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By William D. Kranz

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Is approved by the final examining committee:

John V. Goodpaster

Chair

Nick Manicke

Rajesh Sardar

Christine Picard

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Approved by: Eric Long

Head of the Departmental Graduate Program

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Date

ADVANCES IN SOLID PHASE MICROEXTRACTION FOR THE ANALYSIS OF VOLATILE
COMPOUNDS IN EXPLOSIVES, TIRE TREATMENTS, AND ENTOMOLOGICAL SPECIMENS

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of

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William D. Kranz

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For my Mother and Father,
Who always believed in me.

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LIST OF ABBREVIATIONS

2E1H	2-ethyl-1-hexanol
ADH	accumulated degree hours
AHC	agglomerative hierarchical clustering
ATF	Bureau of Alcohol, Tobacco, Firearms, and Explosives
CAR/DVB	carboxen/divinylbenzene
DA	discriminant analysis
DART	Direct Analysis in Real Time
DMNB	2,3-dimethyl-2,3-dinitrobutane
DNT	dinitrotoluene
EGDN	ethylene glycol dinitrate
ERC	explosive-related compound
GC-MS	gas chromatography-mass spectrometry
HS-SPME	headspace solid phase microextraction
HPLC	high performance liquid chromatography
NESTT	Non-Hazardous Explosives for Security Training
NIST	National Institute of Standards and Technology
NORT	National Odor Recognition Test

PA	polyacrylate
PBX	plastic-bonded explosive
PC	principal component
PCA	principal component analysis
PDMS	polydimethylsiloxane
PDMS/DVB	polydimethylsiloxane/divinylbenzene
PDMS/DVB/CAR	polydimethylsiloxane/divinylbenzene/carboxen
PEG	polyethylene glycol
PMI	postmortem interval
PVC	polyvinyl chloride
qPCR	quantitative polymerase chain reaction
ROC	receiver operating characteristic
SP	smokeless powder
SPME	solid phase microextraction
TATP	triacetone triperoxide
TNT	2,4,6-trinitrotoluene
TV-SPME	total vaporization solid phase microextraction
USAC	United States Auto Club
VOC	volatile organic compound

ABSTRACT

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Solid phase microextraction is a powerful and versatile technique, well-suited to the analysis of numerous samples of forensic interest. The exceptional sensitivity of the SPME platform, combined with its adaptability to traditional GC-MS systems and its ability to extract samples with minimal work-up, make it appropriate to applications in forensic laboratories.

In a series of research projects, solid phase microextraction was employed for the analysis of explosives, commercial tire treatments, and entomological specimens. In the first project, the volatile organic compounds emanating from two brands of pseudo-explosive training aids for use in detector dog imprinting were determined by SPME-GC-MS, and the efficacy of these training materials was tested in live canine trials. In the second project, the headspace above various plasticizers was analyzed comparative to that of Composition C-4 in order to draw conclusions about the odor compound, 2-ethyl-1-hexanol, with an eye toward the design of future training aids. In the third, automobile tires which had participated in professional race events were analyzed for the presence of illicit tire treatments, and in the fourth, a novel SPME-GC-MS method

was developed for the analysis of blowfly (Diptera) liquid extracts. In the fifth and final project, the new method was put to the task of performing a chemotaxonomic analysis on pupa specimens, seeking to chemically characterize them according to their age, generation, and species.

CHAPTER 1. INTRODUCTION

1.1 Solid Phase Microextraction

The twin disciplines of analytical chemistry and forensic science are ever-evolving, and the rapid pace of evolution has fostered a demand for new methods to keep up with laboratories' mounting efficiency and sensitivity requirements. One such method that has gained interest in recent years is solid phase microextraction (SPME), an off-column pre-concentration technique that is adaptable to gas chromatography-mass spectrometry (GC-MS) analysis. This technique utilizes a fiber to adsorb analyte molecules from its environs, gathering them and accumulating them on the fiber surface before transferring them to the injection port of the GC. As shown in Table 1.1, the thickness and chemistry of the fiber can be tailored to the specific needs of the analyst, with non-polar fiber coatings such as polydimethylsiloxane (PDMS) proving adept at collecting non-polar analytes, and polar coatings such as polyethylene glycol (PEG) showing preference to polar compounds. The fiber can be directly immersed in a liquid solution, or it can be suspended in the headspace above a liquid or solid sample, where it serves to collect the off-gassed volatile organic compounds (VOC's) [1-4].

Table 1.1. Summary of common commercially-available SPME fibers. [5]

Fiber	Polarity	Max Operating Temp	Target Compounds
Polydimethylsiloxane (PDMS)	Non-Polar	280 °C	Volatiles and non-polar semivolatiles
Polydimethylsiloxane-Divinylbenzene (PDMS/DVB)	Bipolar	270 °C	Polar volatiles
PDMS-Carboxen (PDMS/CAR)	Bipolar	320 °C	Gases and volatiles
Polydimethylsiloxane-Divinylbenzene-Carboxen (PDMS/DVB/CAR)	Bipolar	270 °C	Odors and flavors
Carbowax-Divinylbenzene (CAR/DVB)	Polar	265 °C	Polar analytes (alcohols)
Polyethylene Glycol (PEG)	Polar	250 °C	Polar analytes (alcohols)
Polyacrylate (PA)	Polar	320 °C	Polar semivolatiles (phenols)

Since its introduction in the late 1980s, SPME has garnered popularity for its sensitivity, with SPME-coupled GC-MS systems showing detection limits down to the sub-ppb level [5, 6]. Furthermore, SPME is amenable to the analysis of samples with little or no workup, and various studies have demonstrated the feasibility of analyzing samples directly. Among the myriad applications that have been published on to date, the technique has found use for the assay of samples as diverse as foods [6, 7], drugs [8-12], ignitable liquids [13-15], explosives [16-20] and environmental pollutants [6, 21, 22],

often in the native state. This eliminates the need for liquid-liquid extraction, solid-phase extraction, and many of the other arduous, time-consuming sample preparation procedures that are endemic to traditional analysis methods. As a result, many types of samples can be run immediately, with sensitivity equal to or greater than what liquid injection would confer, and—due to SPME’s ability to bypass the sources of error that tend to crop up in multi-step workups—often with superior accuracy [4, 5].

In light of its many advantages, there has been an interest in introducing SPME to forensic laboratories. The technique is highly-appropriate to the analysis of many of the types of evidence that crime labs process—especially solid samples, which can require a laborious, error-prone extraction prior to GC-MS analysis; as well as biological samples, which are frequently inconvenienced by the matrix effects brought on by proteins and other macromolecules. SPME avoids these pitfalls entirely. The technique is non-destructive, allowing for the preservation of the evidence in its original form. Moreover, the technique is simple, fast, and easy, offering the potential to expedite the work done by crime labs, helping to alleviate backlog. Finally, the technique is easily-adaptable to existing GC-MS systems, providing an opportunity for crime labs to improve efficiency at relatively low cost, with minimal additional investment in additional instrumentation or infrastructure.

What has been missing is a comprehensive body of literature demonstrating the usefulness of SPME technology and its applicability to forensic evidence. Thus, this dissertation seeks to explore the various ways SPME can be utilized for the analysis of samples in a forensic context. A newly-pioneered variant of the traditional SPME

technique, total vaporization solid phase microextraction (TV-SPME), was also utilized, and multivariate statistical techniques applied to aid in the discrimination of samples.

1.2 Mathematical Description of Multiphase Equilibria in a SPME System

In a closed system, such as the sealed interior of an autosampler vial that is undergoing extraction by a SPME fiber, there are multiple equilibria at work. One equilibrium occurs between the sample and the headspace encompassing it, and a second equilibrium occurs between the headspace and the polymer coating of the fiber. The mathematical description of this multiphase system begins with the understanding that the initial mass of analyte in the sample is equal to the mass of analyte distributed in all three phases once the system has reached equilibrium. This can be expressed:

$$C_0V_s = C_f^\infty V_f + C_h^\infty V_h + C_s^\infty V_s \quad (\text{Equation 1.1})$$

where C_0 indicates the initial concentration of the analyte in the sample matrix; C_f^∞ , C_h^∞ , and C_s^∞ are the equilibrium concentrations of the analyte in the fiber, the headspace, and the sample, respectively; and V_f , V_h , and V_s are the volumes of the fiber, the headspace, and the sample, respectively [4].

The fiber/headspace distribution constant, K_{fh} , and the headspace/sample distribution constant, K_{hs} , are defined [4]:

$$K_{fh} = C_f^\infty / C_h^\infty \quad (\text{Equation 1.2})$$

$$K_{hs} = C_h^\infty / C_s^\infty \quad (\text{Equation 1.3})$$

The mass of analyte distributed in the fiber, n , can be expanded as follows [4]:

$$\begin{aligned} n &= C_f^\infty V_f \\ &= \frac{C_f^\infty V_f C_0 V_s}{C_0 V_s} \\ &= \frac{C_f^\infty V_f C_0 V_s}{C_f^\infty V_f + C_h^\infty V_h + C_s^\infty V_s} \\ &= \frac{C_f^\infty V_f C_0 V_s}{C_s^\infty \left(\frac{C_f^\infty}{C_s^\infty} V_f + \frac{C_h^\infty}{C_s^\infty} V_h + V_s \right)} \\ &= \frac{\frac{C_f^\infty}{C_s^\infty} V_f C_0 V_s}{\frac{C_f^\infty}{C_s^\infty} V_f + \frac{C_h^\infty}{C_s^\infty} V_h + V_s} \\ &= \frac{\frac{C_f^\infty}{C_h^\infty} \frac{C_h^\infty}{C_s^\infty} V_f C_0 V_s}{\frac{C_f^\infty}{C_h^\infty} \frac{C_h^\infty}{C_s^\infty} V_f + \frac{C_h^\infty}{C_s^\infty} V_h + V_s} \\ n &= \frac{K_{fh} K_{hs} V_f C_0 V_s}{K_{fh} K_{hs} V_f + K_{hs} V_h + V_s} \quad (\text{Equation 1.4}) \end{aligned}$$

Note that this is an idealized equation. In actuality, the true expressions for the distribution constants K_{fh} and K_{hs} should include the chemical activities of the species under scrutiny. However, Equation 1.2 and Equation 1.3 are good approximations as long as the level of analyte is trace and the sample matrix is pure and homogenous. In almost all cases, these factors are irrelevant in SPME-GC-MS experiments, and therefore Equation 1.4 holds valid [4].

The chemical potentials of an analyte in the fiber, in the headspace, and in the sample are denoted by the following equations:

$$\mu_f = \mu^0(T) + RT \ln\left(\frac{p_f}{p_0}\right) \quad (\text{Equation 1.5})$$

$$\mu_h = \mu^0(T) + RT \ln\left(\frac{p_h}{p_0}\right) \quad (\text{Equation 1.6})$$

$$\mu_s = \mu^0(T) + RT \ln\left(\frac{p_s}{p_0}\right) \quad (\text{Equation 1.7})$$

where $\mu^0(T)$ is the chemical potential of the analyte at standard temperature T and pressure p_0 ; μ_f , μ_h , and μ_s signify the chemical potentials of the analyte in the fiber, in the headspace, and in the sample, respectively; p_h is the vapor pressure of the headspace; and p_f and p_s are the vapor pressures of the analyte in equilibrium with the analyte contained in the fiber and contained in the sample, respectively [4].

The chemical potentials of the analyte in all three phases are equal when the system is in equilibrium. Therefore [4]:

$$\mu_f = \mu_h = \mu_s \quad (\text{Equation 1.8})$$

From Equations 1.5–1.8, it can be rationalized that [4]:

$$p_f = p_h = p_s \quad (\text{Equation 1.9})$$

It is possible to redefine p_f , p_h , and p_s in more useful terms by invoking Henry's Law and the Ideal Gas Law. Henry's Law states:

$$p_f = K_F C_f^\infty \quad (\text{Equation 1.10})$$

$$p_s = K_S C_s^\infty \quad (\text{Equation 1.11})$$

where K_F and K_S are the Henry's Law constants of the analyte in the fiber and the analyte in the sample [4].

Note that Henry's Law expects that the vapor phase behaves as an ideal gas, while the liquid phase behaves as an ideal solution. In practice, neither of these assumptions is realistic. When performing SPME, the solvent vapor frequently interacts with the fiber in an inelastic way, partitioning into the film coating along with the

analyte. This often causes it to swell, which may alter the fiber volume, V_f . Likewise, the ideality of the liquid phase may be undermined if the attractive intermolecular forces between the solute and the solution are prohibitive to the solute's disposition to the vapor phase. The invocation of Henry's Law is therefore an approximation of convenience.

By applying the Ideal Gas Law, $p_h V_h = n_h RT$, where n_h is the moles of analyte molecule in the headspace, p_h can be defined [4]:

$$p_h = C_h^\infty RT \quad (\text{Equation 1.12})$$

From Equations 1.9–1.12, K_{fh} and K_{hs} can be redefined as [4]:

$$K_{fh} = \frac{C_f^\infty}{C_h^\infty} = \frac{RT}{K_F} \quad (\text{Equation 1.13})$$

$$K_{hs} = \frac{C_h^\infty}{C_s^\infty} = \frac{K_S}{RT} \quad (\text{Equation 1.14})$$

Equation 1.8 states that at equilibrium, the chemical potential of analyte in the fiber is equal to the chemical potential of analyte in the sample. Equation 1.9 elaborates that at equilibrium, the vapor pressure of the analyte in these phases is also equivalent.

Bearing these facts in mind, it is possible to define a new distribution constant, K_{fs} , which is given by $K_{fs} = C_f^\infty / C_s^\infty$. From Equations 1.10 and 1.11 [4]:

$$\frac{p_f}{p_s} = \frac{K_F C_f^\infty}{K_S C_s^\infty}$$

$$\frac{p_f K_S}{p_s K_F} = \frac{C_f^\infty}{C_s^\infty}$$

Furthermore, by Equation 1.9, $p_f = p_s$. Hence, these terms can be eliminated, and the above expression can be simplified [4]:

$$K_{fs} = \frac{K_S}{K_F} = \frac{C_f^\infty}{C_s^\infty} \quad \text{(Equation 1.15)}$$

From Equations 1.13–1.15, the term $K_{fh}K_{hs}$ can be redefined [4]:

$$K_{fh}K_{hs} = \frac{K_S}{K_F} = K_{fs} \quad \text{(Equation 1.16)}$$

Then, by Equation 1.16, Equation 1.4 can be simplified [4]:

$$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + K_{hs} V_h + V_s} \quad \text{(Equation 1.17)}$$

Equation 1.17 signifies that location of the fiber in the system is irrelevant to the amount of analyte that is extracted. The fiber can be placed in the headspace or directly immersed in the sample, and it will not matter; when the system is at equilibrium, the amount that is extracted will always be the same, provided conditions are not perturbed by volume changes in the fiber, the headspace, or the sample [4]. Note, however, that in practice, volume changes in the fiber do occur, particularly upon interaction with the solvent, which can cause the polymer coating to swell. This usually translates to a higher V_f , and resultantly, a higher overall sensitivity—though this may occur at the expense of the sample-to-sample precision of the fiber.

