathion SIM cannot be malathion. The ratios observed, respectively, for the 8.92 peak were 658 and 18%, while for the peak at 9.08 the same observed ratios were 48 and 0.3%.

Figure 3-10: Chromatogram of Larval Homogenate from Malathion Treated Carcass

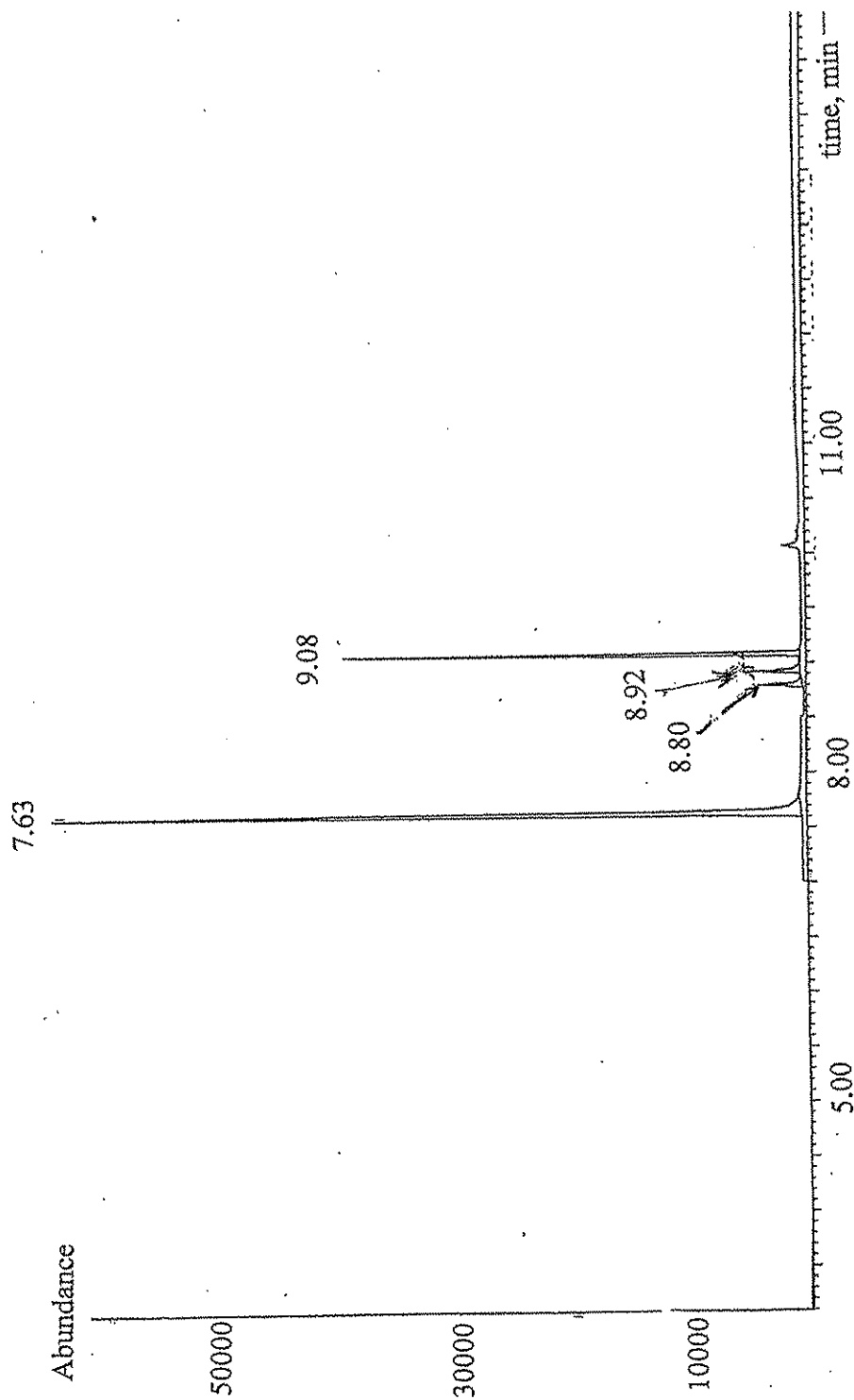


Figure 3-11: SIM Spectrum for Peak @ 8.92 Minutes (Unknown)

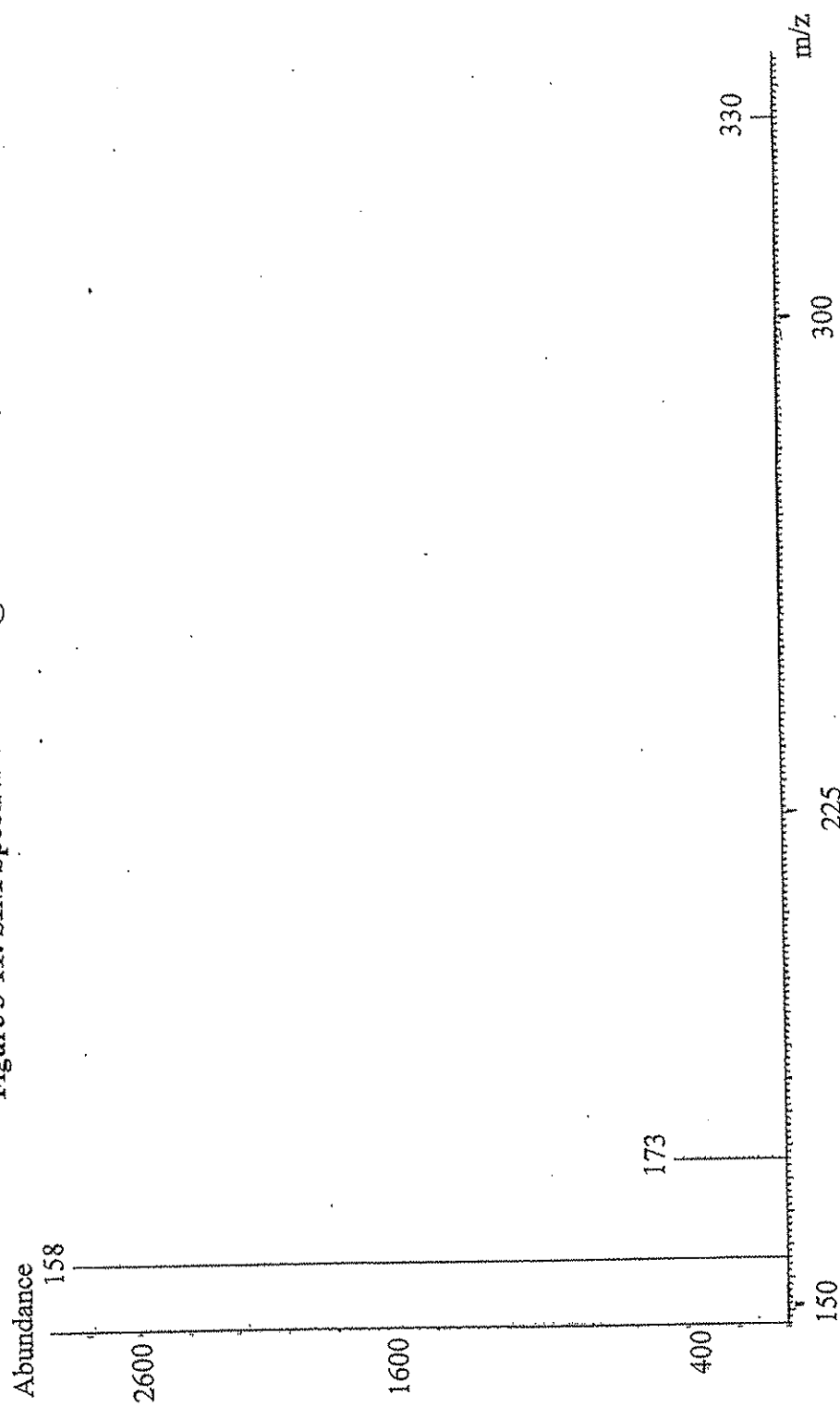


Figure 3-12: SIM spectrum for peak @ 9.08 minutes (Malathion)

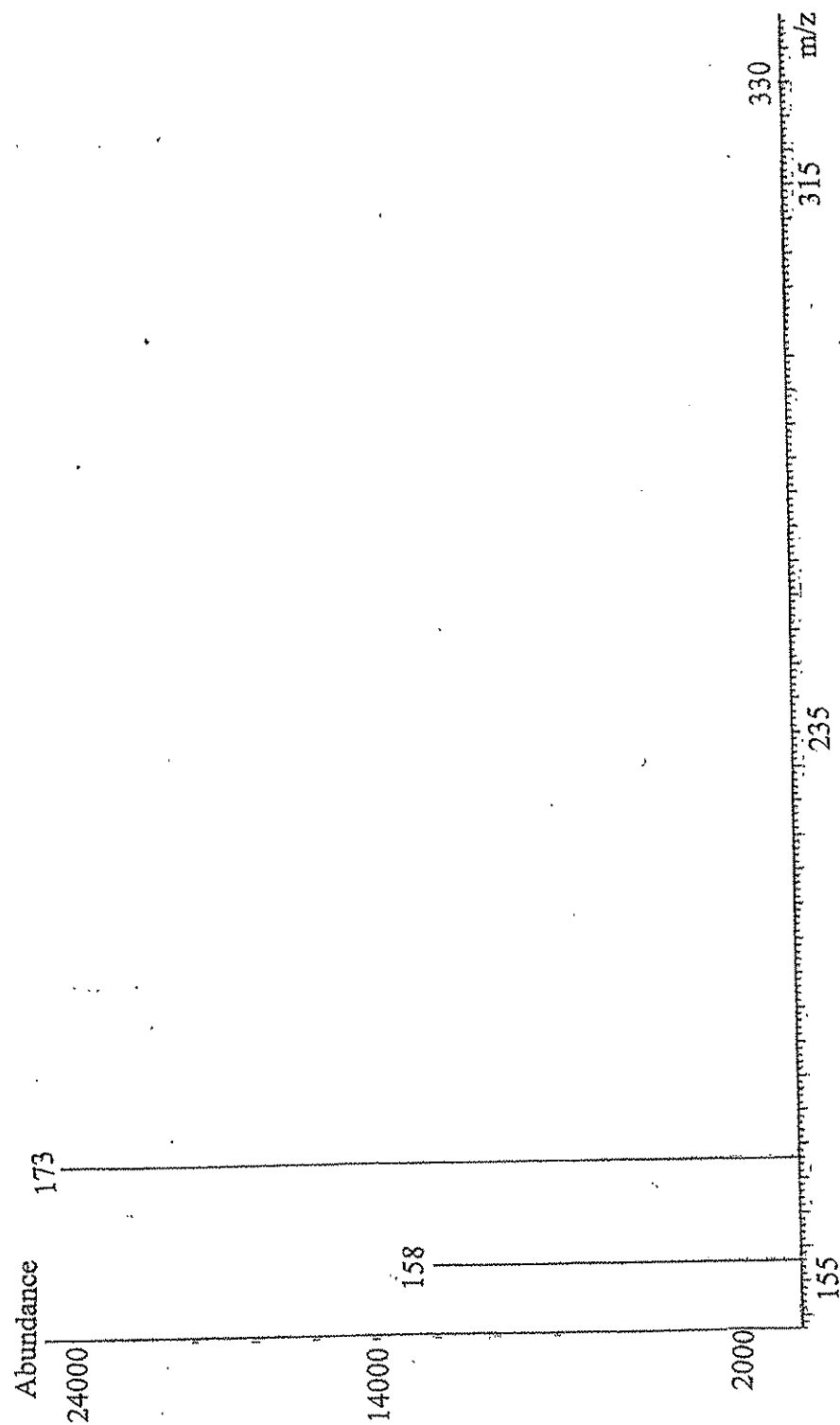


Figure 3-13 was taken from an analysis of carbaryl in the nonviable eggs from a carbaryl treated carcass, i.e. the carcass shown later as belonging to Experiment 1, Day 2. The SIM peak spectra for the chromatographic peaks seen in Figure 3-13 at 8.64 and 8.91 minutes, respectively, are shown in Figures 3-14 and 3-15. The ratios of the 127:115 and 201:115 SIM ions for Figure 3-14 were 7.1 and 8.0%. The same ratios, respectively, for Figure 3-15 were 154 and 0.8%. Comparison to the previously reported ratios of these SIM ions for the standard carbaryl made it easy to identify the peak eluting at 8.64 minutes to clearly be that of carbaryl, while the peak at 8.91 minutes was assigned as an unknown.

Figure 3-13: Chromatogram of Ova Homogenate from Carbaryl Treated Carcass

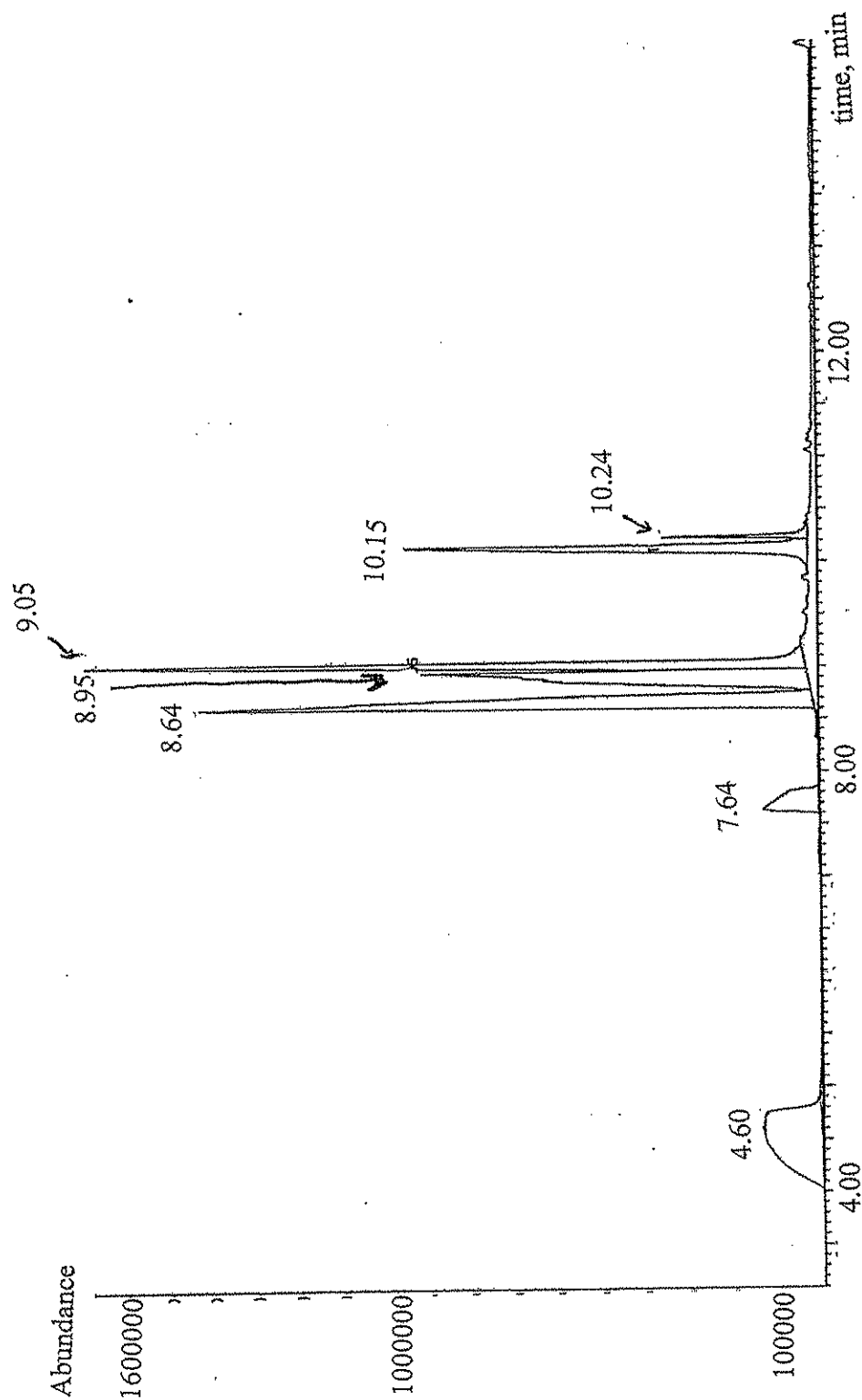


Figure 3-14: SIM Spectrum for Peak @ 8.64 minutes (Carbaryl)