In cases where the fiber is directly immersed in a liquid sample, the headspace term can be eliminated from the denominator, resulting in the simplified equation:

$$n = \frac{K_{fs} V_f C_0 V_s}{K_{fs} V_f + V_s} \quad (\text{Equation 1.18})$$

1.3 Total-Vaporization SPME

Total vaporization solid phase microextraction (TV-SPME) is a new innovation on traditional SPME that has recently been pioneered by our laboratory. Whereas normal headspace SPME (HS-SPME) is characterized by two sets of partitioning equilibria—one equilibrium condition between the sample and the headspace, and a second equilibrium

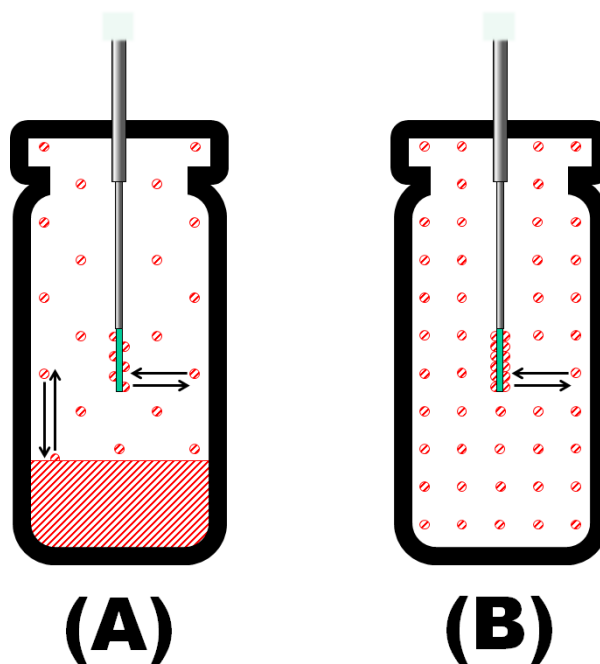


Figure 1.1. Comparison of HS-SPME and TV-SPME. Vial A depicts a HS-SPME system with two chemical equilibria, while Vial B depicts a TV-SPME system where all sample has been brought to the gas phase, eliminating the K_{hs} equilibrium.

condition between the headspace and the polymer coating of the SPME fiber—TV-SPME avoids this complexity by bringing the sample entirely into the gas phase, thereby simplifying the overall system. Figure 1.1 offers a visual comparison, with the traditional paradigm shown in A and the total vaporization version in B.

Mathematically, the TV-SPME system can be rationalized using Equation 1.18, where the vapor that fills the inside of the vial is treated as the sample rather than the headspace, as in direct immersion SPME. Because the volume of the sample is generally much larger than the volume of the fiber (i.e. $V_s \gg V_f$), and because the depletion of

analyte molecules in the sample matrix is relatively low, Equation 1.18 can be approximated:

$$n = \frac{K_{fs}V_sV_fC_0}{K_{fs}V_f + V_s} \approx K_{fs}V_fC_0 \quad (\text{Equation 1.19})$$

The equation most relevant to TV-SPME builds off of Equation 1.19, with a slight modification to the C_0 term:

$$n \approx K_{fs}V_fC_v = K_{fs}V_fC_0 \frac{V_0}{V_v} \quad (\text{Equation 1.20})$$

where C_v is the concentration of the totally-vaporized analyte contained within the vial, V_0 is the volume of the liquid sample, and V_v is the volume of the vial. This expression is intuitive: when some volume (V_0) of an analyte with some concentration (C_0) is allowed to fully vaporize, the new concentration of that analyte in the vapor phase (C_v) will be equal to the original liquid concentration multiplied by the ratio of the liquid volume to the vial volume (V_0/V_v). The amount of liquid that can be brought to the vapor phase can be estimated using the Ideal Gas Law:

$$V_0 = \left(\frac{PV_v}{RT}\right)\left(\frac{M}{\rho}\right) \quad (\text{Equation 1.21})$$

where V_0 is the volume of liquid sample (mL), P is the vapor pressure of the solvent (bar), V_v is the volume of the vial (L), R is the Ideal Gas Constant (L bar/K mol), T is the temperature (K), M is the molar mass of the solvent (g/mol), and ρ is the density of the solvent (g/mL) at temperature T . Note that T must be high enough to fully vaporize the sample, as well as any analytes.

In turn, the vapor pressure of the solvent is governed by temperature, which can be solved for using the Antoine equation:

$$\log_{10} P = A - \frac{B}{T + C} \quad (\text{Equation 1.22})$$

where A , B , and C are the Antoine constants for the solvent.

The full TV-SPME equation can be gleaned by substituting Equation 1.22 into Equation 1.21:

$$V_s = \left(\frac{10^{A - \frac{B}{T+C}} V}{RT} \right) \left(\frac{M}{\rho} \right) \quad (\text{Equation 1.23})$$

Therefore, given a liquid sample consisting of an analyte dissolved in solvent, the volume of sample that should be dispensed into a vial in order to totally vaporize it, V_s , can be calculated using Equation 1.23, provided that the temperature, the vial volume, and the density, molar mass, and Antoine coefficients of the solvent are known [23, 24].

TV-SPME offers several benefits over HS-SPME, foremost among them an improvement in the analytical figures of merit. Experiments have shown that totally vaporizing the analyte tends to yield data with higher precision values and mean recovery rates than what can be obtained by HS-SPME, while also tending to improve the extraction of high-boiling compounds. Sensitivity is also high. A TV-SPME study of nitroglycerine achieved limits of detection as low as 100 pg/mL, with calibration curve slopes and signal-to-noise ratios an order of magnitude higher than those attained using liquid injection [24]. Moreover, TV-SPME enables the analysis of analytes dissolved in organic solvents—something which is difficult to impossible in HS-SPME, where analytes in organic media tend not to partition into the headspace, and troublesome and inefficient in immersion SPME, where equilibration rates are hindered by low diffusion coefficients. Other benefits include quicker extraction times, added chemical selectivity, and the capacity to analyze larger overall volumes of sample [23, 24].

1.4 Project Overview

The research detailed hereafter encompasses the application of SPME-GC-MS to various samples of forensic interest.

Chapter 2 presents a study on pseudo-explosive training aids, a novel commercial product intended to simulate the odor of genuine explosives without incorporating any actual explosive material. Such a product would be invaluable to the

trainers and provisioners of explosives-detecting canines, enabling them to condition dogs to detect Composition C-4, trinitrotoluene (TNT), smokeless powder, and other hazardous materials of interest while alleviating a great deal of the monetary cost, licensing inconveniences, transportation difficulties, storage and security requirements, and other problems attendant to the use of real explosive ordnance. Two brands of pseudo-explosives were chemically evaluated by HS-SPME GC-MS to determine the principal VOC's emitted by each, and the efficacy of both brands was tested in a series of canine trials, where dogs trained on pseudo-explosives were tested for their performance on genuine explosives (and vice-versa). The statistical relevance of results was also verified by innovative use of Fisher's exact test and McNemar's test.

Chapter 3 details a study on the odor of Composition C-4, and in particular the odor compound 2-ethyl-1-hexanol (2E1H). The base explosive was chemically analyzed by HS-SPME-GC-MS, and its odor profile was compared to the headspace odor profiles of three common plasticizers: bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) sebacate, and bis(2-ethylhexyl) phthalate. In a follow-up study, the headspace profiles of many common plastic items were collected for comparison. This data served to facilitate a discussion on C-4's odor, with accompanying speculation on the design of future pseudo-explosive training aids.

Chapter 4 encompasses work done for the United States Auto Club (USAC), a professional automobile racing body. After various race events, USAC took cuttings from the tires of first, second, and third place finishers and submitted them for laboratory

testing. These cuttings were analyzed using HS-SPME-GC-MS, and the presence of illicit tire treatments was confirmed by comparison to known standards.

Chapter 5 describes the development of a TV-SPME-GC-MS method for the analysis of lipid extracts from biological specimens. Experiments were conducted to determine the most well-suited solvent and duration for the liquid extraction, whether any improvement in sensitivity was afforded by heating or ultra-sonicating the sample under liquid extract, and the most appropriate SPME fiber for the analysis. The method was further improved by the inclusion of an off-column derivitization step, allowing for the determination of free fatty acids and sterols by TV-SPME with high sensitivity and chromatographic efficiency.

Chapter 6 applies that method to the analysis of blowfly pupae belonging to the species *Phormia regina*, *Cochliomyia macellaria*, *Lucilia cuprina*, and *Lucilia sericata*. Samples were collected at various timepoints after pupation had taken place and frozen for preservation. Later, these pupae were extracted as per the previously-developed method and submitted to analysis by TV-SPME-GC-MS. The chromatographic profiles were integrated, and multivariate statistics were applied to attempt to discriminate between age, generation, and species based solely on the chemistry of the extracted lipids and hydrocarbons.

Chapter 7 comments on future directions for these lines of research: new projects with new aims, improvements and investigations on old techniques, and entirely new frontiers utilizing new forms of instrumentation.

CHAPTER 2. CHEMICAL AND BEHAVIORAL STUDIES OF PSEUDO-EXPLOSIVE CANINE TRAINING AIDS

2.1 Introduction

For thousands of years, the canine sense of smell has been of unrivaled utility, and mankind has harnessed it for a vast array of purposes, including hunting game, providing security, facilitating search-and-rescue operations, and—in the last century—sniffing out the presence of dangerous explosives. Canines have a superior ability to detect and differentiate odors [16]. This, combined with the exceptional sensitivity of the canine’s nose and the impressionable nature of its temperament, has made canines a valuable tool when it comes to sweeping for hidden bombs and explosives, even in the face of competition from man-made sensors that attempt to do the same.

Among the inherent challenges in training canines to detect explosives are the proper use and storage of explosive training aids. Until now, it has been standard practice to train canines on genuine explosive materials. For example, a canine trained to detect Composition C-4 (an RDX-based plastic explosive) would be exposed to genuine C-4 and then rewarded (e.g. by food or a toy) repeatedly until it alerts reliably, following the tenets of Pavlovian psychological conditioning.

This introduces a number of inconveniences. The training materials themselves are inherently hazardous and prone to contamination, and they require specialized storage and handling. As a result, there has been interest in developing non-explosive training aids that properly induce canine alerts. Approaches to solving this problem have included the following formulations to substitute for an actual explosive:

1. Genuine explosive compounds that have been desensitized, yet maintain their chemical identity.
2. Explosive-related compounds (ERC's), which are impurities and/or degradation products that are more volatile than the parent explosive. A well-studied example is the emission of 2,4-dinitrotoluene (2,4-DNT) by 2,4,6-trinitrotoluene (TNT) [25-29].
3. Energetic volatile compounds ("taggants") such as ethylene glycol dinitrate (EGDN), an explosive in its own right, which is often added to Semtex explosives, and 2,3-dimethyl-2,3-dinitrobutane (DMNB), which is currently added to plastic-bonded explosives (PBX) such as Composition C-4 to increase the likelihood that it can be detected by the vapor it emits [30].
4. Volatile solvents, additives, and impurities associated with the explosive formulation rather than the explosive itself [16, 31, 32]. Examples include cyclohexanone, 2-ethyl-1-hexanol (2E1H), diphenylamine, and ethyl centralite.

The classic example of the first approach is the product known as Non-Hazardous Explosives for Security Training and Testing, or NESTT [33]. This product consists of powdered silica which has been coated with layer of actual explosive but remains inert. A similar approach has been used to train canines to detect explosives like triacetone triperoxide (TATP), which is highly volatile [34] but also highly sensitive to heat, shock, and friction. In these cases, only small (mg) quantities of the explosive deposited onto filter pads or cotton balls have been used for canine training [35]. Other desensitized explosives are commercially available, and information on their composition and effectiveness for canine training and testing has been published [36, 37].

For the purposes of this research, only the formulations detailed in 2, 3, and 4 can be classified as “pseudo-explosives” in that they do not contain an actual explosive, but they do contain compounds intended to mimic the scent of an explosive. The main advantage of these materials is that they are safe, stable, non-hazardous and do not have any special shipping or storage requirements. Prior publications have claimed that canines trained on authentic explosives will respond to such substances [16, 31, 32]. However, response rates to solutions of 2,4-DNT and TNT were low. Canine trials designed to evaluate the response of canines to potential smokeless powder odor compounds were similarly mixed. There was no response to diphenylamine and low response to ethyl centralite and nitroglycerine tablets [32]. Taggants such as DMNB generated no response. The solvent cyclohexanone generated only limited response. The suspected odor compound 2E1H generated the largest canine responses; however, response rates ranged from 10 to 89 % in various trials depending upon the amount of

chemical used [31, 32]. Generally, the only consistent canine responses originated from authentic training aids such as detonating cord, TNT, TNT NESST, and RDX NESST [31, 32].

In the case of Composition C-4, prior headspace studies have identified DMNB, 2E1H, and cyclohexanone [31, 32, 38]. DMNB serves as a taggant, a chemical deliberately added to the explosive composition to aid in detection. It has no known commercial use beyond providing an additional chemical signature for canines and instruments [39]. 2E1H is believed to be a decomposition product of the plasticizers included in the explosive mixture [38], originating from the spontaneous hydrolysis in the presence of humidity or other moisture [40], whereas cyclohexanone is a residual solvent.

Smokeless powders are principally composed of nitrocellulose and nitroglycerine (for double-base smokeless powders), along with a wide range of additives to modify the stability, lifespan, or burn properties of the end product. Additive formulations vary from brand to brand and from manufacturer to manufacturer. However, past experiments have shown the stabilizer compounds diphenylamine and ethyl centralite to be the most common volatiles in the headspace [32]. In some smokeless powder products, it is also common to see 2,4-DNT included as a burn rate modifier [41, 42].

As in the case of C-4 and smokeless powder, the principal energetic compound in TNT, 2,4,6-trinitrotoluene, is not the compound most readily observed in the headspace. Instead, 2,4-DNT, a much more volatile impurity, has been identified as the most abundant off-gassed compound, as well as the compound suggested to be responsible for canine alerts [43].

What has been lacking is a systematic and scientific investigation of the efficacy of products that contain these compounds. In particular, we conducted laboratory and canine testing on pseudo-explosive training aids designed to mimic RDX-based explosives, smokeless powder, and TNT.

2.2 Materials and Methods

2.2.1 Training Aids

In this project, two different brands of pseudo-explosives for RDX-based plastic explosives, smokeless powder, and TNT were investigated. The first pseudo-explosive products (“product A”) consisted of fine-grained amorphous silica powder coated with volatile and semi-volatile compounds, all held inside a cotton pouch. Product A tagged RDX (t-RDX), untagged RDX (u-RDX), smokeless powder (SP), and TNT varieties were tested. The second pseudo-explosive products (“product B”) consisted of liquid or solid material contained within a semi-permeable polyethylene bag, with volatile odors allowed to effuse outward through micropores in the plastic. Product B tagged plastic-bonded explosives (t-PBX), untagged PBX (u-PBX), smokeless powder 1 (SP1), smokeless powder 2 (SP2), and TNT were tested. Each product was analyzed to determine the nature of the volatiles they off-gassed, and then subjected to single-blind canine testing to test their effectiveness as training aids.

2.2.2 Other Materials

Samples of Composition C-4, TNT, and IMR 4064 single-base SP were procured from Omni Explosives (Memphis, TN, USA) and L-Tech Enterprises (Eubank, KY). All explosives were procured, stored, and used in accordance with federal and state regulations as enforced by the Bureau of Alcohol, Tobacco, Firearms and Explosives (ATF) and the Indiana State Fire Marshal's Office. Methylene chloride was obtained from Fisher Scientific (Hampton, NH, USA). Solid phase microextraction (SPME) fibers were obtained from Sigma- Aldrich (St. Louis, MO, USA). Chemical standards of cyclohexanone, 2,3-dimethyl-2,3-dinitrobutane, 2,6-dinitrotoluene, ethyl centralite, 2E1H, and octane were also obtained from Sigma-Aldrich (St. Louis, MO, USA). A chemical standard of 2,4-dinitrotoluene was obtained from Spectrum Chemicals (New Brunswick, NJ, USA). A chemical standard of diphenylamine was obtained from Acros Organics (Geel, Belgium).