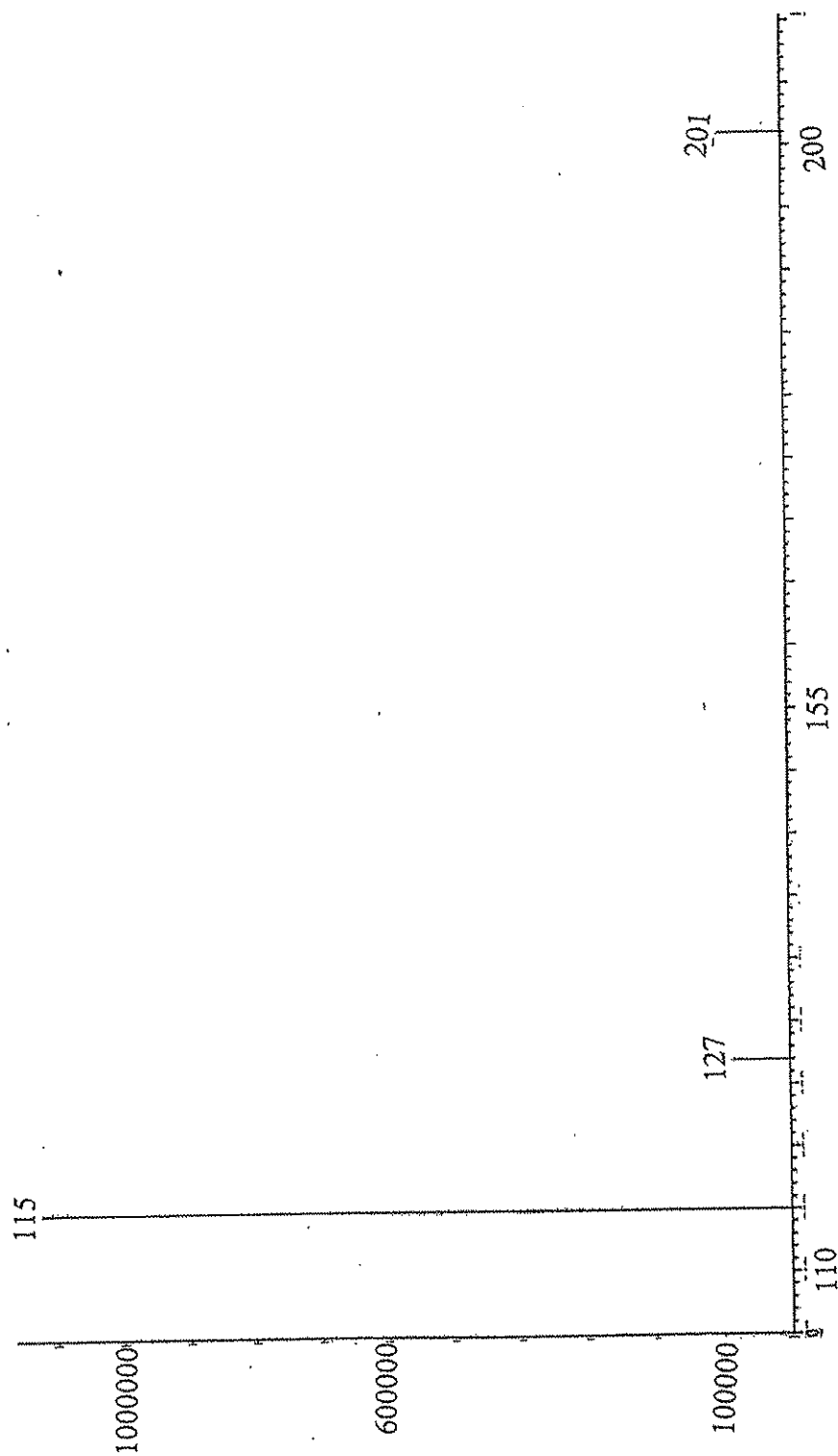
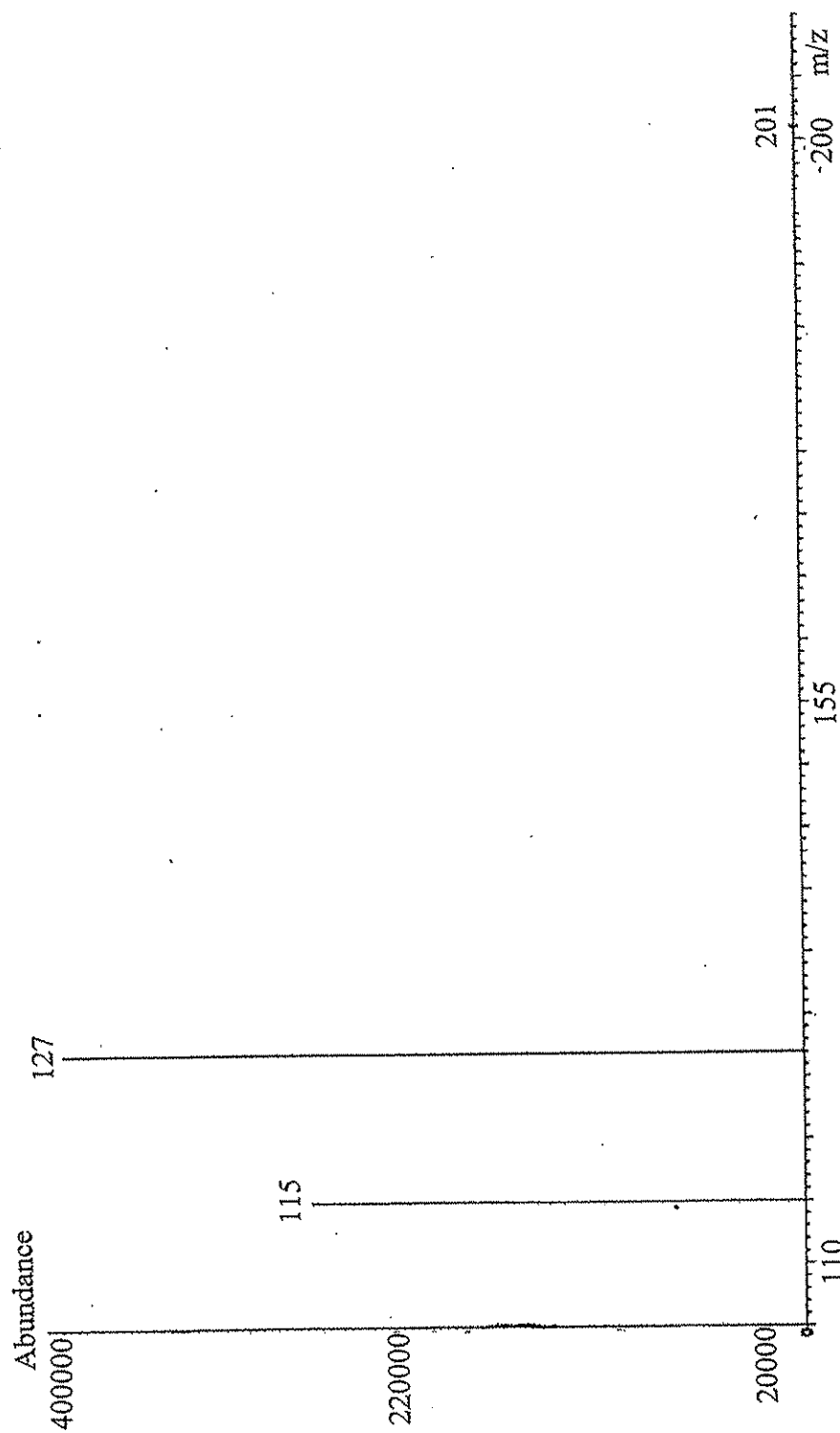


Figure 3-15: SIM Spectrum for Peak @ 8.91 Minutes (Unknown)



The chromatogram shown in Figure 3-16 represents a sample of larval homogenate derived from a permethrin treated pig carcass. Figure 3-17 exhibits an expansion of the two permethrin peaks observed to elute at 11.90 and 11.96 minutes, while Figure 3-18 represents a SIM spectra for the peak eluting at 11.90 minutes. A SIM spectra for the peak eluting at 11.96, incidentally, is essentially identical to that shown for Figure 3-18. The most interesting characteristic of the larval sample taken from the permethrin treated pig compared to the standard permethrin runs is the ratio of the cis- and trans-isomers of this compound. We are not able to identify which of the two peaks shown in the chromatogram in Figure 3-16 is cis- and which is trans-permethrin. Thus, we have simply labeled them as peak 1 and peak 2, in order of their chromatographic elution. The ratio of peak 1 to peak 2 is typically substantially less than one in the standard samples (see, e.g., Figure 3-8), whereas the same ratio in larval homogenates (e.g., Figure 3-17) was routinely greater than one. We will return to this point later.

Figure 3-16: Chromatogram of Larval Homogenate from Permethrin Treated Carcass

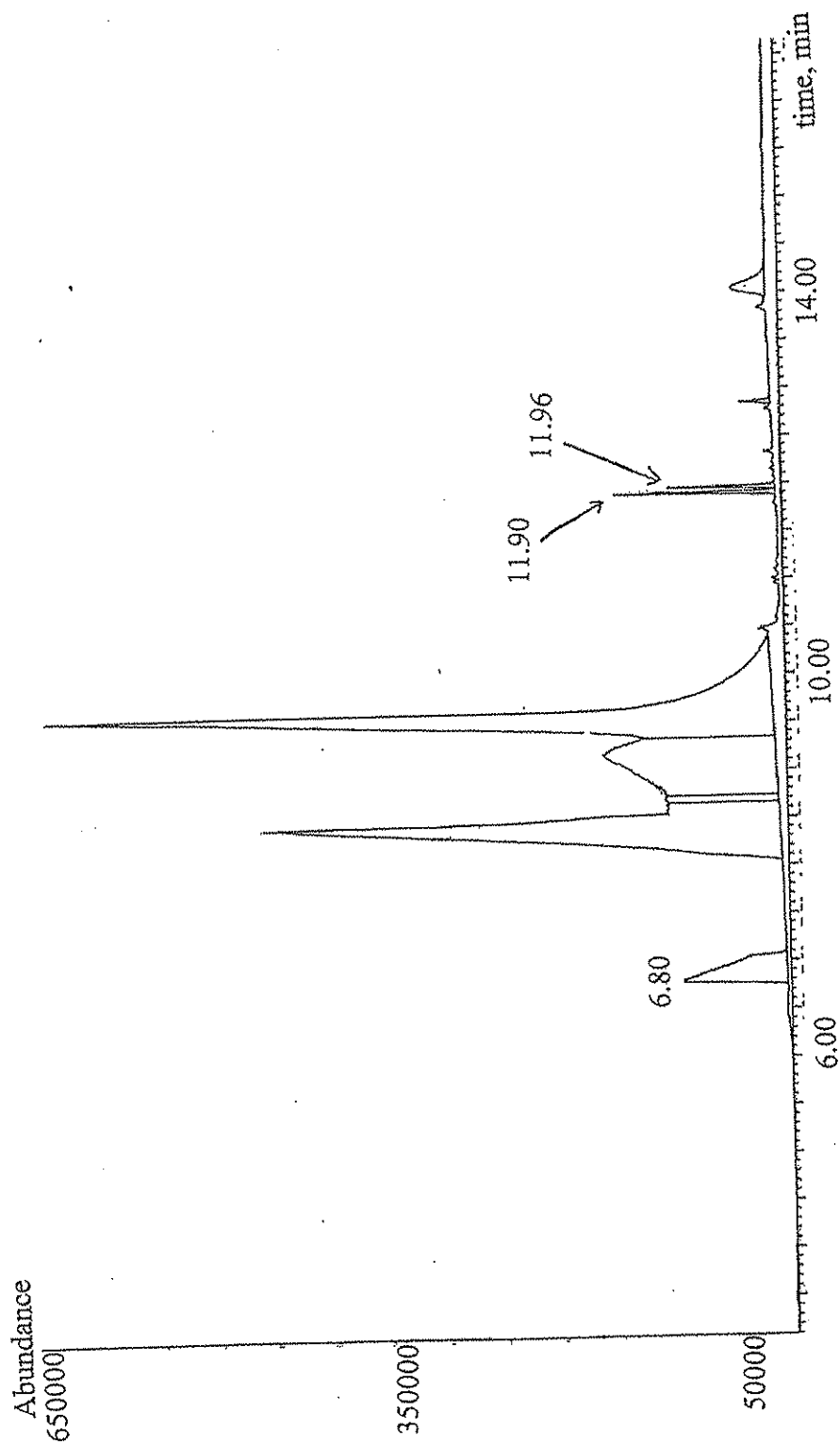


Figure 3-17: Chromatogram of Permethrin Peaks

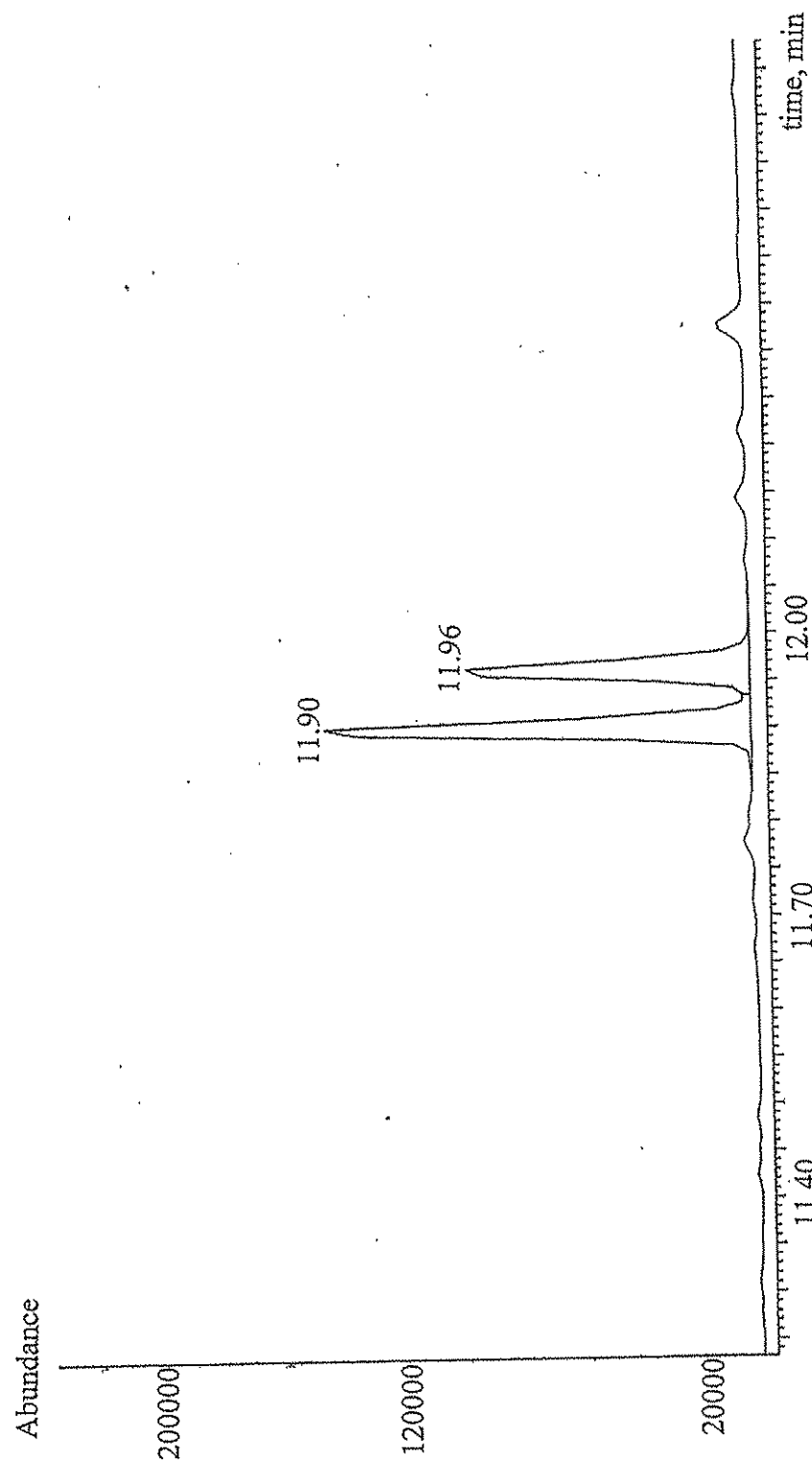
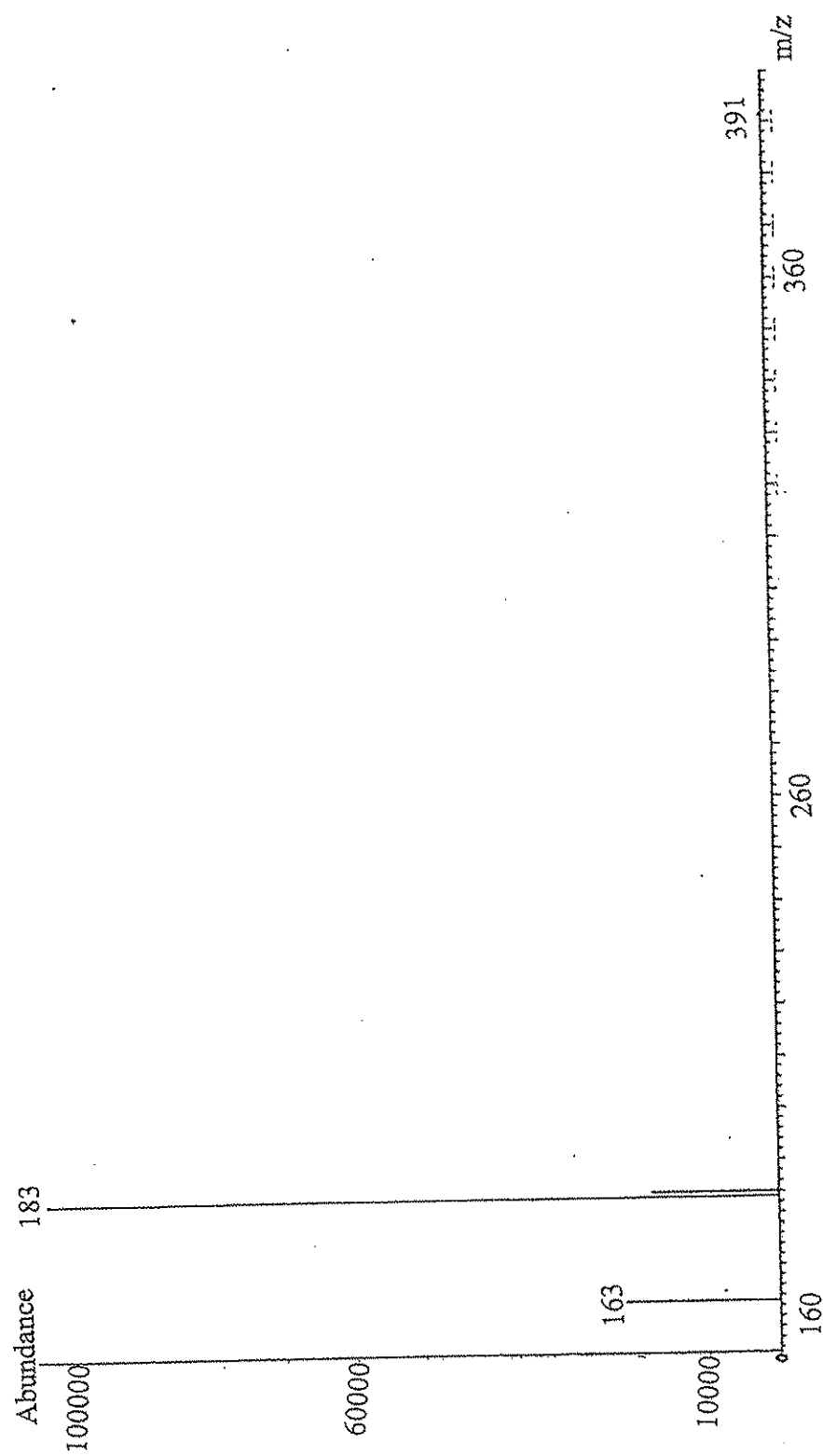


Figure 3-18: SIM Spectrum of Peak @ 11.90 Minutes

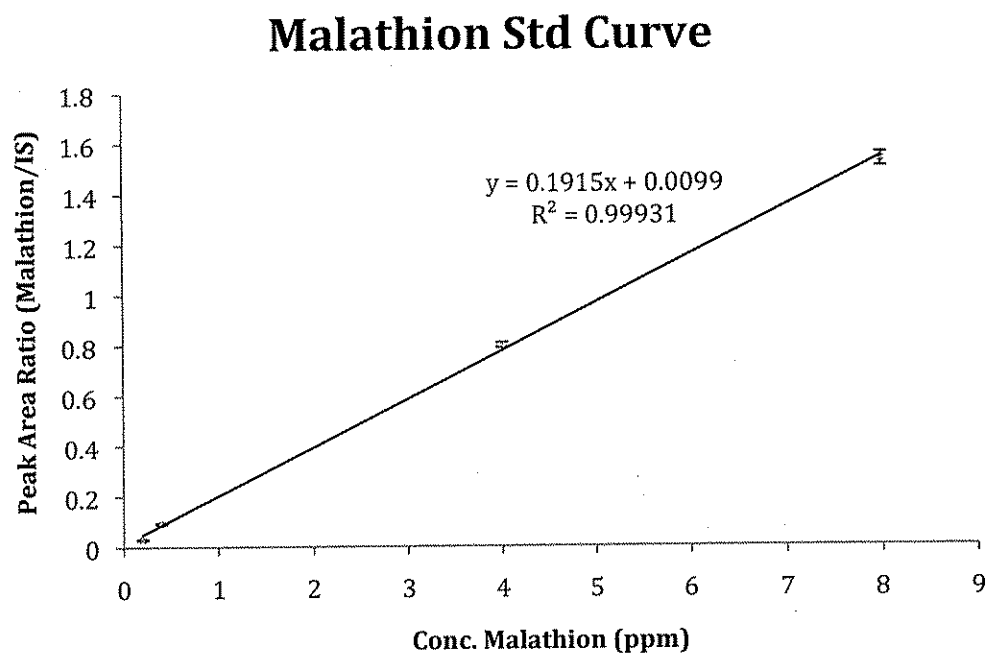


A. 3. Typical Calibration Curves for Analysis of Unknowns

Calibration curves were established on the day of analysis for the insecticide of concern. Thus, if we were determining malathion samples on a given day, a malathion calibration curve would be established for the quantitation of all samples subjected to analysis that same day.

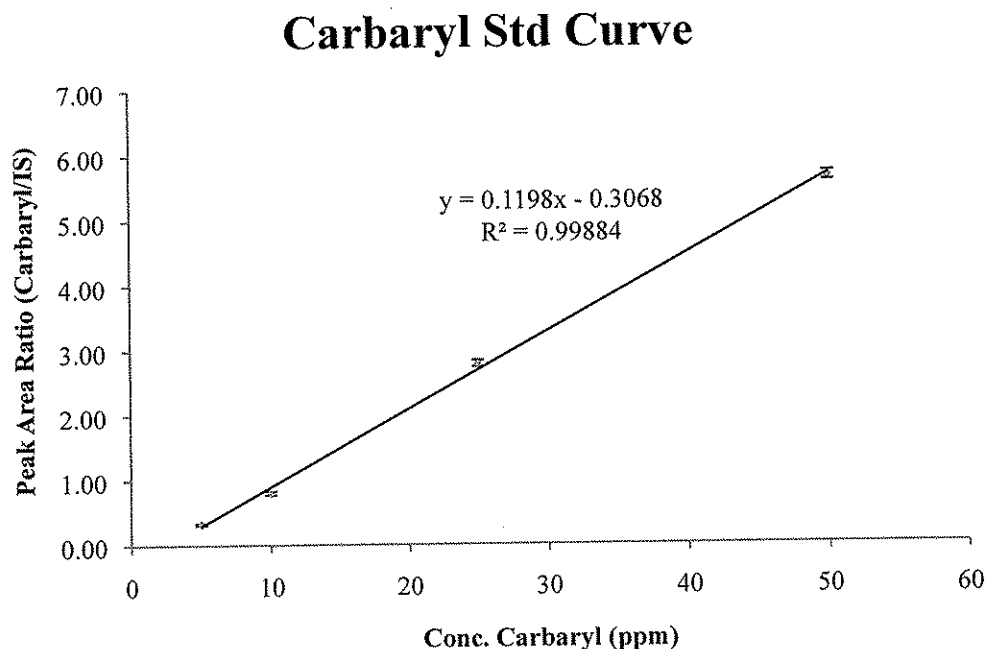
In the particular case of malathion, preliminary investigations utilized a calibration curve spanning the lower ppm range, since that was the anticipated values to be found in the unknown samples. However, actual samples revealed malathion levels typically lower than originally anticipated. Thus, we adjusted the range of the malathion standard curves to be in the upper ppb, or lower fractional ppm, range. A typical malathion standard curve incorporated the following concentrations of malathion: 0.20, 0.40, 4.0, and 8.0 ppm. This linear graph, shown in Figure 3-19, produced an R^2 value of 0.999.

Figure 3-19: Typical Calibration Curve for Malathion



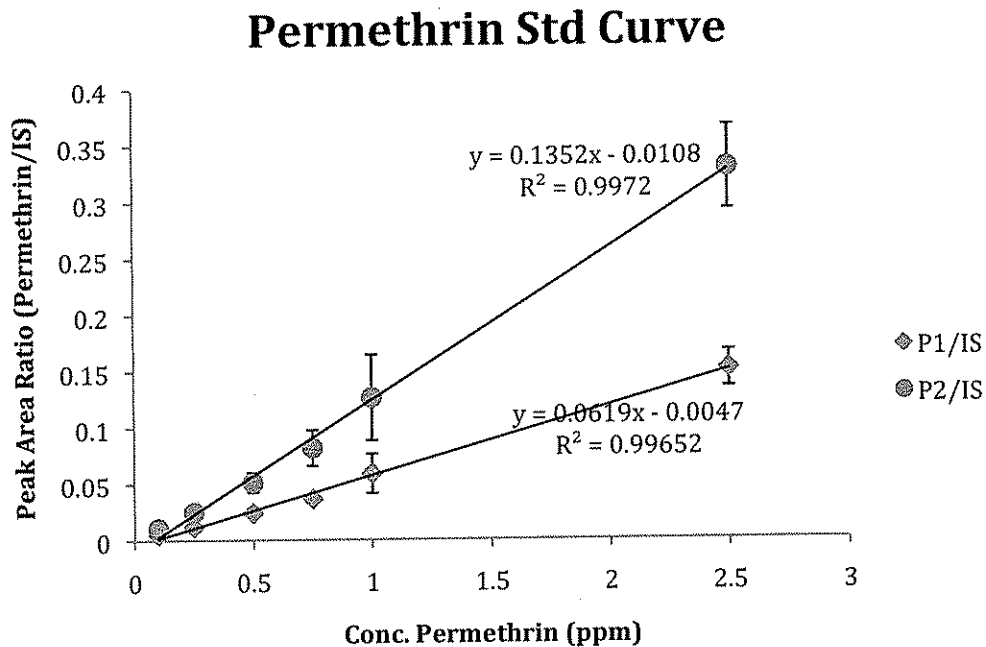
The carbaryl standard curve employed four concentrations: 5, 10, 25, and 50 ppm. A typical standard curve for carbaryl is shown in Figure 3-20, where the R^2 value for this linear graph was 0.999.

Figure 3-20: Typical Calibration Curve for Carbaryl



Two separate standard curves were constructed for permethrin. The first was for Peak 1, while the second was for Peak 2, which, as mentioned above, correspond (not necessarily respectively) to the cis- and trans-permethrin isomers. Concentrations of the standard mixture used for the construction of a typical permethrin standard curve are shown in Figure 3-21. The R^2 values for the individual linear graphs are 0.997 and 0.997.

Figure 3-21: Typical Calibration Curve for Permethrin



B. Determination of the Half-Lethal Concentration of Insecticide

The half-lethal concentration of the individual insecticides was determined to provide a rough estimation of the value to be used in the field studies that were to follow. This was done for each of the three insecticides: malathion, carbaryl, and permethrin. The half-lethal concentration is defined as the dose, given in units of ng/cm^2 , which led to death in one-half of the treated adult flies. The dose was coated on the inside wall of the vials, and the adult

flies were exposed to this environment and allowed to continuously contact the coated layer for 24 hours.