2.2.3 Chemical Analysis

A Trace GC Ultra gas chromatograph coupled to a DSQ II mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used to analyze the headspace above each explosive and pseudo-explosive training aid. One gram of material was removed from each training aid, placed inside a 20 mL headspace vial, and allowed to equilibrate for at least 1 h at room temperature. The headspace was then sampled at room temperature

using a Gerstel MPS2 autosampler (Gerstel, Inc., Linthicum, MD) equipped with a 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) SPME fiber. Extraction occurred for 30 min prior to injection. The column was a J&W Scientific Durabond DB-5ms (Agilent Technologies, Santa Clara, CA) with the following specifications: 30 m column length, 0.25 mm internal diameter, 0.250 μm film thickness. The inlet was a PTV inlet held at 220 $^{\circ}\text{C}$ with a split flow of 10 mL/min and a split ratio of 10:1. The oven temperature program utilized an initial temperature of 40 $^{\circ}\text{C}$ held for 1 min, a ramp of 20 $^{\circ}\text{C}/\text{min}$, and a final temperature of 300 $^{\circ}\text{C}$ held for 1 min. The MS transfer line temperature was 250 $^{\circ}\text{C}$. The mass spectrometer utilized an electron impact detector with a 1.40 min solvent delay and a scan range of m/z 40 to 550. The components of each sample were identified based upon comparison of their retention times and mass spectra to known chemical standards. All mass spectra were also matched to the National Institute of Standards and Technology (NIST) version 11 mass spectral database.

Methylene chloride extracts of the training aids were also analyzed by liquid injection GC-MS. To perform the extractions, 100 mg of each training aid was extracted with 10 mL methylene chloride, and the extract was passed through a 0.45 μm filter. The injection volume was 1.0 μL . The PTV temperature was 220 $^{\circ}\text{C}$ with a split flow of 50 mL/min and a split ratio of 50:1. The oven temperature program, the MS transfer line temperature, and the mass spectrometer parameters were the same as listed previously.

2.2.4 Canine Testing

Three groups of dogs participated in the canine testing phase of the experiment, with five to six dogs assigned to each group. All of the dogs were between one and three years old, and none of them had any previous training on explosives or pseudo-explosive materials. They were trained specifically for this experiment.

Over the course of months, each group of canines was imprinted on a different set of target odors. The first group on the three genuine explosives (Composition C-4, TNT, and IMR 4064 single-base smokeless powder), the second group on the four product A pseudo-explosives (t-RDX, u-RDX, SP, and TNT), and the third group on the five product B pseudo-explosives (t-PBX, u-PBX, SP1, SP2, and TNT). All canines were trained by a private sector subcontractor (Vohne Liche Kennels, Denver, IN).

The canines were then tested to competency on the type of material for which they received training. This test was modeled after the National Odor Recognition Test (NORT), which was developed by the ATF. This protocol uses a randomized, single-blind test method where the handler does not know where the target odor samples are placed. This helps to verify that the canine is actually recognizing target odors and not responding to any other cues. An independent test administrator was responsible for selecting and recording the placement of all sample containers and evaluating the test results. The ratio of target odors (i.e. explosives or pseudo-explosives), distractors (i.e. non-explosive materials such as canine food, chalk, rubber bands, etc.), and blanks (i.e. empty containers) was 1:1:1 for all canines tested. The distractors were everyday

materials that may be encountered during a canine search. As such, they were acquired ad hoc from various grocery and office supply stores in and around Indianapolis, IN. The quantity of each explosive was 20 g and the pseudo-explosives were utilized as received (i.e., cloth bag or plastic pouch). Each sample was placed inside a sealed quart-sized paint can with a perforated lid, and this can was then placed inside an unsealed gallon-sized paint can that had been secured to a 1' x 1' square of 3/4" plywood. All cans were identical, clean, unlabeled, and previously- unused.

The containers were arranged in a circle with at least 4' between them. All containers were in place for a minimum of 30 min prior to testing. During testing, the canine team was allowed to progress around the circle twice. When the canine alerted by sitting down next to a container, the alert was reported to the test administrator. The test administrator then recorded the placement of the container where each alerting response was identified. During proficiency testing, the canine was only rewarded for correct alerts made on positive samples identified by the handler and confirmed by the test administrator. To successfully pass the proficiency test, the canine had to make at least one positive response on all target odors (i.e., live or pseudo-explosives) for which the canine had been imprinted. The canine failed the test if it missed any target odor.

After all of the canines were proven to be competent, they were tested again using two circles of cans that contained actual explosives as well as product A and product B pseudo- explosives. This test was meant to determine response rates to the material the canines were trained on (target odors) and the response rate to materials for which they did not receive training (test odors). These "cross-tests" were otherwise

the same as the proficiency test, with one major exception: an intermittent reward system was employed. In this system, canines were not rewarded on test odors. Furthermore, the canines were rewarded 75 % of the time when they alerted to a training aid on which they had been imprinted (target odors). Intermittent reward systems avoid artificially reinforcing odors, regardless of past training methods. However, consistent rewards for target odors and consistent non-rewards on test samples would risk discouraging the canine from alerting to the test substance; hence, a given percentage of target odors are rewarded. Note that the intermittent reward system is not compatible with a double-blind arrangement given that the test administrator must know where the imprinted odors are located so that the decision to reward or not reward is made clear to the handler.

2.3 Results and Discussion

2.3.1 Chemical Analysis

Analysis of the training aids by headspace SPME GC-MS identified the principal volatiles emitted from the test materials, with liquid injection serving as an additional means of confirmation. Chromatograms for the C-4-related pseudo-explosives, the TNT-related pseudo-explosives, and the smokeless powder-related pseudo-explosives are displayed in Figure 2.1, Figure 2.2, and Figure 2.3, respectively. Only the data for the SPME study is shown, as data from liquid injection yielded the same information.

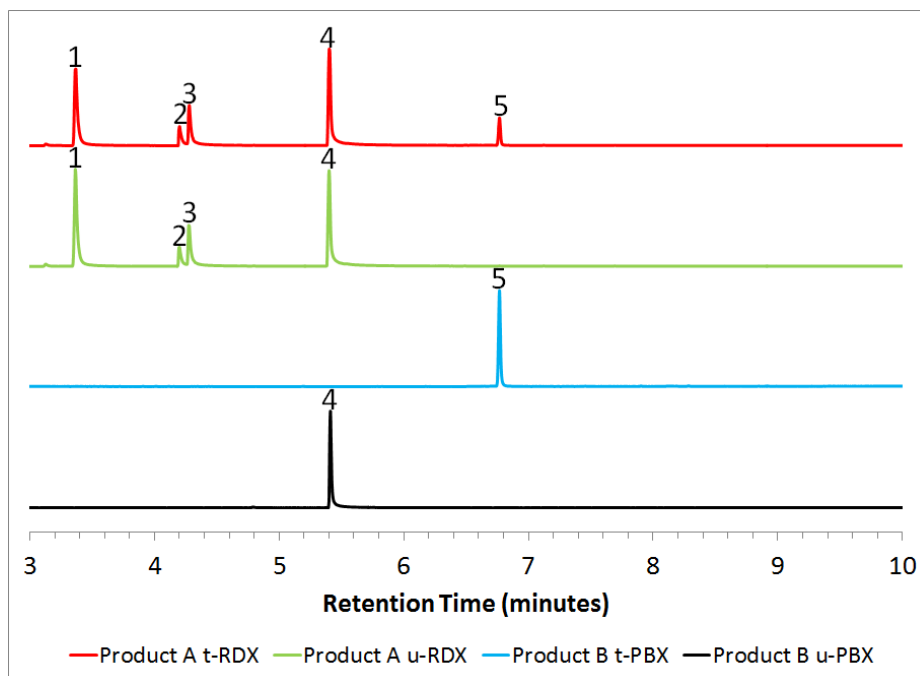


Figure 2.1. Chromatograms for C-4 related pseudo-explosives. The labeled compounds are 1: octane, 2: cyclohexanol, 3: cyclohexanone, 4: 2E1H, and 5: DMNB.

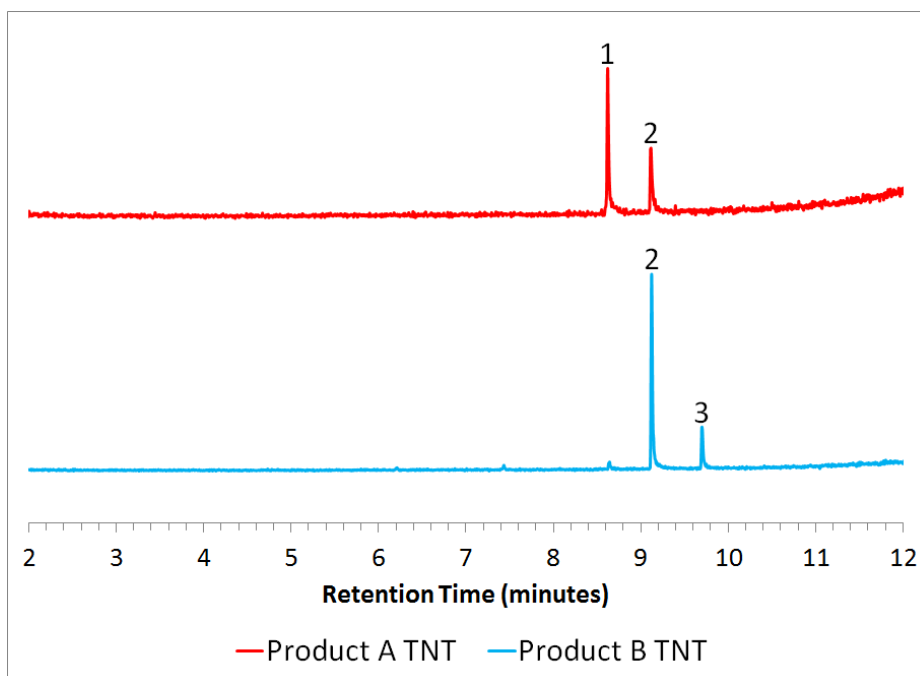


Figure 2.2. Chromatograms for TNT-related pseudo-explosives. The labeled compounds are 1: 2,6-DNT, 2: 2,4-DNT, and 3: diphenylamine.

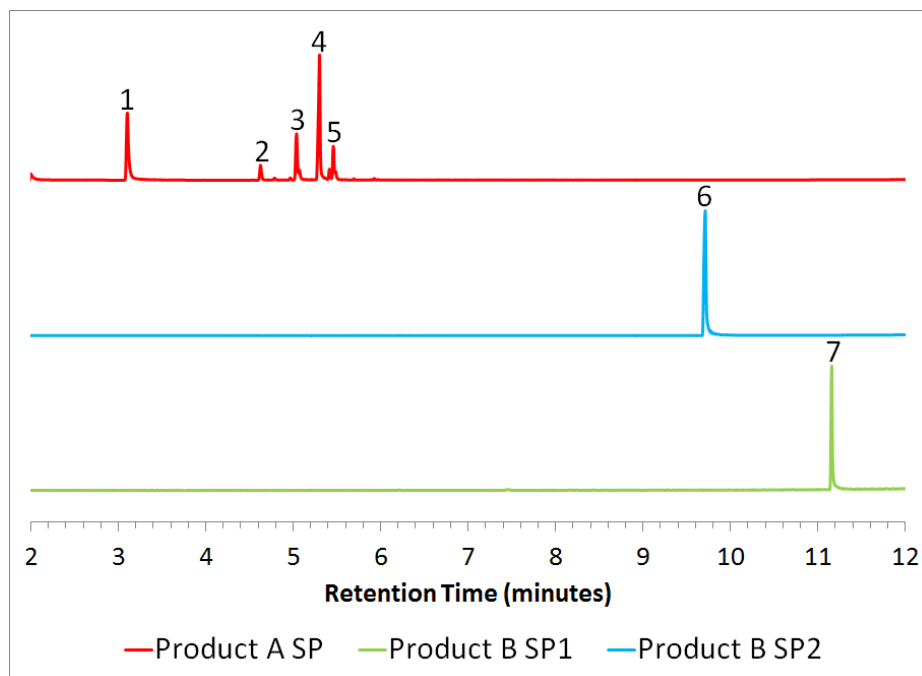


Figure 2.3. Chromatograms for smokeless powder-related pseudo-explosives. The labeled compounds are 1: toluene, 2: alpha pinene, 3: beta pinene, 4: 3-carene, 5: limonene, 6: diphenylamine, and 7: ethyl centralite.

It is readily apparent that product A and product B have a different philosophy when it comes to designing pseudo-explosives, as each has taken a very different approach to solving the problem. Product A provides a product laced with a mixture of different volatile and semi-volatile compounds, seeking to replicate the chemical profiles of genuine C-4, smokeless powder, and TNT. Both of the product A C-4 training aids feature the key volatile compounds cyclohexanone and 2E1H, differing only by the inclusion of DMNB in the tagged variety (Figure 2.1). Octane has not been previously identified in the headspace above Composition C-4; hence, it may be a residue from the solvent used to coat the silica particles. The product A smokeless powder chromatogram identifies a variety of terpenes, which are consistent with compounds known to

emanate from the Vinsol resin that coats some brands of smokeless powder (Figure 2.3) [44]. However, ethyl centralite and diphenylamine, two very common stabilizers found in smokeless powder, are both notably absent from the product A product. Finally, the product A TNT mimic is relatively simple, emitting 2,4- and 2,6-DNT, which are commonly found in actual TNT (Figure 2.2).

Whereas product A bases their training aids on a bouquet of different volatile chemicals, product B does quite the opposite. Most of the product B training aids consist of pure chemicals rather than mixtures. The product B design reflects the view that when a canine hits on an explosive, the canine is not actually alerting to a combination of different volatile chemicals, but rather to one or two dominant chemicals. To this end, the product B tagged PBX training aid contains pure DMNB; the product B untagged PBX training aid contains pure 2E1H (Figure 2.1). Likewise, the product B smokeless powder #1 and smokeless powder #2 training aids contain ethyl centralite and diphenylamine, respectively (Figure 2.3). The product B TNT mimic clearly aims to provide a source of 2,4-DNT for canines to imprint on (Figure 2.2). However, diphenylamine is also present in the headspace. Additional testing of the material that is used in the product B TNT training aid was carried out, including a visual/microscopic exam, ignition testing, and solvent extraction followed by FTIR analysis (data not shown). The results of these tests identified the material as a tube-type single-base smokeless powder, which is based on nitrocellulose and contains 2,4-DNT and diphenylamine.

2.3.2 Canine Testing

Ultimately, all of the canines (16 canines split into three groups) passed the proficiency testing criteria. The only odor that was not detected with 100 % reliability during this phase of the project was the product A pseudo-TNT product, which had proven difficult for the canines to detect throughout the training of the product A group. False positives (alerts to distractors and blanks) were monitored throughout the test. The mean false positive rate for any group of dogs on any test ranged from 0 to 9 %.