B. 1. LC₅₀ Determination for Malathion

The determination of the contact LC₅₀ for the blow fly commonly identified as the blue bottle fly, *Protophormia terra-novae*. The data collected are shown in Table 3-6 below. The analysis of this data to provide the LC₅₀ used the simplified approach of Reed and Muench.⁸⁵

Table 3-6: Dose Response for Malathion LC₅₀ Determination

Dose (ng/cm²)	Malathion Deaths	Total No. of Flies Tested
0.68	25	90
1.36	36	90
2.72	45	90
3.39	62	90
4.07	67	90

B. 2. LC₅₀ Determination for Carbaryl

The determination of the contact LC₅₀ for the blow fly commonly identified as the blue bottle fly, *Protophormia terra-novae*. The data collected are shown in Table 3-7 below. The analysis of this data to provide the LC₅₀ used the simplified approach of Reed and Muench.⁸⁵

Table 3-7: Dose Response for Carbaryl LC₅₀ Determination

Dose (ng/cm ²)	Carbaryl Deaths	Total No. of Flies Tested
0.68	15	90
1.36	50	90
2.72	52	90
3.39	57	90
4.07	58	90

B. 3. LC₅₀ for Permethrin

The LC₅₀ values for permethrin for another blow fly commonly identified as a black bottle fly, *Phormia regina*, had already been reported using a method similar to that employed in the present investigation. This report showed an LC₅₀ of 1.6 ng/cm².(J.Buley⁸⁴) We thus decided to use this value directly.

B. 4. LC₅₀ Summary

The LC₅₀ values for the three insecticides are summarized below in Table 3-8.

Table 3-8: LC₅₀ Values for Malathion, Carbaryl and Permethrin

Insecticide	LC ₅₀ (ng/cm ²)
Malathion	2.5
Carbaryl	1.8
Permethrin	1.6 ^a

^aPermethrin data for fly from same genus, but different species, determined by J. Buley.⁸⁴

C. Extraction Efficiency

Larvae in the third instar were injected directly with various volumes of insecticide, homogenized as described in Materials and Methods, and the homogenate taken to dryness. The concentrations of the injected insecticide were 50 ppm for malathion, 50 ppm for carbaryl, and 20 ppm for permethrin. Reconstitution with the appropriate solvent (dichloromethane for malathion and carbaryl; ethyl acetate for permethrin) was followed by injection into the

GC/MS for analysis by GC-SIM-MS. The results are presented below in Table 3-9.

Table 3-9: Extraction Efficiency

	Volume Injected		
Insecticide	2 μL	4 μL	6 μL
Malathion	71 \pm 6%	79 \pm 2%	82 \pm 2%
Carbaryl	87 \pm 2%	100 \pm 30%	90 \pm 10%
Permethrin ^a	110 \pm 30%	110 \pm 10%	118 \pm 3%
	90 \pm 30%	100 \pm 10%	102 \pm 3%

^aPermethrin is a mixture of cis- and trans- isomers. Extraction efficiencies are shown for each isomer in elution order, although assignment of each is uncertain. Actual masses of the malathion and carbaryl injected, respectively, were 100, 200, and 300 ng; for permethrin, masses were 40, 80, and 120 ng.

D. Laboratory Studies of Insecticides and Fly Larvae

Larvae were raised from hatching throughout the larval developmental stages in a laboratory setting upon a 10.0 g chicken liver food source which had been bathed in 1.00 mL of a solution containing 10 μ g of an insecticide. After

hatching, a fresh portion of liver, freshly treated with insecticide was used to replace the old. Larvae raised entirely upon the insecticide treated liver source were labeled chronically treated subjects. Upon hatching, some of the larva were transferred to a separate rearing chamber and subsequently raised on an insecticide free liver source. These were labeled as acutely treated subjects. When the chronic larva reached the second instar stage, a selected portion were transferred to a separate rearing chamber and subsequently raised on an insecticide free liver source. These were labeled as extended acutely treated subjects. The experiments carried out using permethrin were, unfortunately, not successful. We never succeeded in getting any of the eggs to hatch into larva. Thus, there are no permethrin results shown. However, reasonable results were obtained for malathion, shown in Table 3-10 below, and for carbaryl, shown in Table 3-11 below.

Table 3-10: Malathion Concentrations (ng/mg) in Larvae

Collected in the Lab

Life Stage	Chronic Exposure	Acute Exposure	Extended Acute
First Instar	3.1 ± 0.1	N/A	N/A
Second Instar	1.69 ± 0.08	4.0 ± 0.1	N/A
Third Instar	4.5 ± 0.1	0.42 ± 0.02	0.64 ± 0.05

Table 3-11: Carbaryl Concentrations (ng/mg) in Larvae

Collected in the Lab

Life Stage	Chronic Exposure	Acute Exposure	Extended Acute
First Instar	49 ± 7	N/A	N/A
Second Instar	42 ± 4	< 5	N/A
Third Instar	68 ± 14	< 5	< 5

Basically, the data for both malathion and carbaryl point to relatively constant levels of the insecticide in the chronically exposed group. Modest variations in this statement can probably be accounted for by the fact that the

average instar lifetime is about 16 hours under normal circumstances whereas the sampling time for these experiments occurred only once every 24 hours. Thus, although the instar stage was identified, the presence of the given larva at the beginning, middle or end of that stage is not precisely known. We would expect considerable variability in the content of endogenous insecticide as the molting processes and/or preparation for pupation were in process as opposed to active growth within an instar stage.

E. Field Studies of Interruption of Normal Decay by Insecticides

Dead pigs were treated by coating with solutions of insecticides. They then were placed in groups of four in a remote wooded area to allow normal decay processes to occur. Each experiment involved four pigs: control, malathion treated, carbaryl treated, and permethrin treated. One experiment was started in the winter. Unfortunately, the weather was too cold for any flies to effectively locate the carcasses and/or too cold for the larvae to survive. The winter experiment essentially ended with the carcasses experiencing decay, eventually, by autolysis and microbial degradation; no flies were ever observed to be involved. Two experiments, labeled as Trial 1 and Trial 2 in Table 3-12 below, were performed in the summer months and were more successful.

E. 1. Prior Treatment of Carcasses with Insecticides

The weight, volume and dose of insecticide used on each carcass are given below in Table 3-12. Calculations for the surface area of each carcass and the dose on each carcass were based on equations from Plumb and are shown below.⁸⁶

$$\text{Surface Area (cm}^2\text{)} = (10.1 \times \text{weight(g)} \times \frac{2}{3})$$

$$\text{Dose} = \text{volume applied (mL)} \times \text{concentration (g/100mL)} \times \frac{1}{\text{SA}}$$

Table 3-12: Prior Treatment of Carcasses

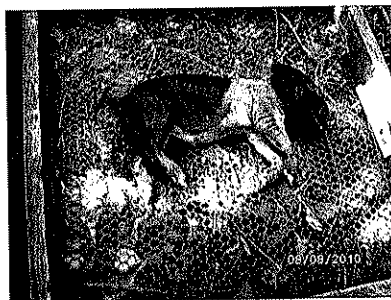
Treatment	Weight (kg) Trial 1	Volume (mL) Trial 1	Dose ($\mu\text{g/cm}^2$) Trial 1	Weight (kg) Trial 2	Volume (mL) Trial 2	Dose ($\mu\text{g/cm}^2$) Trial 2
Control	4.649	0	0	6.208	0	0
Malathion	4.621	15.0	2.41	7.088	17.0	1.78
Carbaryl	4.734	16.0	2.44	4.649	8.0	1.28
Permethrin	3.090	6.5	1.56	7.428	14.0	1.40

E. 2. Decay Processes of Carcasses in Field After Insecticide Treatment

The decay of the treated and control carcasses were followed daily in the field experiments. The results are shown in the pictorial review that follows with the appearance of the carcass being documented on, typically, a daily basis after placement in the field. The various stages of decay are quite evident in the two summer experiments, labeled in these photos as Experiment 1 and Experiment 2. The day the carcasses were initially treated with the insecticide and placed in the field was labeled as Day 0. Samples of larva, flies, pupa, and beetles were subsequently collected, as appropriate and available, on a daily basis.

**Figure 3-22: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 1, Day 0**

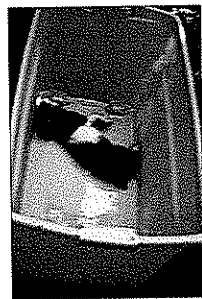
A. Control



B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated



**Figure 3-23: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 1, Day 3**

A. Control



B. Malathion Treated



C. Carbaryl Treated

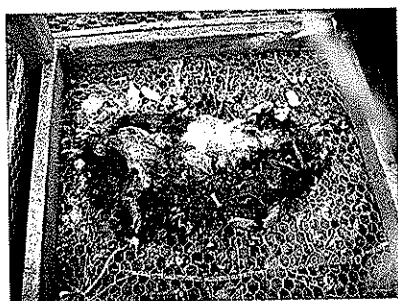


D. Permethrin Treated

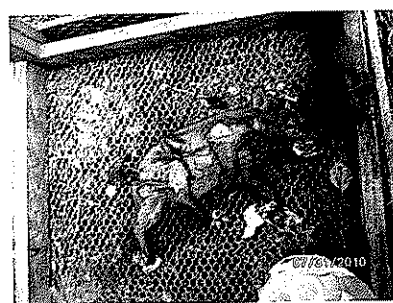


**Figure 3-24: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 1, Day 4**

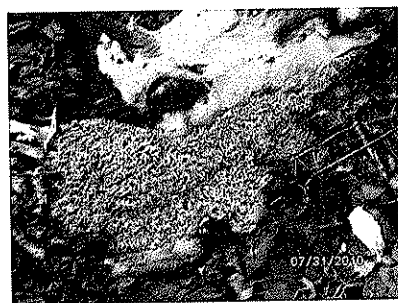
A. Control



B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated



**Figure 3-25: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 1, Days 5, 9, and 19**

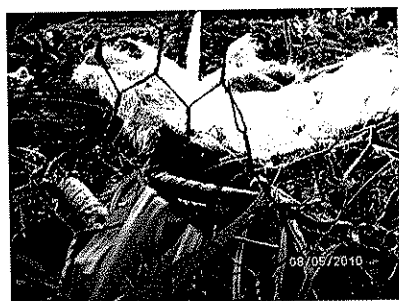
A. Carbaryl Treated Day 5



B. Permethrin Treated Day 5



C. Permethrin Treated Day 9



D. Permethrin Treated Day 19



**Figure 3-26: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Day 0**

A. Control



B. Malathion Treated



C. Carbaryl Treated

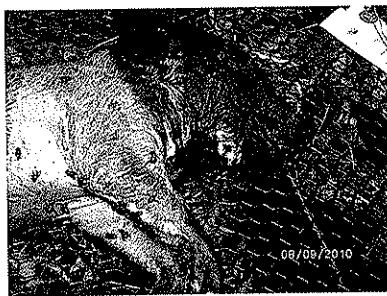


D. Permethrin Treated



**Figure 3-27: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Day 1**

A. Control



B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated



**Figure 3-28: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Day 2**

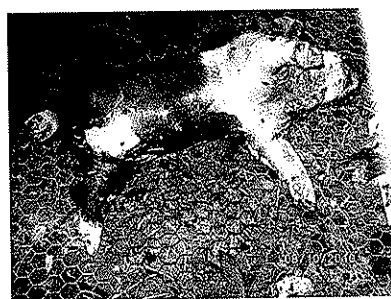
A. Control



B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated



**Figure 3-29: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Day 3**

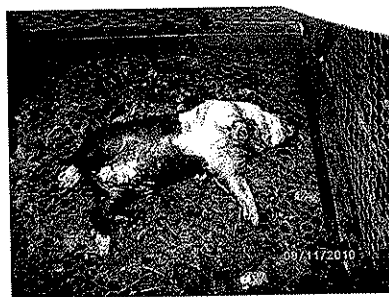
A. Control



B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated



**Figure 3-30: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Day 6**

A. Control

No picture for this pig
on this day.....

B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated

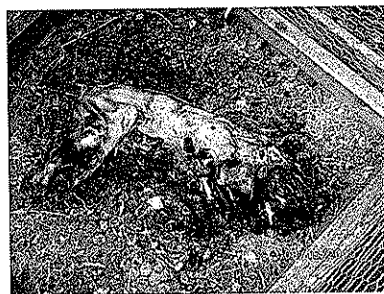


**Figure 3-31: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Day 7**

A. Control



B. Malathion Treated



C. Carbaryl Treated



D. Permethrin Treated



**Figure 3-32: Pictorial Examination of Decay Processes for Pig
Carcasses Experiment 2, Days 7 and 10**

A. Permethrin Treated Day 7



B. Permethrin Treated Day 10



The exact categorization of the decay stage as a function of the day the carcasses were involved in the field studies are given below in Tables 3-13 and 3-14. As can be seen from the progression from fresh to bloat to active decay to post decay to skeletal, there is a fairly consistent pattern that shows up for the individual carcasses in a group of four in each of the two experiments. For example, the control pig is clearly decaying at a more rapid rate than any of the

insecticide treated pigs, which were each treated with the slightly different volumes of the same concentration (0.5%) of their respective insecticides. Then,

Table 3-13: Stages of Decomposition in Experiment 1

Day	Control	Malathion	Carbaryl	Permethrin
0	Fresh	Fresh	Fresh	Fresh
1	Active Decay	Bloat	Fresh	Fresh
2	Active Decay	Active Decay	Bloat	Bloat
3	Post-decay	Active Decay	Active Decay	Active Decay
4	Skeletal	Post-decay	Active Decay	Active Decay
5	Skeletal	Post-decay	Post-decay	Active Decay
6	Skeletal	Skeletal	Post-decay	Active Decay
7	Skeletal	Skeletal	Skeletal	Active Decay
8	Skeletal	Skeletal	Skeletal	Active Decay
9	Skeletal	Skeletal	Skeletal	Active Decay
10	Skeletal	Skeletal	Skeletal	Active Decay
11	Skeletal	Skeletal	Skeletal	Active Decay

in order, we observe increasingly rapid decay in the cases, respectively, of malathion treated, carbaryl treated, and permethrin treated animals. While not always readily apparent in the fresh to bloat to active decay transitions, this

Table 3-14: Stages of Decomposition in Experiment 2

Day	Control	Malathion	Carbaryl	Permethrin
0	Fresh	Fresh	Fresh	Fresh
1	Fresh	Fresh	Fresh	Fresh
2	Active Decay	Bloat	Bloat	Bloat
3	Active Decay	Active Decay	Bloat	Active Decay
4	Post-decay	Post-decay	Active Decay	Active Decay
5	Post-decay	Post-decay	Active Decay	Active Decay
6	Post-decay	Post-decay	Active Decay	Active Decay
7	Post-decay	Post-decay	Post-decay	Active Decay
8	Skeletal	Post-decay	Post-decay	Active Decay
9	Skeletal	Post-decay	Post-decay	Post-decay
10	Skeletal	Post-decay	Post-decay	Post-decay

trend is clearly observed in the progression to the longer-term transitions, i.e., from active decay to post decay and, finally, from post decay to skeletal decay.

E. 3. Monitoring of Adult Flies in the Vicinity of Pig Carcasses Used in Field Experiments 1 and 2

To gain some insight into the possible adult fly sources of the eggs layed on the pig carcasses and, thus, the nature of the subsequent larvae involved in the decay process, we monitored the adult flies in the area for the first two days following insertion of the dead pigs into the remote site. A sticky strip was used to capture the adults; other insects were collected as well. The individual insects captured are identified in Table 3-15 below. Since we were predominantly concerned with the associated fly larvae, we summarized the total number of flies captured on the sticky traps associated with each of the controls, malathion treated, carbaryl treated, and permethrin treated pig carcasses on each of Days 1 and 2 for each of Experiments 1 and 2. We also identified the particular genus and species of the flies for which we had a dichotomous key; these included the majority of the flies found. The collection data for the flies only is summarized in Table 3-16 below.

The results shown in Tables 3-15 and 3-16 provide some interesting general observations. First, the insect species distribution between the various treated carcasses did, indeed, vary somewhat. However, as far as the flies were

concerned, the majority were members identified as calliphorids. The calliphorids were recognized to be from the order and family, respectively, of Diptera Calliphoridae. The two primary calliphorids were further identified (genus and species) as *Cochliomyia macellaria* and *Phaenicia coeruleiviridis*, which are, respectively, commonly recognized as a secondary screwworm and a blow fly known as a green bottle fly. The remaining, less abundantly found, flies were identified as either sarcophagids or unknown.

Table 3-15: Insect Species^{a,b,c,d} Captured on Sticky Traps

Near Pig Carcasses in Field Experiments

	Control	Malathion	Carbaryl	Permethrin
Exp't 1 Day 1	8 Cm ^a	5 Cm	5 Cm	0 Cm
	24 Pc ^b	1 Pc	16 Pc	0 Pc
	4 sarc ^c	1 sarc	2 sarc	0 sarc
	0 unk ^d	0 unk	0 unk	4 unk
Exp't 1 Day 2	19 Cm	23 Cm	48 Cm	57 Cm
	32 Pc	56 Pc	13 Pc	45 Pc
	2 sarc	3 sarc	4 sarc	4 sarc
	8 unk ^d	24 unk	2 unk	0 unk
unk	9 Cm	16 Cm	3 Cm	6 Cm
	93 Pc	114 Pc	108 Pc	8 Pc
	5 sarc	2 sarc	1 sarc	1 sarc
	5 unk	2 unk	0 unk	3 unk
Exp't 2 Day 2	43 Cm	28 Cm	13 Cm	119 Cm
	32 Pc	59 Pc	73 Pc	19 Pc
	20 sarc	2 sarc	5 sarc	13 sarc
	9 unk	10 unk	2 unk	9 unk

^a Cm is an abbreviation for *Cochliomyia macellaria*

^b Pc is an abbreviation for *Phaenicia coeruleiviridis*

^c sarc is an abbreviation for sarcophagid

^d unk is an abbreviation for unknown

**Table 3-16: Total Numbers of Adult Flies on Sticky Traps
Near Pig Carcasses in Field Experiments**

	Control	Malathion	Permethrin	Carbaryl
Exp't 1 Day 1	39	7	4	23
Exp't 1 Day 2	61	106	106	67
Exp't 2 Day 1	112	134	18	112
Exp't 2 Day 2	102	99	160	93

A pertinent piece of information related to the collection of insects on the sticky traps is the average daily temperature associated with each of the four collection days. We actually recorded the temperature hourly for the entire Experiment 1 and Experiment 2, as seen in Appendix C. For each of the four days associated with the adult insect capture on sticky traps, the hourly temperatures were summed for the 24 hourly data points in the day; this sum was divided by 24 to yield the average daily temperature. The average daily temperature was 24°C, 25°C, 31°C, and 28°C, respectively, for Experiment 1 Day 1, Experiment 1 Day 2, Experiment 2 Day 1, and Experiment 2 Day 2.

The clearly noticeable features associated with the flies collected on sticky traps are (1) the relatively low values for all locations on Day 1 of Experiment 1, (2) the typically lower numbers of Day 1 and Day 2 flies collected for Experiment 1 as compared to Experiment 2, (3) the low value for Day 1 in Experiment 1 for flies collected near the malathion treated animal, and (4) the particularly lower number of flies collected near the permethrin treated animals on Day 1 of both Experiments 1 and 2. We will address each of these points in order.

The relatively lower numbers of collected flies on Day 1 of Experiment 1 compared to Day 2 of the same experiment are not readily explained at the current time. Since the exact time of day the traps were checked was not recorded, however, it is possible that the total collection time for Day 1 of Experiment 1 was somewhat less than the total collection time for Day 2. The typically lower values for both collection days for Experiment 1 compared to Experiment 2 would possibly be due to the differences in temperature. The higher temperature could be responsible for the increased abundance of flies experienced in Experiment 2 as compared to Experiment 1. The quite low number of collected flies for the Experiment 1 Day 1 malathion treated carcass is simply unexplained; this is particularly true since it was not strongly reproduced in Experiment 2. Perhaps the single most interesting piece of information derived from Table 3-16 is the extremely low number of collected

flies for the first day of both Experiments 1 and 2 in the vicinity of the permethrin treated pig carcass. This may simply be a reflection of the fact that all three insecticide treated carcasses were treated with the same concentration of insecticide, namely 0.5%. However, as seen previously in the LC_{50} studies, permethrin is substantially more lethal to flies, in general, than are the other two insecticides examined. Permethrin exhibited an LC_{50} value of 1.6 ng/cm^2 , whereas malathion and carbaryl exhibited values of 2.53 and 1.80 ng/cm^2 , respectively. This relatively excessive potency of permethrin may explain the lower values of collected flies nearby in the early stages of the two experiments.

E. 4. Insecticide Levels at Various Life Stages of Flies Collected in the Vicinity of Treated and Control Pig Carcasses Undergoing Decay

The four pig carcasses in a given experiment which were placed in the remote site to undergo decay, as described above in section D. 2., were subsequently examined daily for infestation by fly eggs and/or larvae. When ovipositing initially occurred, samples of the eggs were collected from each of the four carcasses. When various stages of larvae appeared, samples of the first, second, and third instar, in sequence throughout the study were taken directly from a carcass to analyze for the particular insecticide and labeled as the chronic

samples. All chronic samples of larvae derived from carbaryl treated animals were below detection limits.

Samples of the first instar larvae were initially to be transferred to a separate rearing chamber and subsequently grown on a insecticide free (cat food) source to form an acute group. Unfortunately, the first instar stage for the malathion treated carcasses occurred so rapidly that initially observed larvae were already in the second instar stage. In the case of carbaryl, the first instar stage was, indeed, transferred. However, the first instar stage and all the subsequently reared larvae exhibited carbaryl values that were below the detection limits. Thus, no results are presented for carbaryl below. And, the permethrin larvae found in the first instar stage were initially very small in number and, when transferred to the insecticide free food source, promptly died, thus terminating the acute group for the permethrin treated animals. In short, all the planned acute groups for each of the three insecticides never materialized due to various different reasons.

Samples of the second instar stage were also transferred to an insecticide free food source in a separate rearing chamber to provide what was labeled as the extended acute group. The extended acute groups were subsequently sampled for analysis of the pertinent insecticide at the third instar stage for the larvae. This analysis of the third instar stage for the extended acute group was achieved for all three insecticides. However, the concentration of carbaryl

determined in these samples was below the detection limit. The values were viable for the malathion and permethrin treated animals. Further, in the cases of both the malathion and permethrin treated carcasses, we were even able to isolate and analyze the pupae resulting from the third instar in the extended acute group. We were also able to isolate and analyze the flies resulting from the pupae in the extended acute group obtained from the malathion treated carcasses, but no adults were found in the extended acute permethrin group.

The analytical results for the larvae, pupae, and flies, where available from the malathion and permethrin treated samples were combined for the two field experiments, and these results are shown in Tables 3-17, 3-18, and 3-19 for, respectively, the malathion peak from the malathion treated carcasses, Peak 1 of permethrin from the permethrin treated carcasses, and Peak 2 of permethrin from the permethrin treated carcasses.

**Table 3-17: Malathion Concentrations (ng/mg) in Larvae
Pupae, and Flies Derived from the Field Studies**

Life Stage	Chronic Exposure	Acute Exposure
Second instar	3.5 ± 0.9	N/A
Third instar	2.1 ± 0.7	2.4 ± 0.3
Pupae	N/A	3.03 ± 0.07
Adult	N/A	2.50 ± 0.05

**Table 3-18: Permethrin Peak 1 Concentrations (ng/mg) in Larvae
and Pupae Samples Derived from the Field Studies**

Life Stage	Chronic Exposure	Acute Exposure
First Instar	44.6 ± 0.2	N/A
Second Instar	9.7 ± 0.5	N/A
Third Instar	8 ± 2	3 ± 2
Pupae	N/A	< 0.1

**Table 3-19: Permethrin Peak 1 Concentrations (ng/mg) in Larvae
and Pupae Samples Derived from the Field Studies**

Life Stage	Chronic Exposure	Acute Exposure
First Instar	36.0 ± 0.1	N/A
Second Instar	7.5 ± 0.3	N/A
Third Instar	3 ± 2	3 ± 1
Pupae	N/A	< 0.1

For the malathion results, it is observed that the concentration of the malathion declines from the second to the third instar stages in the chronic group, indicating the decay and/or loss of the insecticide on the carcass over time. The extended acute group for malathion shows a small value in the third instar stage, but a modestly increased (relative to the third instar stage) level in the pupae and adult. Although this is not readily explained, it is possibly related to the reduction in mass of the larvae in passing to the pupa and fly stages.

For the permethrin results for both of the permethrin peaks, representing the cis- and trans- isomers, again a decline in the concentrations is observed as one progresses from the first to the third instar stages of the larvae in the chronic

group. The third instar stage in the extended acute group shows low values in the third instar when compared to the third instar stage of the chronic group, and the pupae, which never matured into flies, had permethrin levels below the detection limit.

An anomaly in the permethrin results was associated with the ratio of the peak integration for Peaks 1 and 2. The ratio of these peaks was routinely < 1.0 for standard samples, and, simultaneously, the ratio was routinely > 1.0 for field derived samples.

F. Conclusions

Valuable information was gleaned from these experiments. Analysis of forensically obtained larvae for insecticides that could cause interference in PMI determinations has been done before for permethrin. One analysis was reported to be done by contract.⁸⁴ But, this was the first time that malathion and carbaryl were quantitated in fly larvae. Now that the protocols have been examined and established for such studies, future investigations employing various concentrations of these insecticides can be readily conducted. The concentrations in the larvae have been determined and can be readily compared to the known values applied to the carcass to guide future studies of insecticides

or other chemical agents. A potential screening method for a wide variety of chemical substances has been developed and is applicable to a wide range of forensic entomological studies.

These experiments did show, conclusively, that application of modest concentrations of three different insecticides to a dead carcass will result in an alteration in the post mortem interval when compared to untreated carcasses. In the case of carbaryl, not only was the PMI altered, but also the insecticide was eliminated prior to infestation by the blow flies, as witnessed by the inability of this insecticide to be determined in either of the chronically treated carbaryl carcasses. This could cause problems for investigators who are attempting to establish a timeline and/or prove a suspect's guilt in forensic cases.

When the observations of the stages of decomposition are combined with the observations of the appearance of first instar larvae, a definite correlation to PMI can be made. For a carcass treated with 0.5% malathion, the PMI would be calculated as approximately one day shorter than the actual PMI. For a carcass treated with 0.5% carbaryl, the PMI would be calculated as approximately two days shorter than the actual PMI. Since the permethrin treated carcass never achieved the same number of larvae, an accurate correlation cannot be made. However, the PMI calculation would be at least two days shorter than the actual PMI. Presumably, an investigator would notice the significant difference in the

number of larvae present at the stage of decomposition. The values for the decrease in the PMI determination are approximate due to sampling restrictions.

We initially expected one of two things to happen for the larvae transferred to an insecticide free food source: either the larvae would maintain the amount of insecticide they had ingested previously or they would completely eliminate it. The larvae that were initially exposed to malathion, maintained the level of malathion after being transferred to the cat food. This suggests that the malathion is being stored in the organs as opposed to the chitin. The permethrin data, however, suggests that it is being stored in both locations since it decreased slightly, but was not completely eliminated as the larvae progressed through the growth stages. The lab data for the carbaryl clearly show that if a larva ingests carbaryl, it will be eliminated quite rapidly and will become undetectable after a mere 24 hours.

For the larvae that were chronically exposed, we expected the concentration to continually increase until they died. However, we did not find any dead larvae at the field site. What the data suggests, alternatively, is that the amount of a given insecticide in the chronic exposure was decreasing over time due to a combination of ingestion by the collective maggots and degradation by the environment. The progression of maggots thus had less and less insecticide content.