In order for a given pseudo-explosive to be deemed effective, a canine trained on that pseudo-explosive must respond to the analogous scent of a genuine explosive such that a statistically significant relationship exists between their respective response rates. Similarly, a canine trained on a genuine explosive must respond to that explosive's respective pseudo-explosive such that a statistically significant relationship can be shown to exist. These canine trials sought to ascertain the existence or nonexistence of such a relationship. In this endeavor, two statistical tests were employed: Fisher's exact test and McNemar's test.

Consider the following example, where a group of canines that is trained on a target scent (scent #1) is exposed to a series of items that contain a test scent (scent #2) on which the canines have not been trained. The results of that test can be put in the form of a contingency table, as depicted in Table 2.1.

Table 2.1. Example of a 2x2 contingency table adapted for canine testing.

	Hit Target Odor (Scent #1)	Miss Target Odor (Scent #1)	Sum
Hit Test Odor (Scent #2)	a	b	$a + b$
Miss Test Odor (Scent #2)	c	d	$c + d$
Sum	$a + c$	$b + d$	$n = a + b + c + d$

According to Fisher's exact test, the probability of any given outcome (in terms of the contingency table) is given by Equation 2.1:

$$p = \frac{(a + b)!(c + d)!(a + c)!(b + d)!}{a! b! c! d! n!} \quad (\text{Equation 2.1})$$

One then forms the following null (H_0) and alternative (H_a) hypotheses:

H_0 : The variables are independent (e.g. whether a canine alerts to scent #1 does not affect whether a canine alerts to scent #2).

H_a : The variables are dependent (e.g. canines that hit on scent #1 are either more or less likely to hit on scent #2).

In practice, a p -value is then calculated that is the sum of the probability for the particular outcome of the contingency table and the probabilities of all other outcomes that are more extreme. If this p -value is found to be less than 0.05, the null hypothesis can be rejected and the alternate hypothesis accepted with 95 % certainty. Fisher's test

returns its lowest p -values when the variables are either mutually inclusive or mutually exclusive (the data falls evenly between cells b and c or between a and d, respectively). For the purposes of this research, therefore, a successful pseudo-explosive would be reliably detected by the canines, and those canines would also reliably detect the real explosive. As this result would populate cell a (i.e., the data is neither mutually inclusive nor mutually exclusive), a high p -value is expected.

A second statistical test was also applied to the canine testing data, known as McNemar's test. The test statistic for McNemar's test is the following:

$$X^2 = \frac{(b - c)^2}{b + c} \quad (\text{Equation 2.2})$$

The null hypothesis for this test is that the alert rate to a target odor has no effect on the alert rate to a test odor. The minimum p -value for McNemar's test occurs when all of the data are concentrated in cell b (always missing the target odor but alerting to the test odor) or cell c (always alerting to the target odor but missing the test odor). For the purposes of this research, therefore, a successful pseudo-explosive would see most of its observations falling within cell a, with all deviations falling evenly between cells b and c. If deviations are spread unevenly between cells b and c, then that would translate to a high chi-square value with a low p -value, resulting in the rejection of H_0 , indicating minimal association between inclination to hit on pseudo-explosives and inclination to hit on genuine explosives. If deviations are spread unevenly, the chi-

square value is low, yielding a high p -value, signifying that dogs are equally likely to hit on pseudo-explosives and real explosives.

Note that Fisher's exact test and McNemar's test provide different p -values for the same contingency table because they are testing different things. McNemar's test merely looks at the difference between the probability of event A occurring and the probability of event B occurring and computes whether that difference is significant, modeling itself after a standard chi-squared distribution. Fisher's exact test computes the exact probability associated with observing a particular set of four cell values given that the marginal totals remain fixed. These statistical tests have been used to determine the likelihood of trained canines to alert to Composition C-4 versus compounds emitted by that explosive (i.e. 2E1H, DMNB and cyclohexanone) [38].

The results of the canine trials are presented in Table 2.2. For each explosive category, five to six individual canines were tested. Originally, six canines were intended to perform each trial. However, due to death or illness, the number of canines trained and tested on genuine explosives and the number of canines trained and tested on product B was five. As each canine went around two circles of containers twice, each canine had four chances to hit on the hidden target and test odors, yielding a total of 20–24 total opportunities to hit or miss.

In general, it was noted that the canines only demonstrated an affinity for the materials they trained on. For example, the response to real explosives by canines trained on real explosives ranged from 67 to 95 %. The response rate to product A by

Table 2.2. Canine testing results. Response rates for materials on which dogs were trained are presented in bold italics.

Hit On	Trained On		
	Genuine Explosives	Brand A Pseudo-Explosives	Brand B Pseudo-Explosives
Genuine C-4	17/20	0/24	0/20
Product A u-RDX	2/20	24/24	17/20
Product A t-RDX	1/20	22/24	17/20
Product B u-PBX	0/20	14/24	19/20
Product B t-PBX	2/20	2/24	18/20
Genuine SP	19/20	6/24	1/20
Product A SP	4/20	24/24	5/20
Product B SP1	4/20	0/24	13/20
Product B SP2	5/20	0/24	18/20
Genuine TNT	16/24	6/24	6/20
Product A TNT	6/24	21/24	17/20
Product B TNT	9/24	10/24	13/20

canines trained on product A ranged from 88 to 100 %. The response rate to product B by canines trained on product B ranged from 65 to 95 %. Interestingly, the lowest response rates were seen with actual TNT (67 %) and product B pseudo-TNT (65 %). This was consistent with handler observations during training as well as the results of proficiency testing. In general, there is no clear advantage to training aids that contain more volatile compounds (product A) versus less volatile compounds (product B). More direct quantitative measurements of headspace concentrations would be needed to ultimately settle this issue, however.

Regardless, examination of the results in Table 2.2 also demonstrates that canines trained on genuine explosives failed to reliably detect product A and product B, and canines trained on each variety of pseudo-explosive could not reliably detect

genuine explosives. Results were especially poor for those pseudo-explosive training aids meant to emulate the odor of C-4. None of the canines trained on either product A or product B versions of pseudo-explosive was able to hit on 20 g of actual C-4 explosive. Results for the TNT and smokeless powder pseudo-explosives were almost as poor, with at most six dogs trained on pseudo-explosives demonstrating a proclivity for the genuine explosives.

It is worth mentioning there were two success stories: among the five canines trained on product B TNT, there was one canine that hit reliably on genuine TNT on all four attempts. Among the six canines trained on product A double-base smokeless powder, there was one canine that hit reliably on genuine smokeless powder. Other than these two canines, however, all other canines alerted to genuine explosives 25 % of the time at most.

Statistical testing using Fisher's exact test resulted in none of the testing results generating a p -value of less than 0.05. Therefore, H_0 was accepted in all cases. In other words, it is clear that the data are neither mutually inclusive nor mutually exclusive. This result supports the conclusion that the two variables (hit rate on genuine explosives and hit rate on pseudo-explosives) were independent. Furthermore, none of the canine tests resulted in a case where canines that reliably alerted to real explosives reliably alerted to pseudo-explosives, and vice versa.

Likewise, none of the data sets for canines trained on real explosives had a McNemar p -value higher than 0.05, with one exception. As a result, it cannot be said that canines trained on the pseudo-explosives are equally likely to hit on the genuine

explosive analogs of those pseudo-explosives, and vice versa. In the case of canines trained on genuine TNT hitting on product B pseudo-TNT, the McNemar p -value registered 0.0522. As discussed above, product B was a single-base smokeless powder that emitted 2,4-dinitrotoluene.

In general, it was also observed that canines trained on pseudo-explosives were far more adept at hitting on other pseudo-explosives than at hitting on genuine explosives. For example, canines trained on product B (PBX) detected brand A (RDX), and canines trained on product B (TNT) detected brand A (TNT). In both of these cases, the McNemar test was not rejected. Both pairs of training aids share several of the same odor compounds (e.g., 2,4-DNT, 2-E-1H, and DMNB), so it is possible that some amount of “cross training” between pseudo-explosives is occurring.

2.4 Conclusion

The two brands of training aids examined here take a different approach to the problem of engineering an effective pseudo-explosive. Chemical analysis of product A aids shows that they typically incorporate a bouquet of different odors, seeking to include as many of the compounds commonly cited as being responsible for causing canines to alert as possible. Chemical analysis of product B aids shows that they emphasize a single odor—typically, the most abundant odor available in the headspace of the explosive they seek to emulate.

However, our canine trials confirmed that neither brand of pseudo-explosive aid was an effective replacement for genuine explosives. Canines trained solely on either product A or product B performed poorly across the board when faced with authentic explosives, and canines trained solely on authentic explosives showed little interest in the pseudo-explosives. This lack of cross-compatibility was confirmed through the Fisher and McNemar statistical tests. The fact that the canines in this study did not generalize by training on a single odor (e.g., product B) and then alerting to mixtures containing that odor (e.g., actual explosives) agrees with other recent research where canines trained on a single explosive substance did not reliably respond to mixtures of the explosive with other components [45].

Overall, training canines on “signature compounds” such as the various solvents, additives, and impurities associated with explosive formulations did not result in detection of actual explosives, nor did canine trained on actual explosives show interest in these materials. Although these compounds have been proposed as the possible cause of canine alerts, particularly in materials where the explosive itself is essentially non-volatile, this was not the case in this study. Continuing research in this area is clearly warranted as a more thorough understanding of how canines detect explosives will continue to improve their utility.

CHAPTER 3. EXPERIMENTS ON THE ODOR OF COMPOSITION C-4

3.1 Introduction

The use of canines to detect explosives via the characteristic volatile compounds that they emit confers several advantages. This form of detection is inherently non-invasive, and very low levels of explosives can be detected, rivaling even the most sensitive and modern manmade detectors [16]. The volatile species emitted by explosive materials can also be tracked to the source location.

In general, the odor generated by an explosive can be characterized as “simple” or “complex”. Simple odors consist of a single chemical compound. An example of such an odor is nitromethane, an energetic liquid used in binary high explosives that is both volatile and stable. Nitromethane has significant vapor pressure at room temperature, and as such, the explosive itself can be assumed to be the likely cause of a canine alert. Complex odors, conversely, consist of multiple chemical compounds that might originate from multiple species within the sample, or from the degradation products of a single species, or from a combination of the two. Most explosives fall into the “complex” category. Further complicating this issue is the existence of explosives of extremely low vapor pressure, such as RDX, whose concentration in the headspace is so

low that detection of the explosive vapor is extremely difficult at room temperature [46]. In these cases, more volatile solvents, additives, and impurities associated with the explosive dominate the headspace. In turn, these compounds have been suspected as comprising the actual odor “bouquet” of the explosive formulation.

Among the numerous explosive formulations that exist, Composition C-4 has been of particular interest to many authors [16, 31]. This plastic-bonded explosive consists of approximately 91 % by weight RDX—a non-volatile explosive—with the remaining components being polyisobutylene, a polymeric binder; bis(2-ethylhexyl) adipate, a plasticizer; and process oil, a heavy petroleum distillate. SPME experiments to date have revealed that cyclohexanone, DMNB, and 2E1H are the principal volatiles emitted by Composition C-4 [31, 32]. Cyclohexanone is a residual solvent from the manufacture of RDX that disappears with time. Cyclohexanone has also been found in a wide variety of other non-explosive materials, leading to significant false alarm rates when it was used as an analytical target for instrumentation [47]. DMNB, a unique volatile compound known as a taggant, is legally required to be present in all plastic explosives imported into or manufactured within the United States in order to aid in detection and identification [30, 39, 48]. In this case, the taggant becomes a major component of the explosive odor in addition to whatever else may be present from the explosive itself. Taggants have served as convenient targets for instrument techniques such as ion mobility spectrometry [49].

Canines that are trained to detect explosives are therefore exposed to a cocktail of volatile gases emanating from the source explosive formulation. The determination of

which compounds are responsible for causing a canine to recognize an explosive has long been of scientific interest. However, our relatively limited knowledge on this subject stands in the way of the development of new training aids and procedures. More troubling still, it introduces potential legal challenges with respect to the admissibility of evidence whose scientific basis is still coalescing [16]. Laying these problems to rest has fostered a demand for research to discover the nature of the odor generated by explosives, and to cross-reference these findings with observations on canine behavior.

The performance of explosive-detecting canines may be affected by a multitude of variables, including the influence of the dog handler and the animal's own physiology, temperament, and experience. An early study to determine which compounds actually generate the canine's alert response involved training canines exclusively on a single explosive formulation and then monitoring the animal's response to variations of that composition, including single raw ingredients, partial mixtures (with and without the base explosive) and the full intact mixture [50]. In general, canine response increased as the composition of the test sample approached that of the explosive formulation on which the canines were originally trained. Experiments such as this have given rise to the "bouquet theory," whereby canines are seen as responding to a complex mixture of compounds that are indicative of the explosive formulation. In contrast, more recent research has indicated there may be a preference by canines for some of the most abundant volatiles emitted by the materials on which they have been trained [51]. In light of this, it is unsurprising that 2E1H, cyclohexanone, and DMNB have been

suggested as the compounds most likely responsible for canine recognition of plastic explosives, based on laboratory olfactometry experiments [51] as well as field trials utilizing trained law enforcement dogs [31, 32].

In a number of past studies, canines have been presented with isolated explosive-related compounds to gauge their performance on single components in contrast to the full bouquet emanating from a genuine explosive [16, 31, 32]. However, the canines tested have not performed consistently. For example, in a double-blind test of canines currently in service, taggants such as DMNB generated no response (zero alerts out of nine canines). The solvent cyclohexanone has generated only limited responses (0 %, 0 %, 8.3 % and 33 % in various trials). The suspected odor compound 2E1H has generated the largest canine responses; however, response rates have varied from 10 % to 89 %, depending upon the amount of chemical used [31, 32]. Generally, the only consistent canine responses originated from training aids containing the base explosive RDX (83 %).

In this research, we examined the potential of cyclohexanone, DMNB, and 2E1H as canine training aids, with a particular focus on 2E1H. Our findings stem from the chemical analysis of several plasticizers and plasticized items by SPME-GC-MS, with attention to the presence of 2E1H in the headspace, and by comparison to the volatile profile of the plastic explosive, C-4. Also included are the results of our own canine trials, demonstrating the lack of any statistically-significant relationship between canine responses to these odor compounds and canine responses to C-4.

3.2 Materials and Methods

3.2.1 Instrumentation

A Trace GC Ultra gas chromatograph coupled to a DSQ II mass spectrometer (Thermo Scientific, Waltham, MA, USA) was used to analyze a liquid extract of Composition C-4 as well as all headspace samples. The column was a Zebron ZB-5MS (60 m x 0.25 mm x 0.25 μm). Hydrogen served as the carrier gas, with a column flow of 2.0 mL/min. All samples were desorbed in a PTV inlet and run in splitless mode. The oven temperature program utilized an initial temperature of 40 °C held for 1 min, a ramp of 20 °C/min, and a final temperature of 180 °C held for 1 min. The transfer line temperature was 280 °C. The mass spectrometer employed an electron ionization source at 200 °C, with a 3 min solvent delay and a scan range of m/z 40-550. FTIR analysis was completed on a Spectrum One FTIR, manufactured by Perkin Elmer (Waltham, MA, USA) and equipped with an ATR accessory with a diamond crystal. The instrument was operated in absorbance mode from 4000 cm^{-1} to 650 cm^{-1} with a resolution of 4 cm^{-1} .

3.2.2 SPME Optimization

As the ultimate goal of these experiments was to estimate the amount of 2E1H present in the air above a sample under conditions similar to those experienced by

canines, all samples were extracted at room temperature. Prior SPME studies on explosives have used various fiber chemistries, including PDMS [31, 32, 41, 43, 52-54], PDMS/DVB [54], and PDMS/DVB/CAR [54]. Given our particular interest in 2E1H, these three different fiber types were evaluated for their ability to concentrate and desorb this compound. Prior to use, all fibers were conditioned in the injection port for the recommended length of time and at the recommended temperature. 2E1H was purchased from Sigma-Aldrich (St. Louis, MO, USA). A 100 μ L aliquot was taken from the bottle, placed into a 20 mL autosampler vial, and allowed several hours to equilibrate prior to sampling. The same SPME parameters were used for all fiber types: 1 min extraction time, 1 min desorption time, and 240 °C desorption temperature. In this and all subsequent SPME studies, the fiber was exposed to a blank vial in between each sample and analyzed via the same GC-MS method to confirm that there was no carry-over. This method was used to estimate the relative affinities of each fiber type for 2E1H in order to simplify the subsequent optimization. Each fiber sampled the vial three times. A three-dimensional optimization of extraction time, desorption temperature and desorption time was then carried out using 1 mL of a 1 % w/w solution of 2E1H in decane. This determined the most advantageous instrumental conditions for the analysis of 2E1H using the most responsive fiber. The instrumental parameters were varied as follows: extraction time (10–60 min), desorption temperature (220–260 °C), and desorption time (1–5 min).

3.2.3 Plasticizer Study

The plasticizer in C-4 was verified by both FTIR and GC/MS. An extract of the plasticizer was obtained by submerging a 1 g sample of the explosive in pentane, agitating for 60 s, and passing the solution through a 0.45 μm polytetrafluoroethylene filter. For FTIR, a drop of the solution was placed on the ATR crystal, and the solvent was allowed to evaporate, leaving only the extracted plasticizer oil. For GC/MS, the extract was injected as a liquid sample in addition to a known standard of bis(2-ethylhexyl) adipate.

We also compared the availability of 2E1H in the headspace above C-4 to the availability of 2E1H in the headspace above three common plasticizers. These plasticizers were theorized to be potential emitters of 2E1H based on their chemical structures. C-4 was purchased from Omni Explosives (Memphis, TN, USA). The plasticizers bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) sebacate, and bis(2-ethylhexyl) phthalate were purchased from Acros Organics (Geel, Belgium), Sigma-Aldrich, and Spectrum Chemicals (New Brunswick, NJ, USA). Approximately 1 g of each material was placed into individual 20 mL autosampler vials and sealed. Samples were allowed to sit for several hours at room temperature to allow for adequate headspace equilibration. The SPME fiber was exposed to the headspace of each vial for 60 min, followed by a 1 min desorption in the injection port at 240 °C. Standard solutions that included 2E1H, DMNB, and cyclohexanone were analyzed by liquid injection immediately prior to and after every SPME sequence to guarantee the retention time did not drift significantly.

3.2.4 Evaluation of Non-Explosive Sources of 2E1H

Twenty-six everyday items were chosen—some because they were hypothesized to be potential emitters of 2E1H, some purely out of the curiosity of the authors. The items included: five samples of PVC tile ('Chalet', 'Chatsworth', 'Ebony Marble', 'Eurostone', and 'Twilight Blue' designs); three types of PVC pipe (Charlotte, Genova, and Lasco brands); three types of electrical tape (Grainger, Lowes, TrueValue brands); three types of plastic food wrap (Glad Cling Wrap, Saran Wrap, and Ziploc Perfect Portions Wrap); a Glad trash bag; a Ziploc freezer bag; a plastic lid to a soda fountain cup; bubble wrap; a plastic milk jug; a credit card; a lottery ticket; a movie ticket; a plastic notebook divider; a Wal-Mart shopping bag; a shower curtain; and a playing card. A 1 g sample was cut from each item, sealed in a 20 mL vial, given several hours to equilibrate, and analyzed for the presence of 2E1H using the same optimized SPME/GC-MS method described previously.

3.2.5 Canine Field Trials

Thirty-three certified canine/handler teams from eight law enforcement agencies assisted in a field trial conducted in Fort Meade, MD, USA. The purpose of the field trial was to study the predisposition of trained explosives-detecting canines to genuine C-4, as well as several other odor compounds under study. These compounds included cyclohexanone, DMNB, 2E1H, and bis(2-ethylhexyl) adipate. C-4 was provided

courtesy of Rick Strobel from the Bureau of Alcohol, Tobacco, Firearms, and Explosives (ATF). Round, two-ounce “sniffer tins” were purchased from Specialty Bottle (Seattle, WA, USA). Paint cans were purchased from W.W. Grainger, Inc. (Lake Forest, IL, USA). Nineteen small holes, arranged in the pattern of an asterisk, were punched into the lid of each tin to allow the odor to escape. Approximately 10 g of the solid samples (C-4 and DMNB) and 10 mL of the liquid samples (2E1H, cyclohexanone, and bis(2-ethylhexyl) adipate) were placed into separate tins. The tins were then placed inside separate open-top quart-sized paint cans, which were in turn placed inside separate open-top gallon-sized paint cans. Several other sniffer tins, similarly-perforated and designated as distractors (containing common items such as erasers, paper clips, and chalk) or blanks (containing nothing), were placed inside identical quart-sized/gallon-sized paint cans. It is important to note that while all of the canines tested had been previously certified and were familiar with single-blind odor recognition testing, several of them had little to no experience with searching the specific type of container used in this test (cans). Hence, a line-up of several cans containing explosives, distractors and blanks was made available for the teams to do a “warm up” prior to the test.

The test was modeled after the NORT. The test was randomized and single-blind. In this case, single-blind testing means that neither the canine nor the handler knew where the target/test odor samples were placed. This helps verify that the dog is actually recognizing the target/test odors it alerts to, rather than responding to any cues from the handler. An independent test administrator was responsible for selecting and recording the placement of all sample containers (blanks, distractors, and odor samples)

and evaluating the test results. All containers were identical, clean, unlabeled, and unused. The containers were arranged in a circle with at least four feet between them. All containers were in place for a minimum of 30 minutes prior to testing.

During testing, each dog was guided around the circle of cans by his or her respective handler. The dog was presented each can and allowed to sniff it. A positive response was noted when the dog stopped and sat beside a can. A negative response was noted when the dog continued on without pause. Upon reaching the end of the circle, the direction was reversed, and the dog was presented each can a second time, this time going counter-clockwise instead of clockwise. The canine's behavior was then documented, where a "hit" meant that the canine responded to a particular odor at least once and a "miss" meant that the canine did not react to the odor during either pass of the cans.

An intermittent reward schedule was used where the canine was rewarded 75 % of the time on the target odor on which it had trained (C-4) but not rewarded on any test odors (e.g., DMNB, cyclohexanone and 2E1H). Intermittent reward systems are used because consistently rewarding the canines on a target odor while consistently not rewarding the canines on the test odors can cause negative conditioning of the dog to the test odors. Conversely, any reward on the test odors can cause positive conditioning of the dog to the test odors. The dogs were also not rewarded if they alerted to a distractor or blank to prevent inadvertent reinforcement of negative samples.

3.2.6 Analysis of Canine Field Trial Data

As in Chapter 2, the data was compiled into contingency tables conforming to the layout of Table 3.1, with each table depicting the response rate for the test odor (2E1H, cyclohexanone, DMNB, or bis(2-ethylhexyl) adipate) in comparison to the target odor (Composition C-4).

Also as in Chapter 2, these tables were subject to statistical treatment by McNemar's test and Fisher's exact test. The following null (F_0 and M_0) and alternative (F_1 and M_1) hypotheses were established:

F_0	The variables are not mutually-exclusive/inclusive	Accept F_0 when $p > 0.05$
F_1	The variables are mutually-exclusive/inclusive, i.e. the data will fall evenly between two diagonal cells.	Reject F_0 when $p < 0.05$
M_0	The probability of b is equal to the probability of c , i.e. a dog trained on C4 is equally-likely to hit on 2E1H.	Reject M_0 when $p < 0.05$
M_1	The probability of b does not equal the probability of c , i.e. that a dog trained on C4 is not equally-likely to hit on 2E1H.	Accept M_0 when $p > 0.05$

Table 3.1. Example of a 2x2 contingency table adapted for canine testing.

	Hit Target Odor (Scent #1)	Miss Target Odor (Scent #1)	Sum
Hit Test Odor (Scent #2)	a	b	$a + b$
Miss Test Odor (Scent #2)	c	d	$c + d$
Sum	$a + c$	$b + d$	$n = a + b + c + d$

3.3 Results and Discussion

3.3.1 SPME Optimization

Based on the fiber study, the PDMS fiber was found to be the best fiber of the three for the detection of 2E1H. For example, the 2E1H area-under-peak values for the PDMS/DVB fiber were over one hundred times lower than those for the PDMS fiber. The PDMS/DVB/CAR fiber demonstrated sensitivity for 2E1H that rivaled that of the PDMS fiber, however, the standard deviation of the area-under-peak values was far larger. Following an optimization, the parameters for the detection of 2E1H by PDMS were set to 60 min extraction time, 240 °C desorption temperature, and 1 min desorption time.

As discussed above, samples of pure 2E1H were used as test odors. Experiments undertaken during the SPME optimization corroborate the fact that a 1 mL standard, a 1

drop standard, and a 100 μ L standard of 2E1H were all found to have similar area under peak values despite an order of magnitude difference in volume.

3.3.2 Plasticizer Study

The tendency of PVC tiles to emit 2E1H was previously noted by a research group examining indoor environmental pollutants in Japan, whom likewise speculated that the compound had arisen from the natural degradation of bis(2-ethylhexyl) phthalate in the flooring [55, 56]. In this case, we hypothesized a link between the 2E1H given off by C-4 and the plasticizer used in the explosive formulation (bis(2-ethylhexyl) adipate). Figure 3.1 depicts the molecular structures of 2E1H, bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) sebacate and bis(2-ethylhexyl) phthalate. Irrespective of the central diester, the 2-ethylhexyl moiety recurs throughout. Whether 2E1H is a hydrolysis product of a parent plasticizer molecule, or whether it is a chemical precursor in the manufacturing process that is never fully removed from the final product, it seems altogether likely that the 2E1H derives from the plasticizer in some way.

FTIR analysis of a pentane extract of the C-4 used in this study confirmed that the plasticizer was bis(2-ethylhexyl) adipate. In addition, Figure 3.2 portrays the IR spectrum for the extract, as well as the library hit confirming its identity. SPME/GC-MS chromatograms for 1 g samples of the plasticizers are compared against 1 g of C-4 in Figure 3.3. 2E1H is present in the chromatogram for all plasticizers and C-4. Furthermore, the area under-peak values for the 2E1H peak in bis(2-ethylhexyl) adipate and

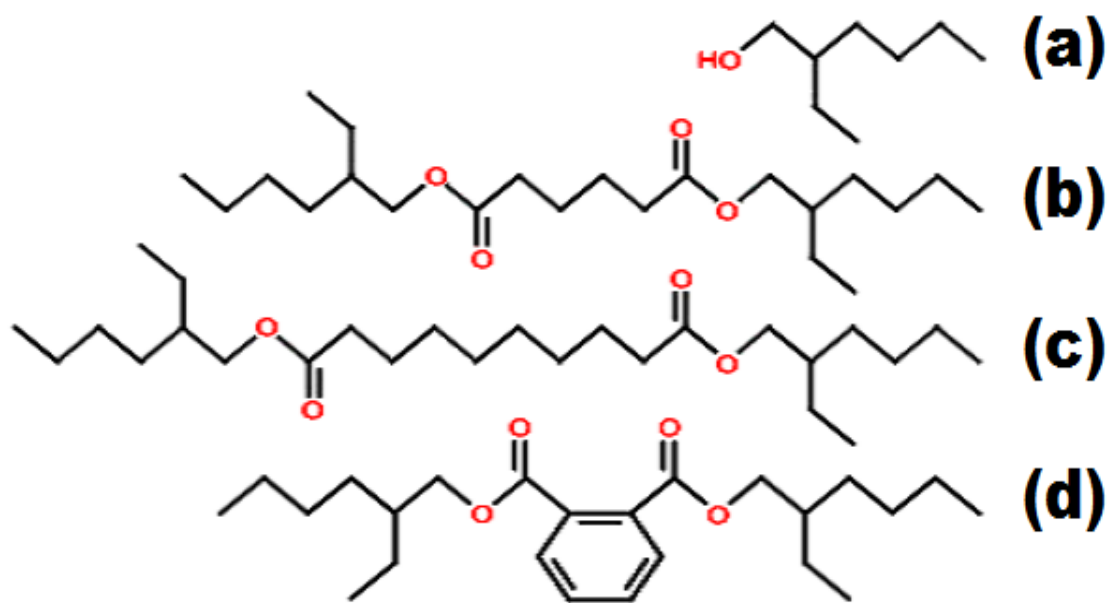


Figure 3.1: Structures of various plasticizers in comparison to 2E1H. (a): 2E1H; (b): bis(2-ethylhexyl) adipate; (c): bis(2-ethylhexyl) sebacate; (d): bis(2-ethylhexyl) phthalate.

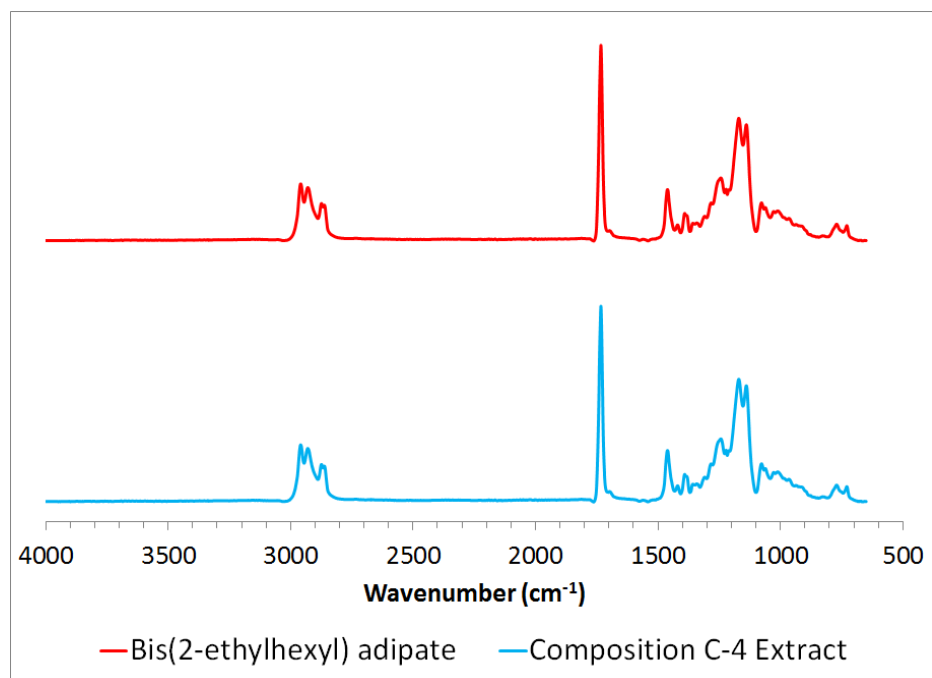


Figure 3.2: FTIR spectra for bis(2-ethylhexyl) adipate and C-4.