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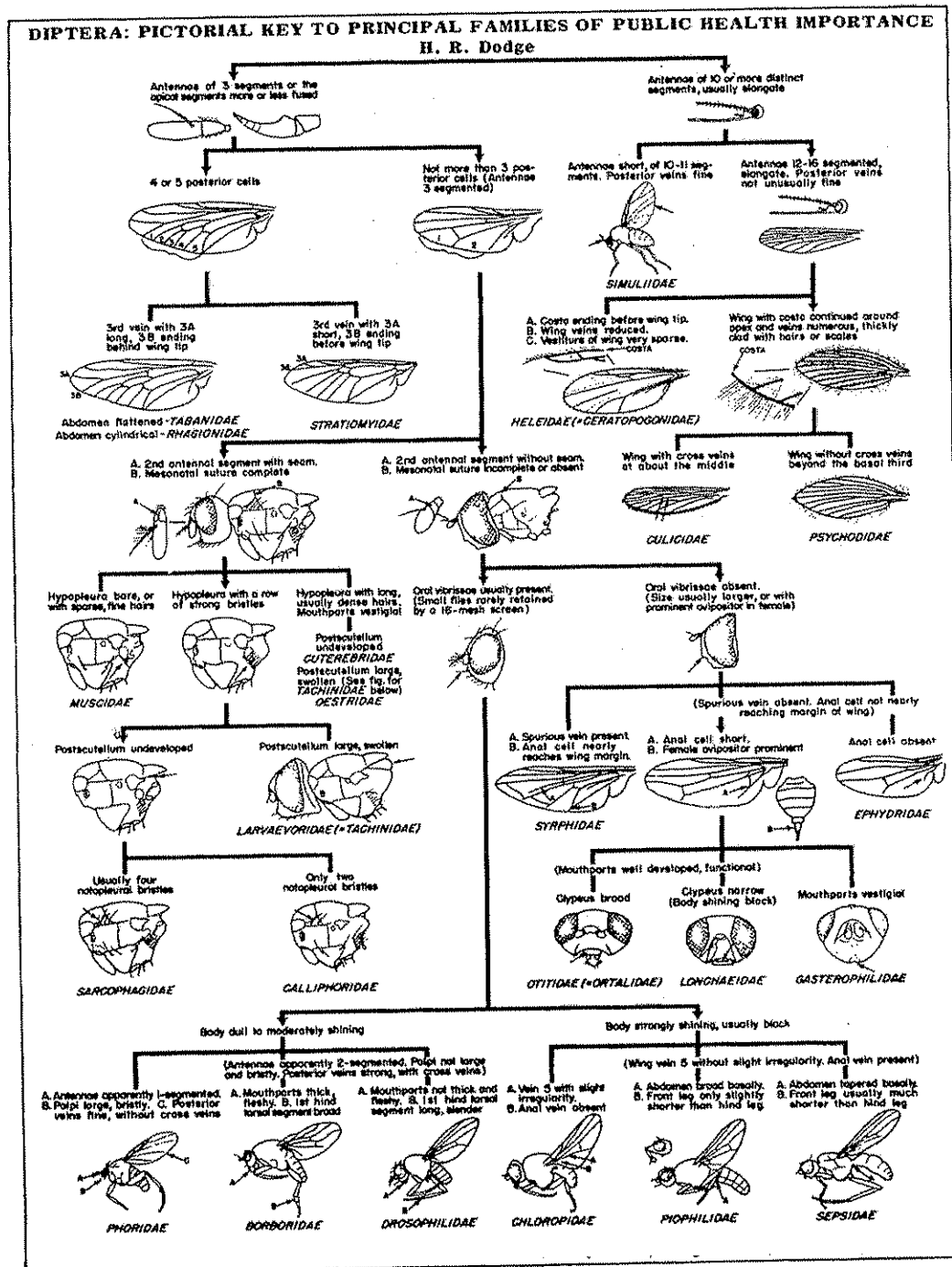
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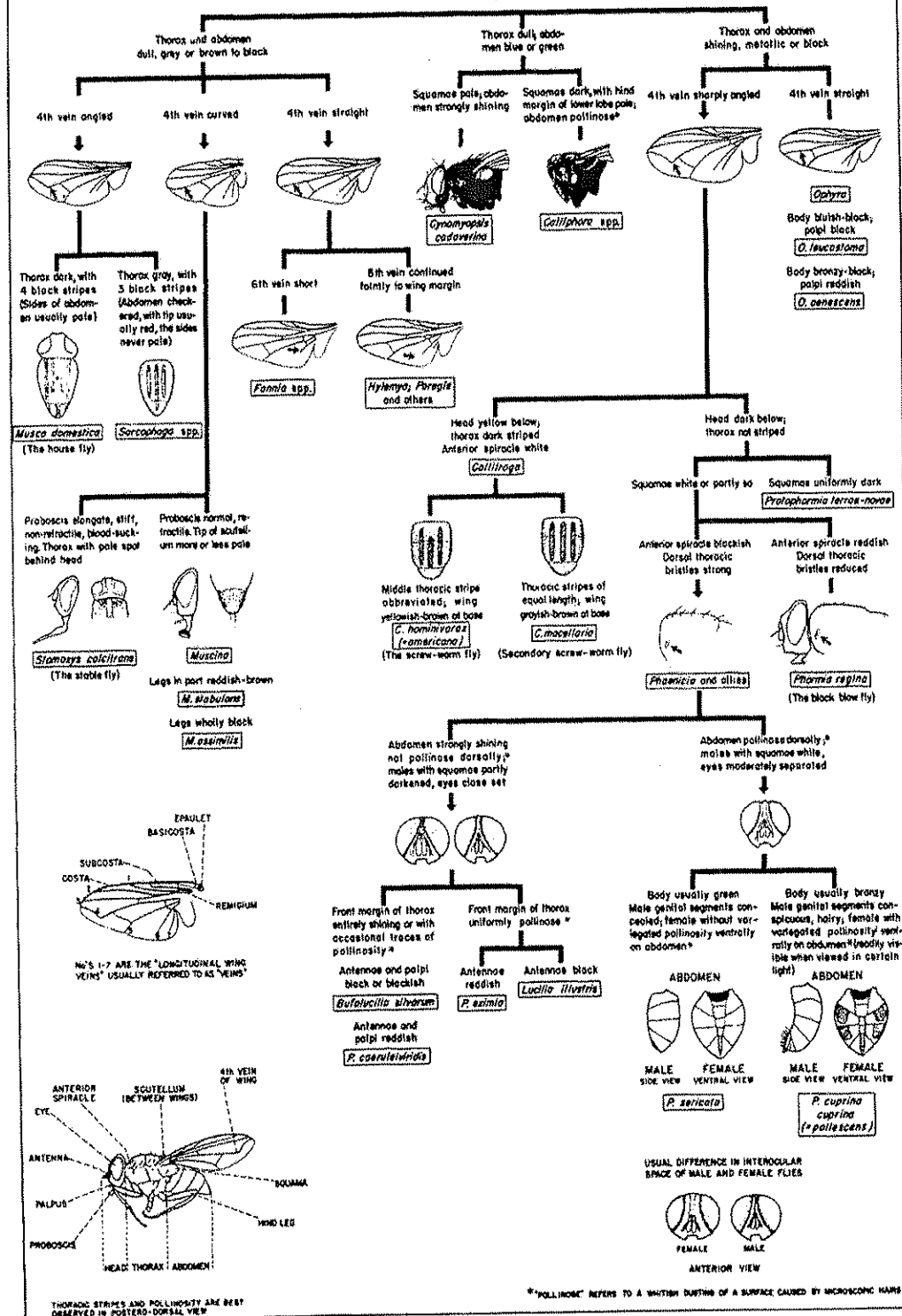
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Appendix A: Dichotomous Key for identifying Diptera⁸⁷

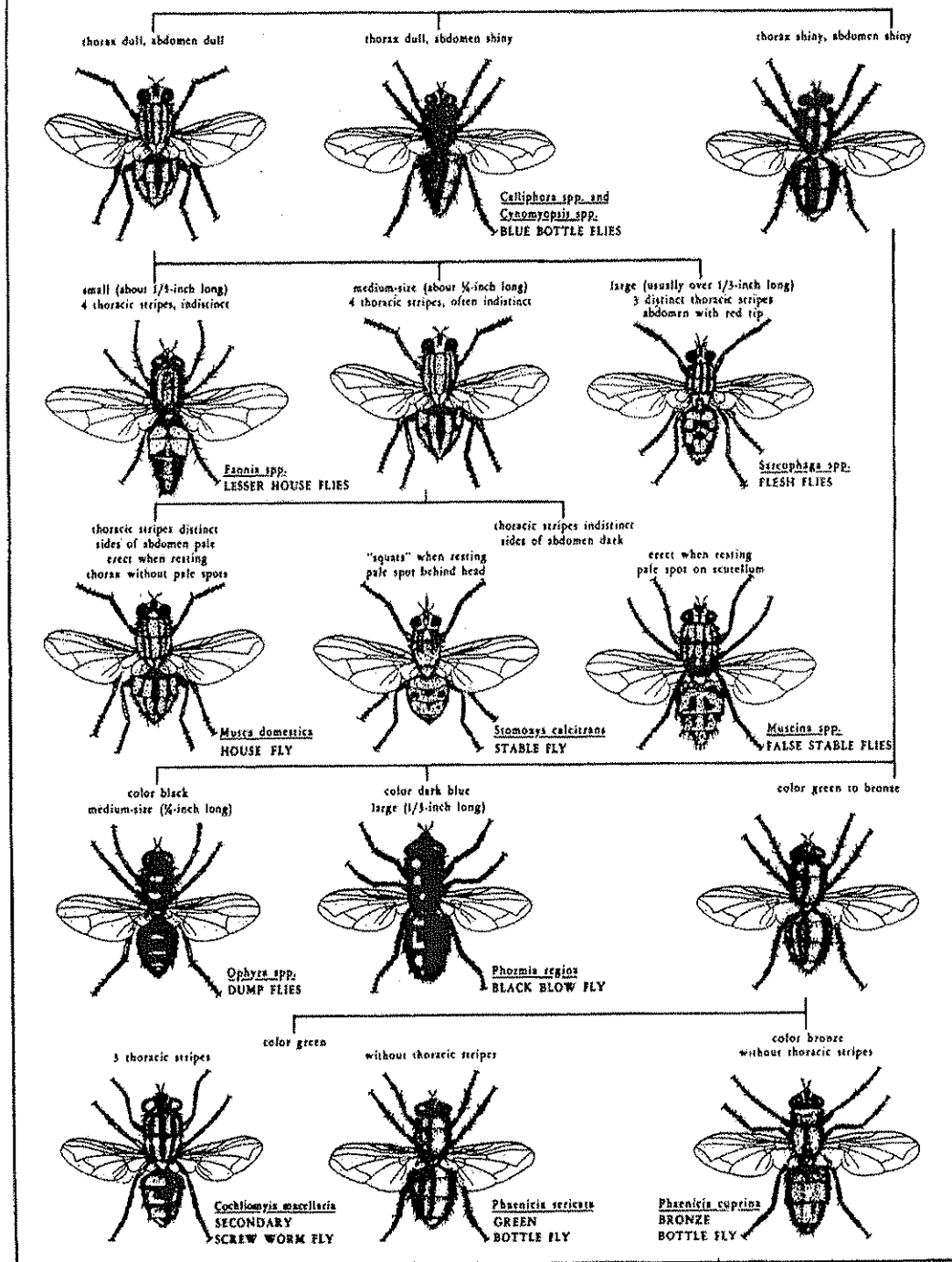


DOMESTIC FLIES: PICTORIAL KEY TO COMMON SPECIES IN THE U.S.
H. R. Dodge



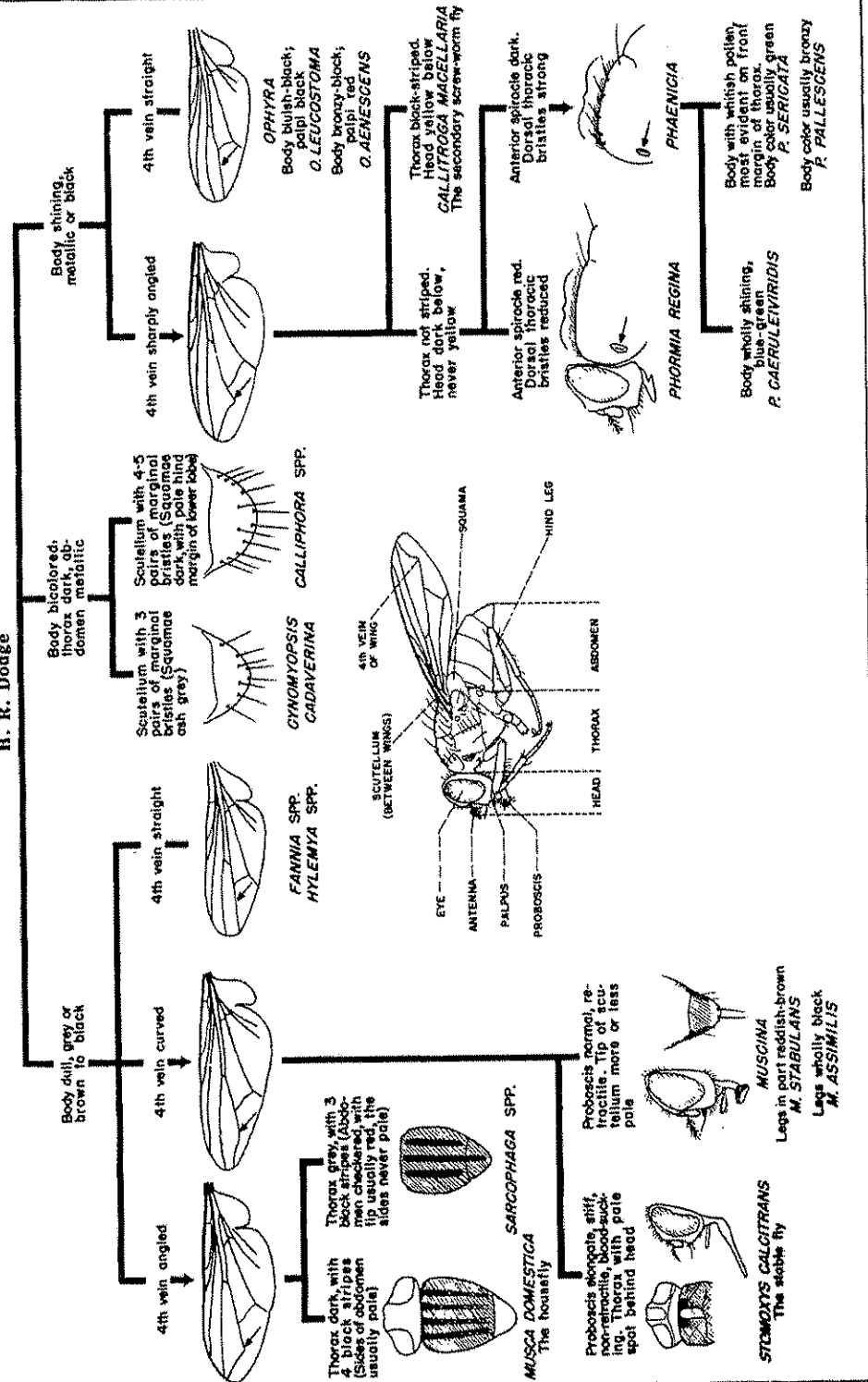
DOMESTIC FLIES: PICTORIAL KEY TO COMMON SPECIES

Harold George Scott and Margery R. Borom

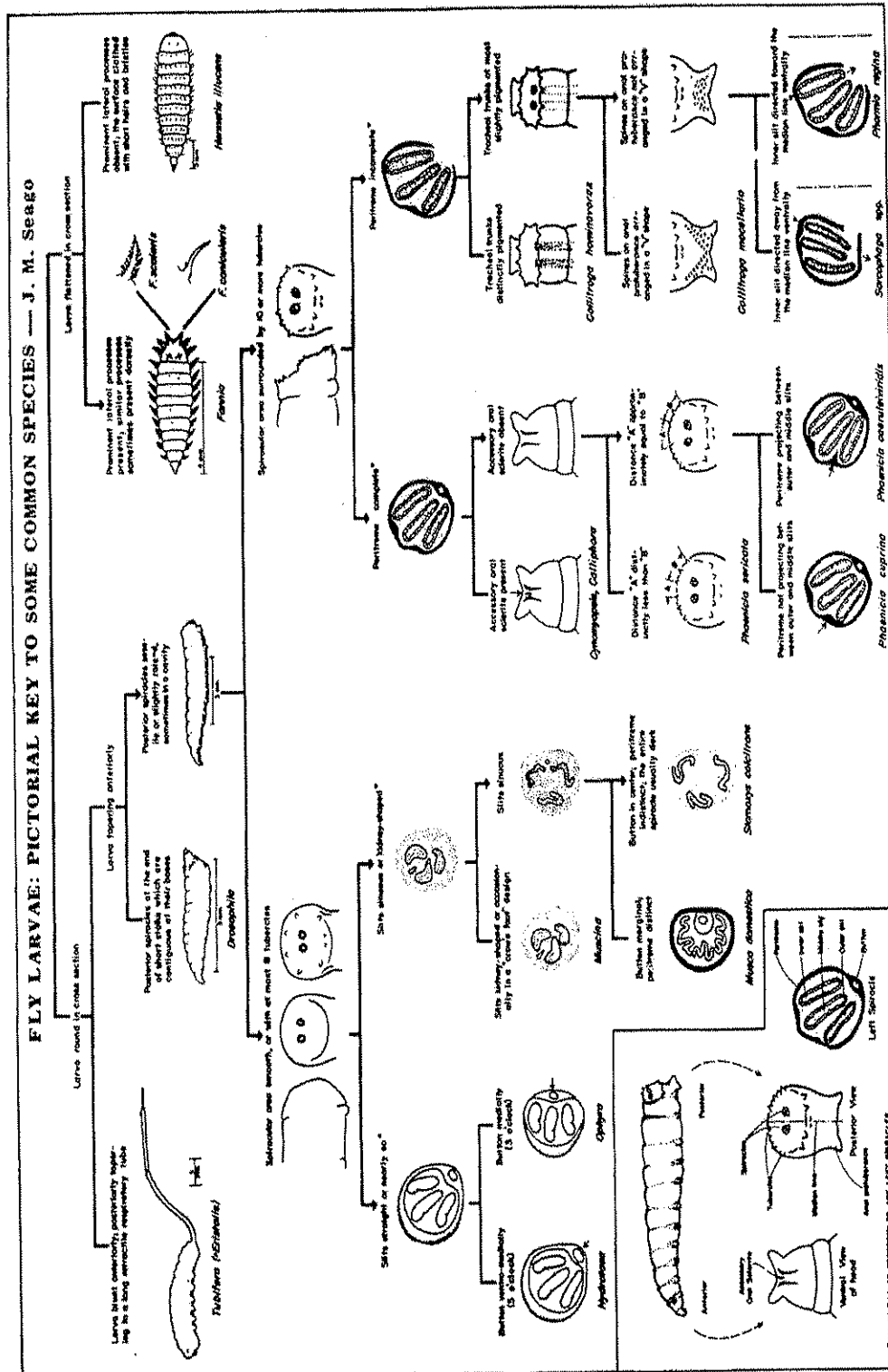


DOMESTIC FLIES: PICTORIAL KEY TO COMMON SPECIES IN SOUTHERN U.S.