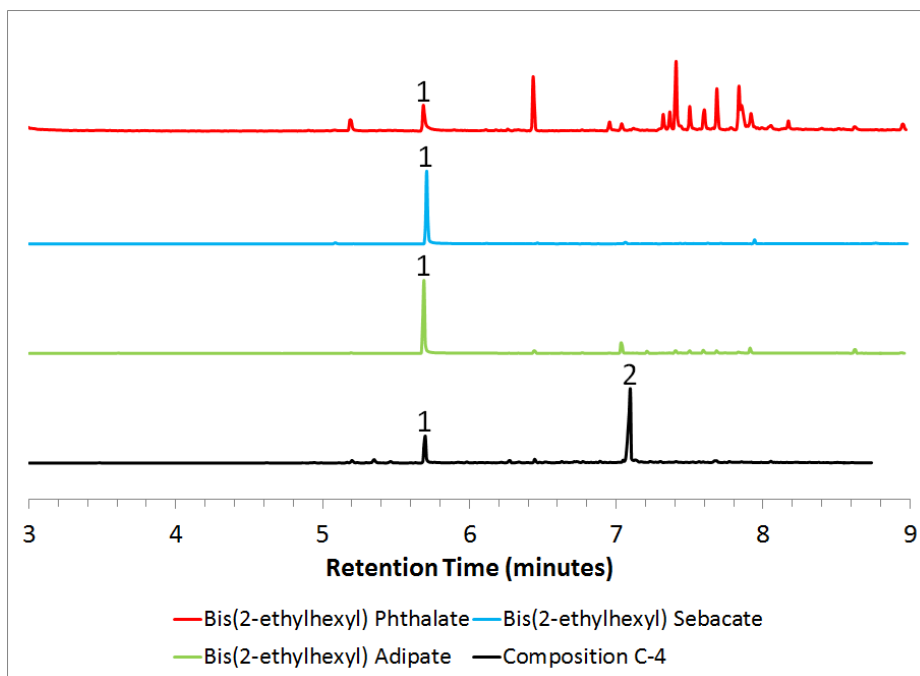


Figure 3.3: Chromatograms for bis(2-ethylhexyl) phthalate, bis(2-ethylhexyl) sebacate, bis(2-ethylhexyl) adipate, and Composition C-4. The labeled peaks are: 1: 2E1H and 2: DMNB.

bis(2-ethylhexyl) sebacate fell within 10 % of the value for authentic C-4. Therefore, on a per gram basis, these samples emitted similar amounts of 2E1H. In contrast, the 2E1H peak for bis(2-ethylhexyl) phthalate was substantially smaller (less than 4 %) than the 2E1H peak for C-4. Regardless of the size of the 2E1H contribution, these results confirm the emission of 2E1H by plasticizers having a 2-ethylhexyl moiety.

3.3.3 Evaluation of Non-Explosive Sources of 2E1H

Of course, these plasticizers do not occur solely in plastic explosives. They have a variety of uses in other commercial and industrial products as well. Bis(2-ethylhexyl)

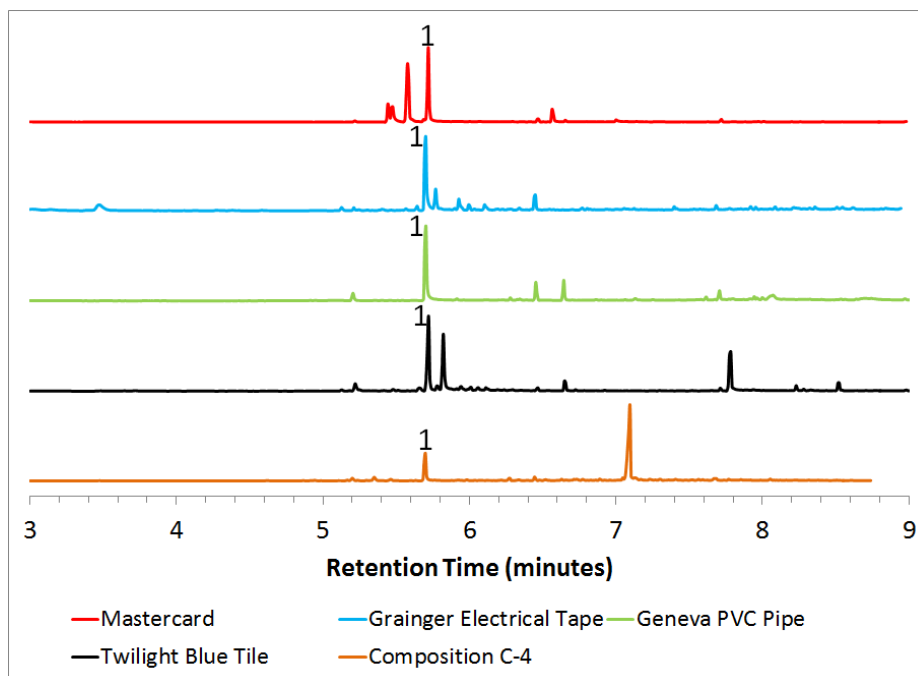


Figure 3.4: Chromatograms for the credit card, Grainger-brand electrical tape, Genova-brand PVC pipe, 'Twilight Blue' PVC tile Composition C-4. The labeled peak is 1: 2E1H.

phthalate, for example—perhaps better known by its trade name, di-2-ethylhexyl phthalate, or DEHP—is the plasticizer of choice in the manufacture of polyvinyl chloride (PVC) items [57]. Likewise, bis(2-ethylhexyl) adipate finds use as an ingredient in PVC-based plastic wraps. Several other plasticizers of similar structure also exist that could conceivably generate 2E1H by off-gassing, including bis(2-ethylhexyl) azelate, bis(2-ethylhexyl) glutarate, 2-ethylhexyl benzoate, and triethylene glycol bis(2-ethylhexanoate). Therefore, the analysis was broadened to include a variety of common plastic items. Of the 26 items evaluated, 11 were found to yield appreciable levels of 2E1H. These items included: all five samples of PVC tile; the Genova and Lasco brands of PVC pipe; all three brands of electrical tape; and the credit card. Representative

chromatograms for these items are compared against C-4 in Figure 3.4. The area-under-peak values for the 'Chalet' PVC tile, the 'Chatsworth' PVC tile, the 'Eurostone' PVC tile, the 'Twilight Blue' PVC tile, the Genova PVC pipe, the Grainger electrical tape, and the credit card all fell within one order of magnitude of C-4's area-under-peak. In the case of the 'Twilight Blue' PVC tile and the credit card, the area-under-peak value was approximately double that of C-4.

3.3.4 Canine Field Trials

Table 3.2 depicts the results of the canine trials with respect to Composition C-4 and 2E1H. Tables 3.3, 3.4 and 3.5 depict the results of the canine trials with respect to cyclohexanone, DMNB, and bis(2-ethylhexyl) adipate, respectively. Over the course of the testing, 33 dogs were observed. Several surprising observations were made during testing. For example, none of the canines tested responded to an inert training aid designed to simulate RDX-based explosives (RDX NESTT). This training aid consists of silica particles with a thin coating of explosive on the outside. As discussed previously, some studies have found that canine response to the base explosive is low relative to the actual explosive formulation, whereas other studies have found that canines respond preferentially to the base explosive. Overall, although many of the canines had previously been exposed to NESTT, the use of NESTT in canine training and testing is not accepted by all canine programs. This is in part due to the issue of low odor availability, which is a concept describing the amount of explosive vapor that is available to the

Table 3.2. Contingency table of Composition C-4 versus 2E1H.

	Hit C-4	Miss C-4	Sum
Hit 2E1H	1	0	1
Miss 2E1H	21	11	32
Sum	22	11	33

Table 3.3. Contingency table of Composition C-4 versus cyclohexanone.

	Hit C-4	Miss C-4	Sum
Hit Cyclohexanone	7	4	11
Miss Cyclohexanone	15	7	22
Sum	22	11	33

Table 3.4. Contingency table of Composition C-4 versus DMNB.

	Hit C-4	Miss C-4	Sum
Hit DMNB	9	1	10
Miss DMNB	13	10	23
Sum	22	11	33

Table 3.5. Contingency table of Composition C-4 versus bis(2-ethylhexyl) adipate.

	Hit C-4	Miss C-4	Sum
Hit Bis(2-ethylhexyl) adipate	1	0	1
Miss Bis(2-ethylhexyl) adipate	21	11	32
Sum	22	11	33

canine's nose [58]. A more ideal sample for future testing would be pure RDX powder, which would presumably have greater odor availability.

While the detection accuracy for C-4 was fair (67 %), it was lower than expected. This may reflect the unfamiliarity of some of the canine teams with the mechanics and containers of the test. Not all canine programs rigorously practice odor recognition testing under the conditions used here. An important point, therefore, is to compare the relative detection rates as well as apply statistical tests to determine any relationships between different materials. For example, the detection rate for 2E1H was far worse than C-4, with only one pass of one dog translating into an alert (3 %). The detection rate for cyclohexanone and DMNB were higher but still quite low overall (33 % and 30 %, respectively)

The output of McNemar's test on Table 3.2 was a chi-square value of 21. This far exceeds the minimum significance value of 3.84. Based on this analysis, M_0 was rejected, indicating a clear preference of canines for C-4 that did not hold true for 2E1H. The

output of the Fisher's exact test on Table 3.2 was a one-tailed p-value of 0.66. This does not meet the minimum significance value of 0.05. Based on this analysis, F_0 was accepted, indicating that there was no association between the detection of C-4 and the detection of 2E1H. As shown in Table 3.3 and 3.4, cyclohexanone and DMNB generated more alerts than 2E1H. However, cyclohexanone and DMNB yield McNemar chi-square values of 6.37 and 10.29, respectively. M_0 is rejected in both cases. Finally, Table 3.5 shows that alert rate to the original plasticizer is also not associated with the alert rate of C-4 (chi-square value of 20).

3.4 Conclusion

These results have implications for the development of new canine training aids that mimic the scent of an explosive yet contain inert ingredients such as 2E1H, cyclohexanone, or DMNB (pseudo-explosives). In particular, chemical analysis following our canine trials has shown that 2E1H is linked to common plasticizers such as bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) sebacate, and bis(2-ethylhexyl) phthalate. In addition, PVC-based items containing these plasticizers emit 2E1H. This raises red flags concerning the use of unadulterated 2E1H as a training aid, as it may lead to alerts on common, everyday items that happen to bear plasticizers with this particular structural characteristic. However, it is also known that canines can be "trained off" of materials that could be false positives.

The canine trials described here address the scenario where compounds like 2E1H could be incorporated into pseudo-explosives such that canines that are already imprinted on actual explosives would respond to the mimic in situations where the use of an actual explosive is inconvenient or even prohibited. However, our testing shows that exposing a dog that has been imprinted on C-4 to either pure 2E1H or the parent plasticizer (which emits 2E1H at levels similar to C-4) results in essentially no response.

Although the basic mechanics and set-up of the canine trials in this study were the same as previous studies, there were differences in the amounts of odor compounds used and the containers in which they were placed (e.g., metal electrical boxes and quart-sized paint cans). What may be needed to truly reconcile different canine studies is not only specifying the amount of odor compound, but also determining the flux of the odor into the environment. This will depend upon quantitating the diffusion of the odors through small holes (such as in this study and [58]). Alternatively, the controlled permeation of odor compounds through polymer films has been described [31, 32, 59]). Additional research in our laboratory has focused on the issue of flux and it will be the subject of future publications in this area.

Further experimentation is also warranted to determine whether the 2E1H in plastic explosives arises from the natural hydrolysis of ester bonds in plasticizers or from the use of 2E1H as a plasticizer precursor. Furthermore, the only plasticizer included in the canine trials was bis(2-ethylhexyl) adipate, which is used in modern C-4. As the SPME analysis of the other plasticizers occurred after the canine trials, they were not included. It would be of interest to include them in future canine trials to see if the

canines have any reaction. However, based on our results for bis(2-ethylhexyl) adipate, we predict that the canines will have little to no interest in these materials.

Although previous field trials conducted by other groups have indicated a possible preference among law enforcement canines for 2E1H, our own experiments in this regard were not nearly as successful. Out of 33 total dogs from 8 different agencies, comprising 66 total passes, only once did a dog hit on 2E1H. Even when taking only successful C-4 passes into consideration and omitting the rest of the data, accuracy on 2E1H amounts to less than 3 %. Statistical analysis by McNemar's test further discounts any association between a trained canine's likelihood to hit on C-4 and its likelihood to hit on 2E1H. Neither is there any association between a trained canine's likelihood to hit on C-4 and its likelihood to hit on bis(2-ethylhexyl)adipate, which is the plasticizer utilized in C-4.

Ultimately, it may be advantageous to pull back the magnifying glass and consider the full suite of volatiles known to emanate from C-4 and other plastic explosives (that is, the "bouquet" theory). 2E1H remains a prime constituent of plastic explosive headspace. However, the contributions of cyclohexanone and DMNB should not be overlooked. There is a possibility that none of these compounds, by themselves, are responsible for triggering a positive response in canines, and that only the combination of all three compounds in proper proportions will elicit recognition.

CHAPTER 4. AN EVALUATION OF COMMERCIAL TIRE TREATMENTS BY SOLID PHASE MICROEXTRACTION

4.1 Introduction

Concerns about cheating in professional automobile racing circuits have been exacerbated in recent years by the introduction of a number of commercial products, which claim to have the ability to illicitly boost performance whilst avoiding traditional means of detection. Commercial tire treatments, in particular, have risen to become a major concern for the administrative bodies that regulate autosports. These formulations are typically applied to the interior and/or exterior surfaces of the tires in the days and weeks leading up to a race. Over the intervening period of time, they absorb into the rubber, purportedly altering it physically and chemically in such a way as to augment the tires' grip on the surface of the racetrack. This, according to the vendors, translates to an improvement in handling, offering a subtle and subversive way to quicken lap times.

Although presently there are only a few commercial entities that specialize in this market, their catalogues are extensive. Several of the available tire treatments come stamped with the guarantee that they are "absolutely undetectable and odorless," specially-engineered to foil any attempt at conclusive analysis. For obvious reasons, the

companies that produce them tend to be highly secretive about their formulations, reluctant to provide materials safety data sheets or to disclose any information whatsoever about their proprietary mixtures. More problematic still, published literature on tire treatments is virtually nonexistent, posing an informational challenge for the aspiring analyst.

The goals of this research, then, were twofold: to offer insight into the yet-unreported nature of the existing tire treatment products, and to describe an effective method for the analysis of questioned tire samples.

Experiments were accomplished using GC-MS, with added sensitivity conferred by SPME. The simplicity and responsiveness of SPME make it well-suited to the ultrasensitive assay of nearly any conventional sample, and it proved especially useful during the course of this study, where the low volatility of the tire rubber provided a clean background for the highly-volatile tire treatment chemicals to propagate. Infrared analysis was also employed for the validation of GC-MS results, with the identities of the principal ingredients in every tire treatment confirmed wherever possible via ATR-FTIR.

The information provided here fills an important niche that has hitherto been vacant. Once again, it must be stated that peer-reviewed, published literature on the analysis of tire treatments is something that is not currently available; to the best of our knowledge, in spite of the urgent needs of the racing industry, there have been no other reported studies or methods. It is our hope that these findings will provide guidance to analysts who seek to evaluate questioned tire samples in the future, and that it will pave the way to a greater understanding of illicit commercial tire treatments.

4.2 Materials and Methods

All tire treatment products and questioned tire samples were provided courtesy of the United States Auto Club.

A 6890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent, Santa Clara, CA, USA) served as the principal instrumentation, with autosampler functionality provided by an MPS2 (Gerstel, Mülheim an der Ruhr, Germany). The column was a J&W DB-5ms (30 m × 0.25 mm × 0.25 μm). The carrier gas was hydrogen, with a constant flow of 2.5 mL/min. The oven temperature program utilized an initial temperature of 40 °C held for 1 min, a ramp of 20 °C/min, and a final temperature of 300 °C held for 1 min. The inlet temperature was 270 °C. The mass transfer line temperature was 280 °C. The mass spectrometer utilized an electron ionization detector at 230 °C, with a scan range of m/z 50-550. All experiments utilized a PDMS SPME fiber with a 5 min incubation time.

Tires were sampled by cutting out a piece of rubber with a mass of 1 g. The sample was then sealed within a 20 mL headspace vial and heated. Following a 5 min incubation time, a PDMS SPME fiber was inserted into the vial. All control and suspect tires were analyzed using a 5 min extraction time in splitless mode. The liquid tire treatment products were analyzed using a 3 min extraction time and a 32 : 1 split ratio.

The sensitivity of the method was gauged by conducting a serial dilution of Tire Treatment B and Tire Treatment J in pentane, then spiking 50 μL of the diluted 10 %, 1 %, and 0.1 % solutions onto 1 g blank tire rubber cuttings and analyzing them using the same instrumental and SPME parameters. Based on the results of these analyses, a

three point calibration curve of signal versus concentration could be constructed, and the effective detection limits for various peaks of interest were calculated using the slope of the calibration curve and the standard error of the y-intercept.

FTIR analysis of the tire treatment products was also completed. The instrumentation was a SpectrumOne (PerkinElmer, Waltham, MA, USA) equipped with an ATR accessory. A drop of each tire treatment product was placed on the diamond crystal and allowed to evaporate, leaving only a thin film. Spectra were collected in absorbance mode. Signal averaging was performed over 16 scans with a scan resolution of 4 cm^{-1} .

4.3 Results and Discussion

4.3.1 Analysis of Tire Treatment Products

Table 4.1 summarizes the results of the GC-MS analysis (Figure 4.1) and the FTIR analysis (Figure 4.2). Wherever possible, the major “red flag” peaks were identified, and an infrared library match was reported.

The tire treatment products can be grouped into two broad classes: products whose headspace is composed of a simple, two-to-four-component mixture of plasticizer-related compounds, which we define as the “plasticizer-based” class; and products that give off a complex suite of chemicals, largely normal and branched alkanes, cycloalkanes, and alkyl benzenes, which we define as the “hydrocarbon-based”

Table 4.1. Table of all commercial tire treatments evaluated in this study, with the major components identified via GC-MS and FTIR highlighted. FTIR match scores are reported in parenthesis.

Tire Treatment	GC-MS Major Compounds	FTIR Library Hits
(A) Tire Tac Blue Tire Wash	1-Propoxy-2-propanol 2-Ethyl-1-hexanol	Unidentified
(B) Tire Tac Tire Tuff	2-Ethyl-1-hexanol Benzoic acid 2-ethylhexyl ester	Unidentified
(C) Tire Tac GK1	Butanedioic acid dimethyl ester Pentanedioic acid dimethyl ester Hexanedioic acid dimethyl ester Benzoic acid 2-ethylhexyl ester	Hexanedioic acid diester (0.831)
(D) Tire Tac STS5 Purple	Butanedioic acid dimethyl ester Pentanedioic acid dimethyl ester Hexanedioic acid dimethyl ester	Hexanedioic acid diester (0.865)
(E) Venom Juice Red Hot	Hexanedioic acid dimethyl ester Pentanedioic acid dimethyl ester Benzoic acid 2-ethylhexyl ester 2-Ethyl-1-hexanol	Hexanedioic acid diester (0.863)
(F) Quick Lap Undetectable	Nonane Decane Undecane Various other hydrocarbons	Bis(2-ethylhexyl) phthalate (0.980)
(G) Hot Lap Undetectable	Nonane Decane Undecane Various other hydrocarbons	Bis(2-ethylhexyl) phthalate (0.995)
(H) Tire Tac PRW	Pentanedioic acid diethyl ester 1-Phenoxy-2-propanol Benzoic acid 2-ethylhexyl ester Various other hydrocarbons	Unidentified
(I) Tire Tac SAA	Benzoic acid methyl ester Pentanedioic acid diethyl ester 1-Phenoxy-2-propanol Various other hydrocarbons	Benzoic acid ester (0.872) Butyl benzoate (0.849)
(J) Pro-Blend Motorsports Tire Cleaner	2-Butoxyethanol Various other hydrocarbons	Diethylene glycol ether (0.779)
(K) Tire Tac BTGP Red	Naphthalene Various other hydrocarbons	Methyl oleate (0.995)

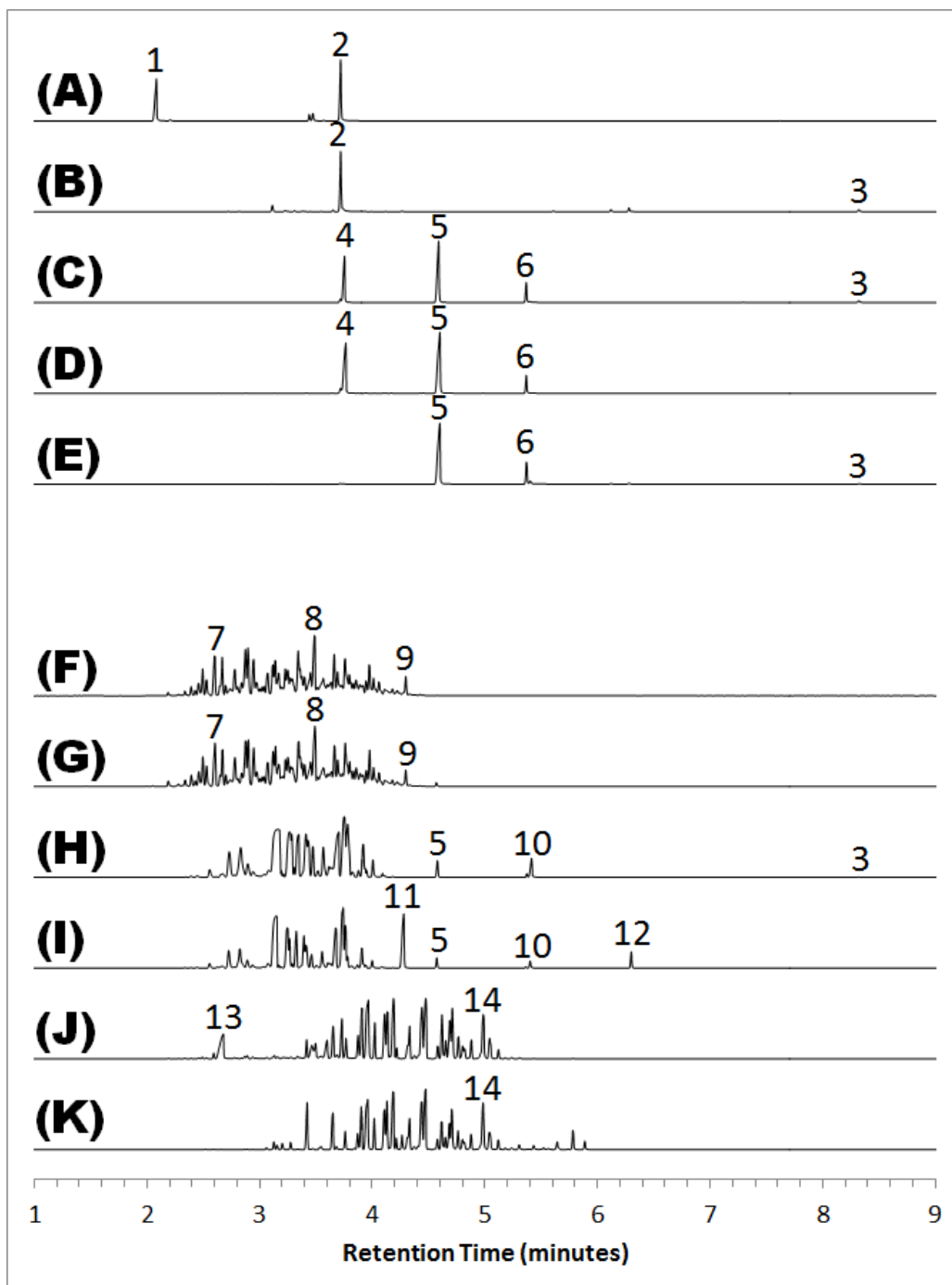


Figure 4.1. Chromatograms for tire treatments listed in Table 4.1. The major library hits are 1: 1-propoxy-2-propanol, 2: 2-ethyl-1-hexanol, 3: benzoic acid 2-ethylhexyl ester, 4: butanedioic acid dimethyl ester, 5: pentanedioic acid dimethyl ester, 6: hexanedioic acid dimethyl ester, 7: nonane, 8: decane, 9: undecane, 10: 1-phenoxy-2-propanol, 11: benzoic acid methyl ester, 12: butyl benzoate, 13: 2-butoxyethanol, and 14: naphthalene.

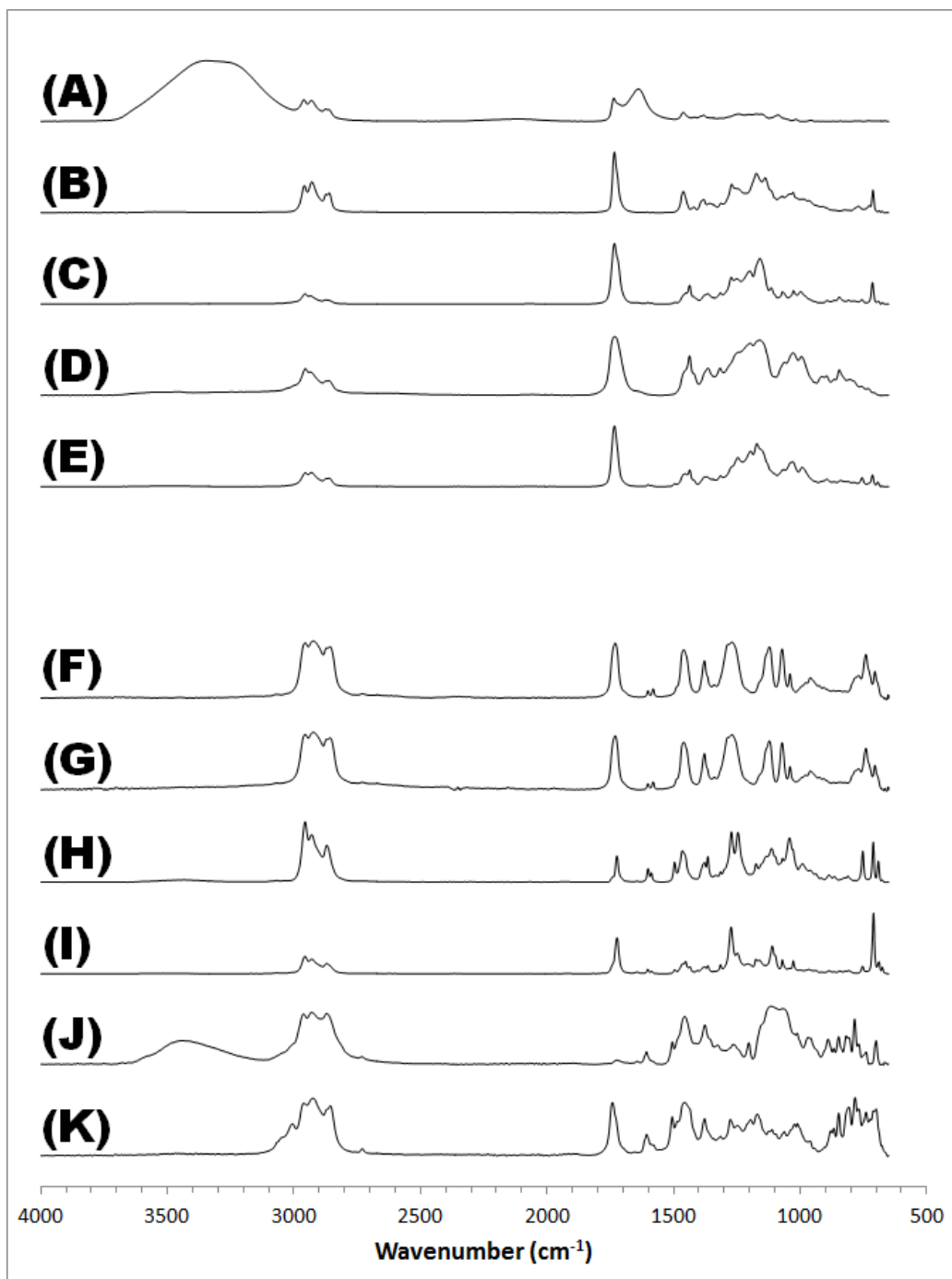


Figure 4.2. FTIR spectra for tire treatments listed in Table 4.1.

class. These classes have been separated on Figure 4.1 and Figure 4.2 to highlight the intrinsic differences between them.

Characteristic compounds for the plasticizer-based tire treatments include butane-, pentane-, and hexanedioic acid diethyl ester. These bear structural similarity to a number of widely-used plasticizers, such as dioctyl adipate and bis(2-ethylhexyl) adipate. 2E1H also registers among the most telltale compounds, and is a known hydrolysis product of the plasticizers bis(2-ethylhexyl) phthalate, bis(2-ethylhexyl) adipate, bis(2-ethylhexyl) sebacate [38, 55, 60, 61]. In a number of products, the 2-ethylhexyl moiety is present in the form of benzoic acid 2-ethylhexyl ester, suggesting a possible source of origin.

The hydrocarbon-based products are more difficult to characterize, for they run the gamut of simple organic molecules, from normal and branched alkanes to alkenes, mono-, di-, tri-, and tert-substituted cycloalkanes, alkylbenzenes, and naphthenics. There appears to be no uniformity among them, aside from their general aspect as an unresolved envelope of peaks. In some formulations, the aliphatics and aromatics are accompanied by a selection of plasticizer-related compounds, such as pentanedioic acid diethyl ester and benzoic acid 2-ethylhexyl ester in Tire Treatment H.

FTIR analysis largely served to confirm the primary ingredients in each tire treatment, although not all treatments could be successfully characterized. The chemical differences between the different classes are manifest in the infrared spectra, with those falling under the plasticizer-based umbrella yielding a relatively simple fingerprint region, and those in the hydrocarbon-based category proving more complex.

Tire Treatments A and J were aqueous-based, as indicated by the hydroxyl peak present in both infrared spectra and the solvents' general reluctance to evaporate, while the rest were all purely organic.

4.3.2 Sensitivity Study

The results of the serial dilution study showed the method was quite sensitive to the presence of illicit tire treatments. Tire Treatment B was an arbitrarily-chosen exemplar from the plasticizer-based class, while Tire Treatment J was an arbitrarily-chosen exemplar from the hydrocarbon-based class. Calibration curves indicated that butanedioic acid diethyl ester could be detected in tires spiked with Tire Treatment B at effective amounts as low as 200 nL tire treatment per gram of tire rubber, while pentanedioic acid diethyl ester could be detected at 500 nL/g. The limits of detection for Tire Treatment J were similar, with nonane, decane, and undecane each detectable at 200 nL/g. Each compound's limit of quantitation is ~3.3 times higher than its respective detection limit, giving a rough quantitation limit range of 600–1600 nL/g.