H. R. Dodge



FLY LARVAE: PICTORIAL KEY TO SOME COMMON SPECIES — J. M. Seago



FLY LARVAE: KEY TO SOME SPECIES OF PUBLIC HEALTH IMPORTANCE
 Chester J. Stojanovich — Harry D. Pratt — Elwin E. Bennington

1. Larva with a definite, hard, sclerotized head capsule (Fig. 1 A).....2
- Larva without a definite, hard, sclerotized head capsule (Fig. 1 B).....3

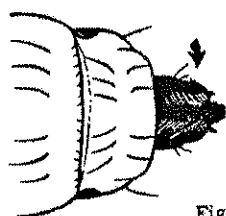


Fig. 1 A

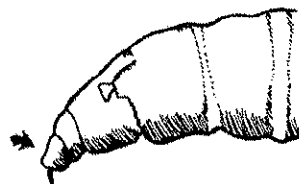


Fig. 1 B

2. Body flattened; large larvae 12-20 mm. long (Fig. 2 A)... (*Hermetia illucens*) SOLDIER FLY
- Body cylindrical with spiracles opening in a tubular segment at posterior end of body, last segment modified into a sclerotized air tube (Fig. 2 B).....
- (Genus *Psychoda* & allies) FILTER FLIES

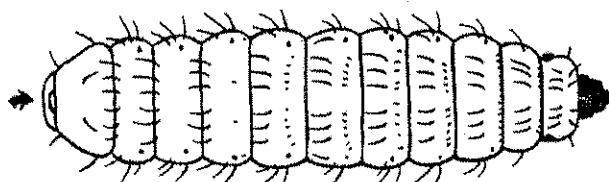


Fig. 2 A



Fig. 2 B

3. Body with spine-like dorsal and lateral processes on each segment; posterior spiracles on small elevations (Fig. 3 A)..... (Genus *Fannia*)...4
- Body smooth, or with short spines, but no long lateral processes (Fig. 3 B)..... 5

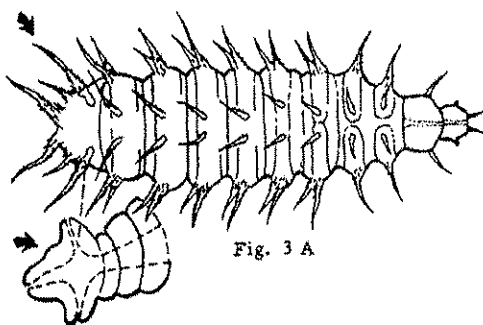
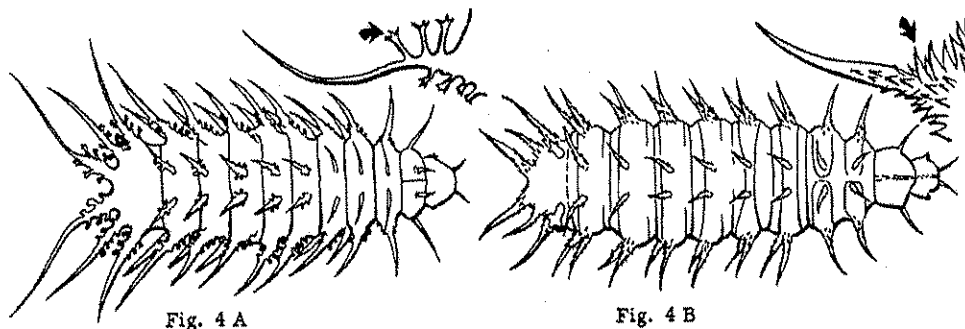


Fig. 3 A

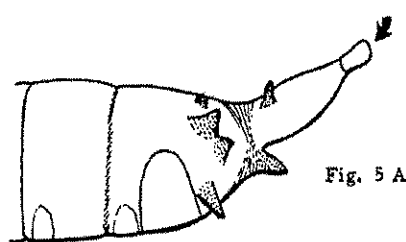


Fig. 3 B

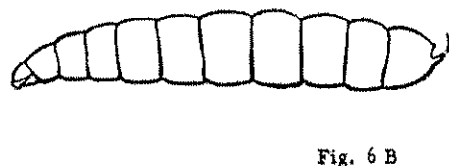
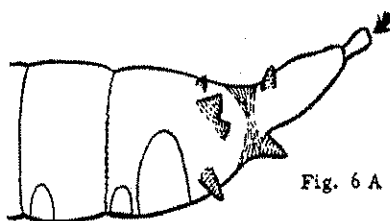
4. Processes branched or feathery (Fig. 4 A).....(*Fannia scalaris*) LATRINE FLY
Processes without branches, spiny (Fig. 4 B)..(*Fannia canicularis*) LESSER HOUSE FLY



5. Posterior spiracles on peg-like tubercles or cones; smaller larvae, usually 6-9 mm. long (Fig. 5 A)..... 6
Posterior spiracles not on peg-like tubercles; larger larvae, usually 9-18 mm. long (Fig. 5 B)..... 7

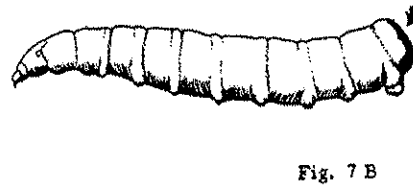
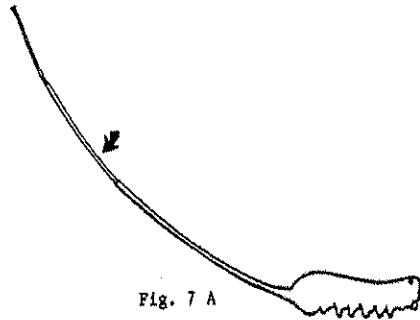


6. Posterior spiracles at ends of long tubercles (Fig. 6 A).....
..... (Genus *Drosophila*) VINEGAR FLIES
Posterior spiracles on short cones, last segment with short finger-like lateral process (Fig. 6 B).....(*Piophilidae casei*) CHEESE SKIPPER



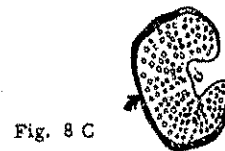
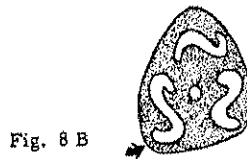
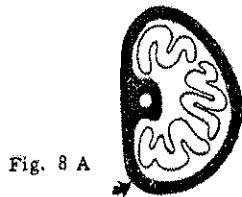
7. Posterior end of body extended to form a tail (Fig. 7 A).....
 (*Eristalis tenax*) RAT-TAILED MAGGOT

Body swollen or tapered posteriorly, but never extended into a tail like process (Fig. 7 B).. 8



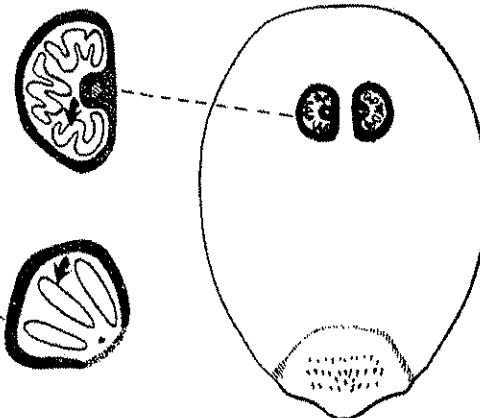
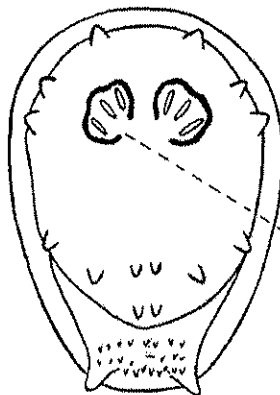
8. Peritreme present, with 3 distinct slits (Fig. 8 A)..... 9

Peritreme absent; or if present without 3 distinct slits (Fig. 8 B & C)..... 23



9. Slits of posterior spiracles straight (Fig. 9 A)..... 10

Slits of posterior spiracles strongly sinuous (Fig. 9 B)..... 22



10. Dorsal and ventral arms of cephaloskeleton almost equal (Fig. 10 A); peritreme with two non-sclerotized areas away from the button (Fig. 10 B).. (Genus Ophyra) DUMP FLY

Dorsal arm of cephaloskeleton longer than ventral arm (Fig. 10 C); peritreme complete or with one weakly sclerotized area (Fig. 10 D & E)..... 11



Fig. 10 A

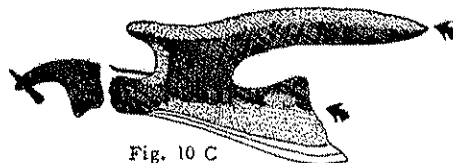


Fig. 10 C

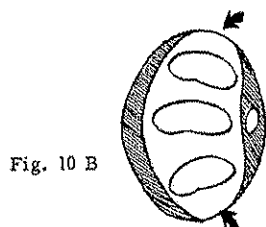


Fig. 10 B

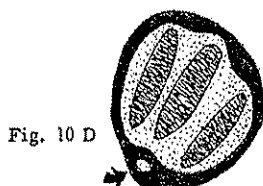


Fig. 10 D



Fig. 10 E

11. Posterior spiracles with peritreme complete, sometimes weak in area of button (Fig. 11 A) 12

Posterior spiracles with peritreme incomplete, not enclosing a sometimes ill-defined button (Fig. 11 B)..... 16



Fig. 11 A

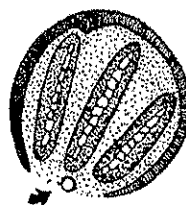


Fig. 11 B

12. Spiracular plate and button heavily sclerotized; accessory oral sclerite present (Fig. 12 A & B)..... 13

Spiracular plate and button not heavily sclerotized; accessory oral sclerite absent (Fig. 12 C & D)..... 14

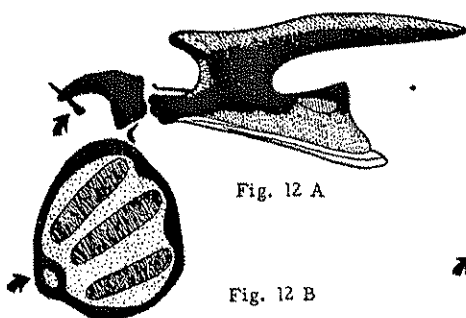


Fig. 12 A



Fig. 12 B

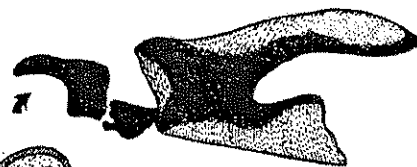


Fig. 12 C

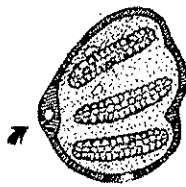
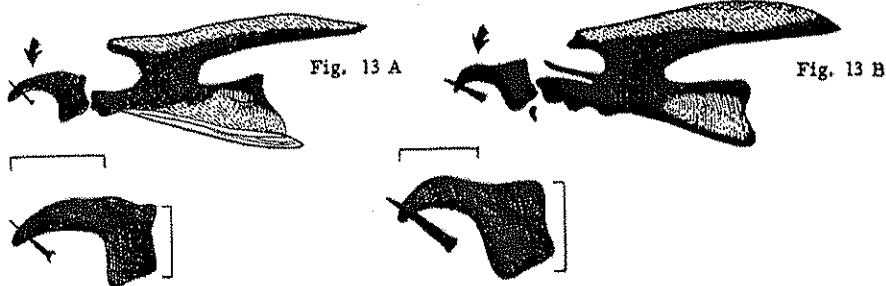


Fig. 12 D

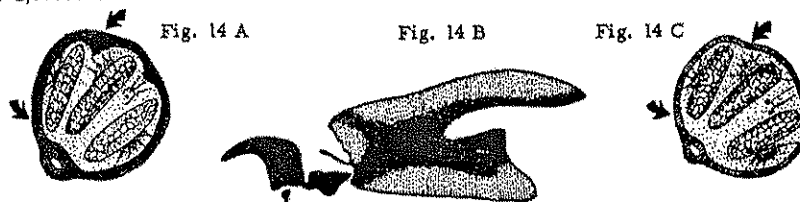
13. Mandibular sclerite with tooth longer than greatest width of basal portion (Fig. 13 A).....
 (*Calliphora vicina*) A BLUE BOTTLE FLY

Mandibular sclerite with tooth only as long as greatest width of basal portion (Fig. 13 B)..
 (*Cynomyopsis cadaverina*) A BLUE BOTTLE FLY



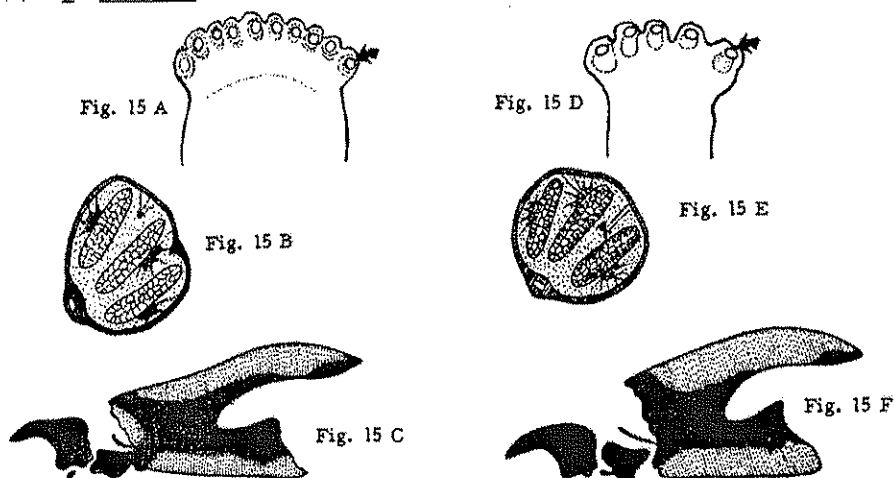
14. Peritreme thick with rounded or sharp projections which extend inward toward spiracular slits (Fig. 14 A); cephaloskeleton as in figure 14 B.....
 (*Phaenicia caeruleiviridis*) A GREEN BOTTLE FLY