4.3.3 Analysis of Questioned Tire Samples

Over the course of one year, over seventy tire samples were submitted for analysis. In that time, ten tires returned a positive result for the presence of illicit tire

treatments. We present three case studies highlighting the analysis of these tires and the conclusions that were drawn.

The results of the first case study are depicted in Figure 4.3. Six questioned tires were submitted for analysis along with a “track tire”—a tire provided by USAC that was run on the same racetrack and race day as the other exemplars, which functioned as a negative control. The track tire bore resemblance to three of the six questioned tires, indicating that those tires had not been treated. The remaining three questioned tires, however, were highly suspect. Signal for these samples was an order of magnitude greater than that of the negative control, and the chromatograms were populated with

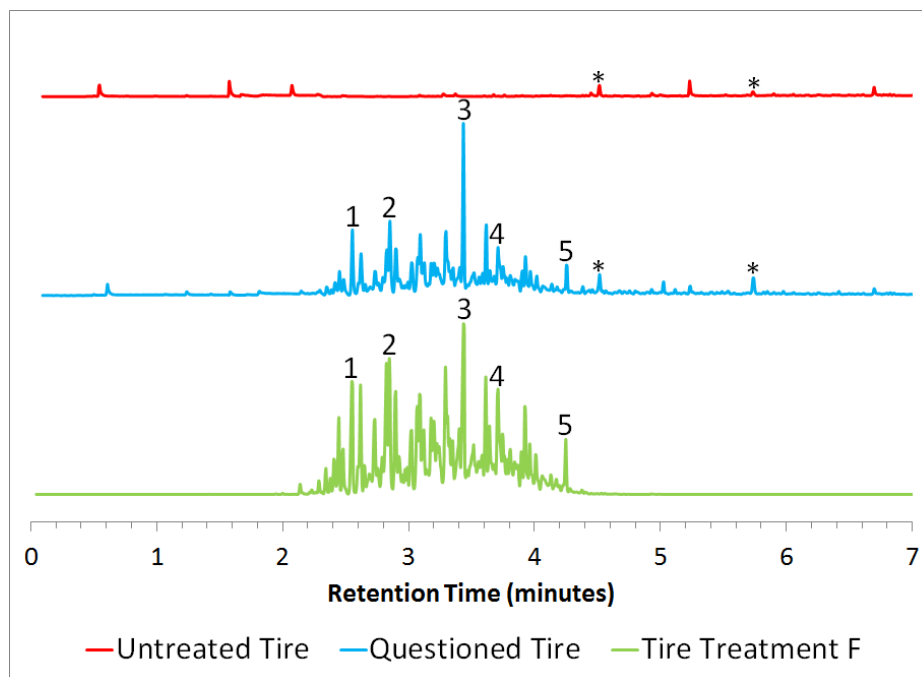


Figure 4.3. Chromatographic comparison between an untreated tire, a questioned tire, and a suspect tire treatment. The labeled peaks are 1: nonane, 2: propylcyclohexane, 3: decane, 4: butylcyclohexane, and 5: undecane.

an abundance of n-alkanes and cycloalkanes, including nonane, decane, undecane, propylcyclohexane, and butylcyclohexane. Furthermore, all three of the suspect tires were chromatographically indistinguishable from one another. Comparison to known commercial products revealed the tires were likely treated with a product like Tire Treatment F, whose distribution of chromatographic peaks was highly consistent.

Another case study is presented in Figure 4.4. Here, the questioned tire displayed a broad envelope of largely aromatic molecules. For the most part, these took the form of mono-, di-, tri-, and tetra-methylated benzene compounds, whose mass spectra were so similar to one another as to impede a conclusive library match. Also identified were the unconventional compounds 2-butoxyethanol and dipropylene glycol monomethyl ether, as well as decane, undecane, and naphthalene. Once again, the chromatogram had little in common with the accompanying negative control that had been submitted, but bore a great deal of resemblance to a known tire treatment.

In this case, Treatment J was found to contain many of the same compounds as the questioned tire, with a highly-similar distribution of peaks. However, several key differences are of note. Nonane is present in the questioned tire, but is absent in the treatment. Likewise, a trio of high-boiling naphthalene-related peaks located between 5.6 and 5.9 minutes are nowhere to be found in the chromatogram for the tire treatment product. Perhaps most telling of all, pentanedioic acid dimethyl ester and hexanedioic acid dimethyl ester (but not butanedioic acid dimethyl ester) occurred in the questioned tire chromatogram. The presence of these additional compounds suggests the possibility that a combination of treatment products may have been

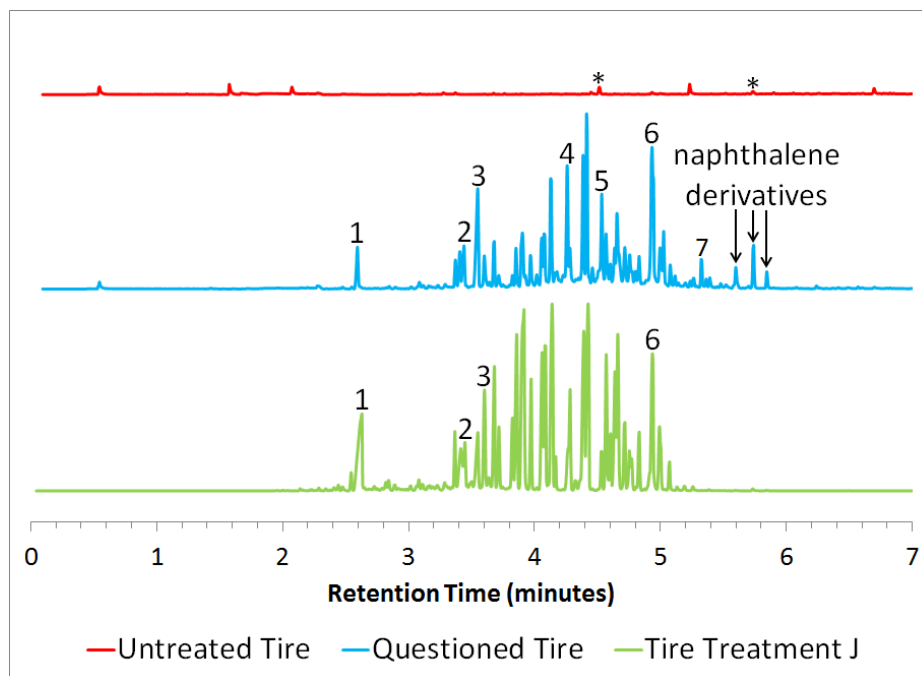


Figure 4.4. Chromatographic comparison between an untreated tire, a questioned tire, and a suspect tire treatment. The labeled peaks are 1: 2-butoxyethanol, 2: decane, 3: dipropylene glycol monomethyl ether, 4: undecane, 5: pentanedioic acid dimethyl ester, 6: naphthalene, and 7: hexanedioic acid dimethyl ester.

applied to the tire, with Tire Treatment J forming the principal residue left behind, along with small traces of another unknown product. Alternatively, a singular treatment may have been used, highly similar to Tire Treatment J in chemical composition, but also incorporating nonane and the assorted naphthalene- and plasticizer-related compounds in its formulation.

In cases where a tire treatment is suspected, important information can occasionally be gleaned by evaluating cuttings from the interior and exterior of a questioned tire independent of one another. A longitudinal bisection of the rubber yields two halves, each of which is submitted to analysis by SPME-GC-MS. Figure 4.5

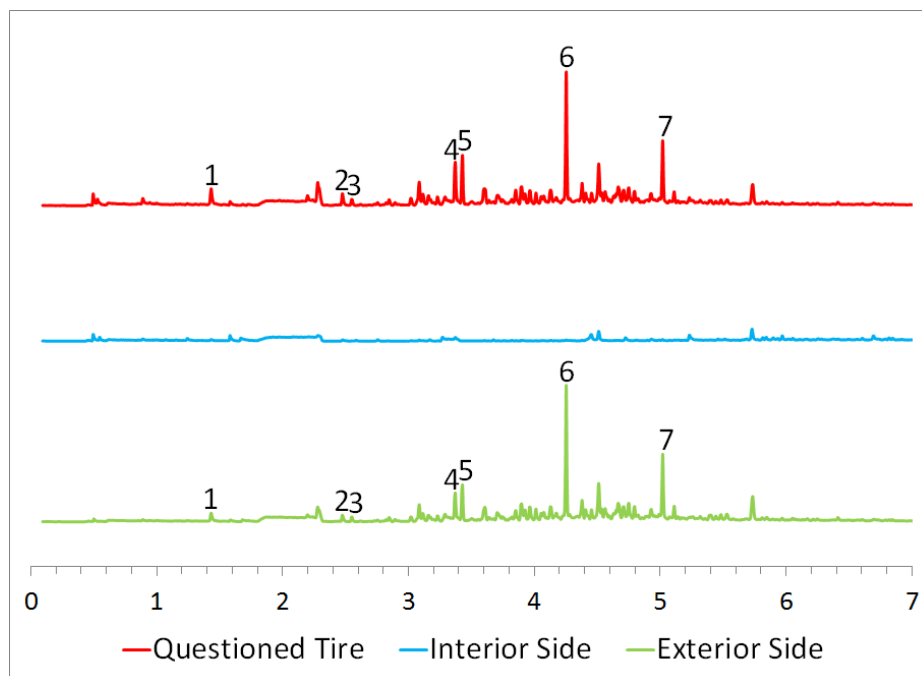


Figure 4.5. Chromatographic comparison between a questioned tire and the front and back sides of the same tire. The labeled peaks are 1: toluene, 2: meta and para xylene, 3: ortho xylene, 4: 1,2,4-trimethylbenzene, 5: decane, 6: undecane, and 7: dodecane.

portrays the results of such an experiment. It is clear from the data shown that the vast majority of the volatiles originate from the outer surface of the tire, not the inner surface. This type information can prove essential in concluding whether a product has been applied: many commercial formulations instruct the end user to coat the interior surface area of the tire with product while leaving the exterior untreated—presumably to avoid detection by race officials, who would not find it difficult to evaluate the easily-accessible outer surface by sight and by touch. A tire sample whose interior surface yields a plethora of volatile compounds in contrast to the exterior, then, is essentially a smoking gun: there is no conceivable way that the sealed interior of any tire could come to be tainted with suspicious volatile material while leaving the exterior unaffected,

except by deliberate action. Conversely, in the case shown in Figure 4.5, the tire interior is blank, whereas the exterior yields a chromatographic profile resembling a mixture of gasoline and medium petroleum distillate. This type of observation is inherently less conclusive, as it leaves open the possibility of contact cross-contamination between the tire and any illicit residue deposited on the racetrack. However, it may still be of use in pinning down a particular treatment product to a questioned tire: if the front side of a tire produces a chromatogram consistent with a “front-side application” product, then that is one more article of evidence in favor of a treatment product having been used.

Above all else, it is important to minimize the risk of false positives as much as possible. The chance of one car’s treated tires spreading their contamination to the wheels of other innocent parties via the racetrack is fully within the realm of possibility. Whenever a tire is evaluated and deemed to be suspicious, it should always be compared to a negative control tire, which has been provided by the race officials or a disinterested third party and raced upon the same track, on the same day as the questioned exemplars.

4.4 Conclusion

In summary, a novel technique was pioneered for the analysis of chemicals applied to automobile racing tires. This represents an innovative and important contribution, for while race events abound throughout the country and the world, published literature on the detection of illicit tire treatments is not at all widespread.

The racing circuits' demands for accountability require sensitive chemical determinations. To this end, further attention is warranted, and suitable methods must be developed.

SPME-GC-MS was found to offer a fast and simple way to evaluate tires for the presence of treatment products, with limits of detection around 200 nL/g. Despite the claims of some commercial entities that their formulations are undetectable, we found the reverse to be true. Products could not only be detected days after they had been applied to the tires, but characterized and discriminated as well. Furthermore, their chromatographic profiles allowed them to be categorized into two broad classes: 'plasticizer-based' tire treatments and 'hydrocarbon-based' tire treatments. Localization of a treatment product to a particular side is possible by bisecting the rubber and submitting each half to independent analysis.

CHAPTER 5. DEVELOPMENT OF A TOTAL VAPORIZATION SPME-GC-MS METHOD FOR THE ANALYSIS OF LIPIDS IN BLOWFLIES

5.1 Introduction

Though the analysis of Diptera and other insects has traditionally fallen within the purview of entomologists and biologists, there has been considerable effort in the chemistry sphere to evaluate these specimens by GC-MS, LC-MS, and similar analytical techniques. Efforts have generally sought to illuminate the chemical profiles of the various species under study, particularly with respect to the compounds that can be passively extracted from the biological matrix of the insect.

The cuticular lipids, hydrocarbons, and other compounds that can be isolated from flies have been of concern to various authors for a wide assortment of reasons. An interest in the biochemistry and physiology of flies has driven much of the research. For example, some scientists have sought a better understanding of the pheromones that drive sexual activity with an eye toward pest control applications [62], while others have devoted themselves to elucidating the antimicrobial and bacteriostatic effects of certain cuticular lipids [63]. Still others have made efforts to catalogue the insects' chemical profiles for chemotaxonomy purposes [64], or to develop new instrumental methods for age and species determination, which would be of tremendous use in forensic

investigations relying on entomological evidence, serving to facilitate better postmortem interval (PMI) estimates [65].

Although techniques exist for collecting the gaseous chemicals given off by the insects—for example, using a volatile trap [62]—the work-up has classically relied upon liquid extraction. Typically, the insect is immersed in an apolar solvent for some length of time, allowing the cuticular and internal lipids to be extracted. These extracts may be derivitized to help improve sensitivity and performance during the subsequent separation steps [65, 66]. Inevitably, one or more rounds of chromatography follows: gas chromatography [63-65, 67-72], liquid chromatography [63, 67-72], and thin layer chromatography [64] are among the various techniques that have been described. This general procedure has been applied to the analysis of pupae [63, 68-70] and puparia [64, 65, 73] alike, and to the analysis of single specimens [63, 65, 68] as well as specimens in bulk [64, 73].

SPME has a published record of use for the determination of insect-related compounds. However, these studies have typically involved the examination of insects other than blowflies [74-79]. Moreover, although there has been published research on the analysis of blowflies by SPME, this research has either relied on direct contact between the insect and the fiber to enable the contact transfer of cuticular lipids [80], or it has relied on the headspace analysis of live samples in bulk [81].

The purpose of this research was to develop a new method for the analysis of *Phormia regina* pupae by SPME-GC-MS. Initial experiments sought to evaluate the VOC's emitted by pupae using headspace solid phase microextraction (HS-SPME) at elevated

temperatures. When this proved unsuccessful, attention turned to the development of a new method for the liquid extraction of pupae in order to isolate the cuticular lipids, followed by the analysis of those liquid extracts by total vaporization solid phase microextraction (TV-SPME). A trimethylsilyl derivitization was also carried out inside the sample vial immediately prior to GC-MS analysis, offering a potential advantage to future analysts seeking to analyze blowfly pupae.

5.2 Materials and Methods

A 6890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent, Santa Clara, CA, USA) served as the principal instrumentation, with autosampler functionality provided by an MPS2 (Gerstel, Mülheim an der Ruhr, Germany). The column was a J&W DB-5ms (30 m × 0.25 mm × 0.25 μm). All GC-MS analyses utilized H₂ carrier gas with a flow rate of 2.5 mL/min operated in splitless mode, with a scan range of *m/z* 40-550.

5.2.1 Rearing of Fly Colonies

A colony of F10 *