Peritreme thin, usually with no projections or if present only slightly sclerotized (Fig. 14 C).....15



15. At least one of the prothoracic spiracles with 8 or more openings (Fig. 15 A); peritreme and cephaloskeleton as in figures 15 B & C. (*Phaenicia sericata*) A GREEN BOTTLE FLY

At least one of the prothoracic spiracles with 6 or less openings (Fig. 15 D); peritreme and cephaloskeleton as in figures 15 E & F.....
 (Syn. *P. pallescens*)..... (*Phaenicia cuprina*) A BRONZE BOTTLE FLY



16. Spiracular slits not pointing toward opening in peritreme (Fig. 16 A).....17
 Spiracular slits pointing toward opening in peritreme (Fig. 16 B)..... 18



Fig. 16 A

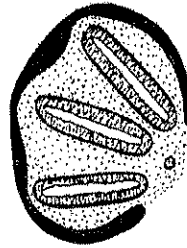


Fig. 16 B

17. Very large size, about 20 mm. long; mandibular sclerite as in figure 17 A.....
(*Sarcophaga clitellivora* or *S. bullata*) A FLESH FLY
 Smaller size, about 10 mm. long; mandibular sclerite as in figure 17 B.....
 (*Sarcophaga haemorrhoidalis*) A FLESH FLY



Fig. 17 A



Fig. 17 B

18. At least one of the prothoracic spiracles with 9 or less openings (Fig. 18 A).....19
 At least one of the prothoracic spiracles with 10 or more openings (Fig. 18 B).....20



Fig. 18 A



Fig. 18 B

19. Mandibular sclerite with tooth longer than width of basal portion (Fig. 19 A).....
(*Wohlfahrtia opaca*) A FLESH FLY
 Mandibular sclerite with tooth only as long as greatest width of basal portion (Fig. 19 B).
 (*Wohlfahrtia vigil*) A FLESH FLY



Fig. 19 A

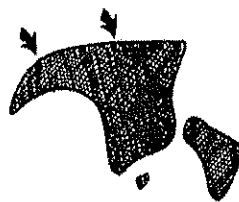


Fig. 19 B

20. Button indistinct or absent; walls of slits with lateral swellings (Fig. 20 A).....21
 Button present; walls of slits without lateral swellings (Fig. 20 B).....
 (Phormia regina) BLACK BLOW FLY



Fig. 20 A



Fig. 20 B

21. Tracheal trunks pigmented (Fig. 21 A).....
 (Cochliomyia hominivorax) PRIMARY SCREW-WORM
 Tracheal trunks not pigmented (Fig. 21 B).....
 (Cochliomyia macellaria) SECONDARY SCREW-WORM

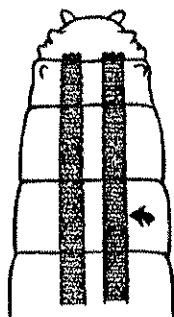


Fig. 21 A

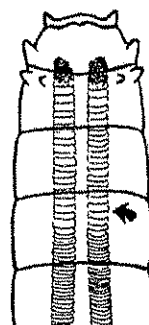


Fig. 21 B

22. Peritreme thick (Fig. 22 A)..... (Musca domestica) HOUSE FLY
 Peritreme thin (Fig. 22 B)..... (Haematobia irritans) HORN FLY



Fig. 22 A



Fig. 22 B

23. Small or slender, round larvae, usually less than 13 mm. long, tapering anteriorly (Fig. 23 A).....24
- Large, robust larvae, over 15 mm long, with very stout spines (Fig. 23 B)..... 26

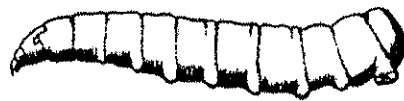


Fig. 23 A

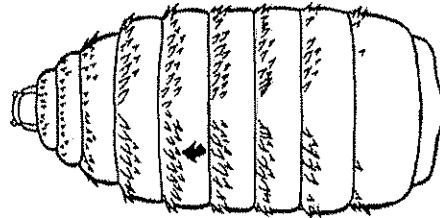


Fig. 23 B

24. Button centrally located (Fig. 24 A)..... (*Stomoxys calcitrans*) STABLE FLY
- Button not centrally located (Fig. 24 B).....25

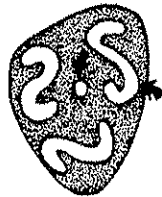


Fig. 24 A



Fig. 24 B

25. Slits of posterior spiracles strongly sinuous (Fig. 25 A).... (*Musca autumnalis*) FACE FLY
- Slits of posterior spiracles not strongly sinuous (Fig. 25 B).....
- (Genus *Mucina*) FALSE STABLE FLY



Fig. 25 A

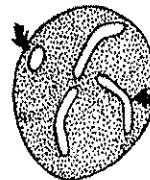


Fig. 25 B

26. Posterior spiracles with 3 distinct slits (Fig. 26 A).....27
- Posterior spiracles without 3 distinct slits (Fig. 26 B).....28



Fig. 26 A



Fig. 26 B

27. Spiracular slits straight and sunken in deep cavity (Fig. 27 A); body shape as in figure 27 B.
(Genus Dermatobia) HUMAN BOT FLY

Spiracular slits curved and at most in shallow cavity (Fig. 27 C); body shape as in figure
 27 D.....(Genus Gasterophilus) HORSE BOT FLY



Fig. 27 A



Fig. 27 C

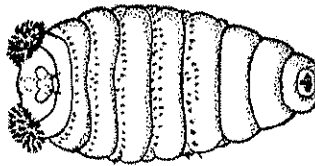


Fig. 27 B

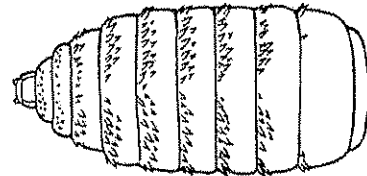


Fig. 27 D

28. Each spiracle divided into several plates (Fig. 28 A).....
(Genus Cuterebra) RABBIT AND RODENT BOT FLY

Each spiracle not divided into several plates (Fig. 28 B).....29

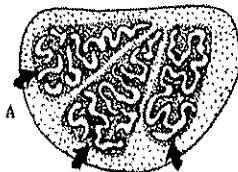


Fig. 28 A



Fig. 28 B

29. Button centrally located (Fig. 29 A).....(Oestrus ovis) SHEEP BOT FLY

Button not centrally located (Fig. 29 B).....30



Fig. 29 A



Fig. 29 B

30. Opening toward button narrow (Fig. 30 A)....(Hypoderma bovis) NORTHERN CATTLE GRUB

Opening toward button wide (Fig. 30 B).....(Hypoderma lineatum) CATTLE GRUB



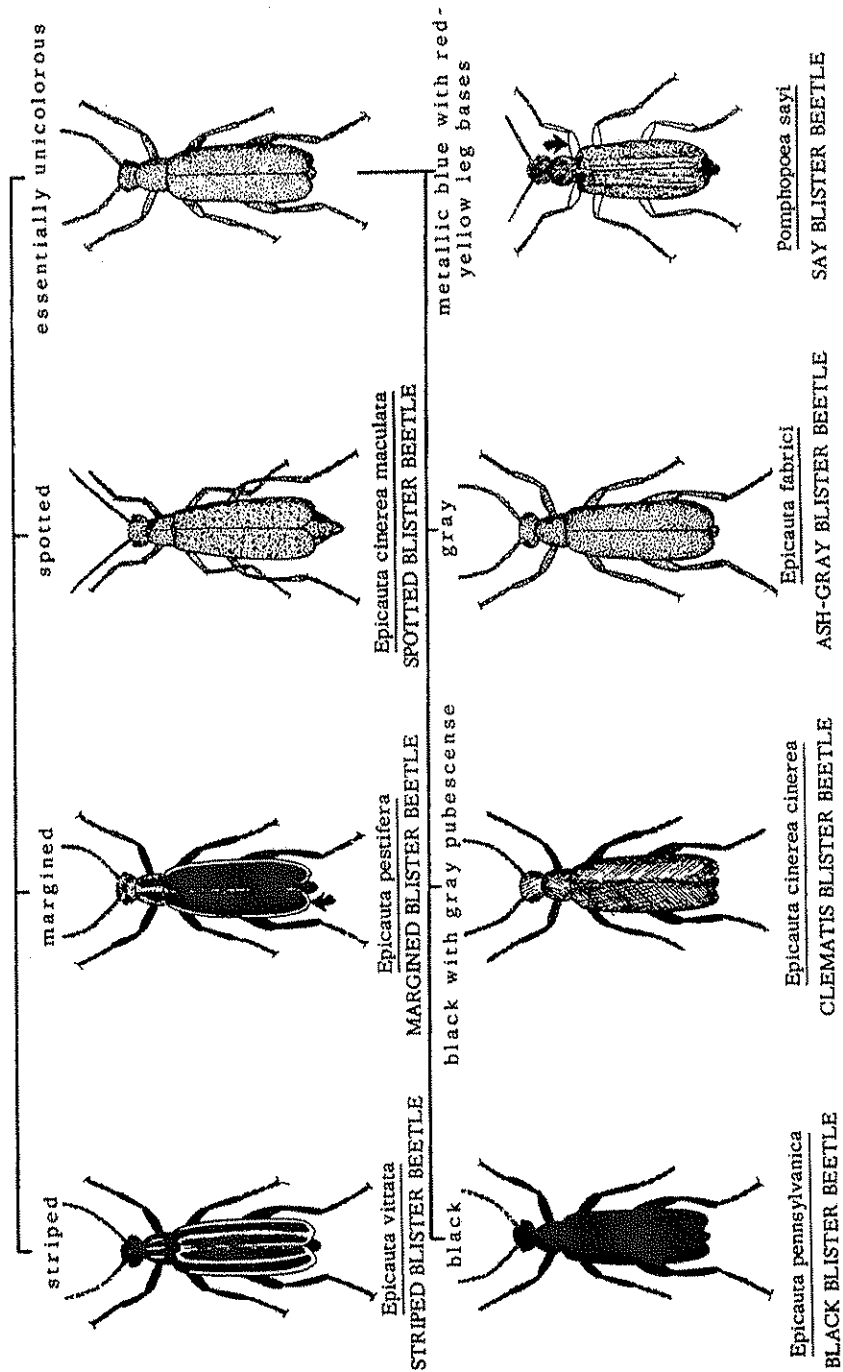
Fig. 30 A



Fig. 30 B

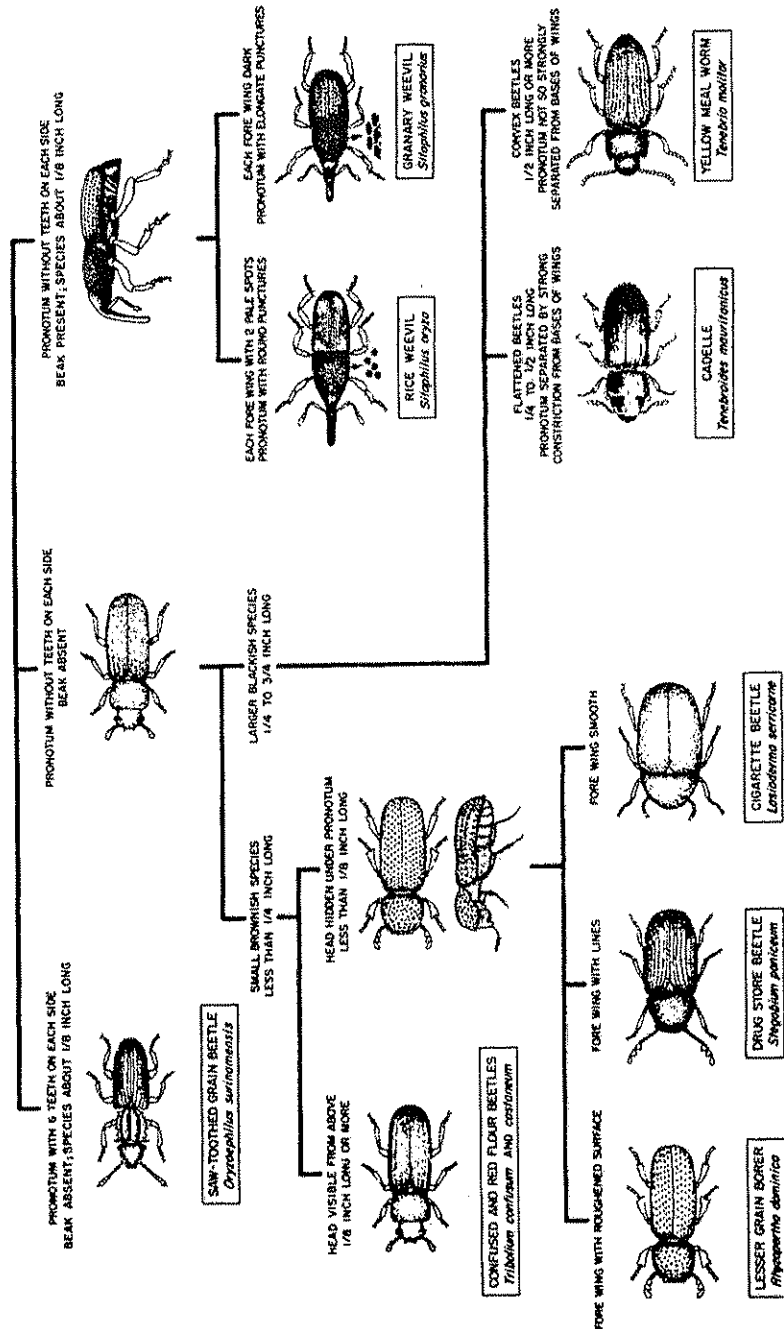
Appendix B: Dichotomous key for identifying Coleoptera⁸⁷

BLISTER BEETLES: KEY TO SOME COMMON UNITED STATES SPECIES Harold George Scott and Chester J. Stojanovich



BEETLES: PICTORIAL KEY TO SOME SPECIES COMMONLY ASSOCIATED WITH STORED FOODS

Harry D. Pratt



Appendix C: Temperature and Relative Humidity for Field

Trials

Temperature was recorded every hour at each of four locations and compared to a central location. Since the results at the four locations were all virtually identical and all were identical to the centrally recorded temperature, only the central location temperature values are reported. The graphs show the weighted average temperature for the duration of each experiment.

The relative humidity was recorded hourly at a central location. The average for each day was calculated for the graphs.

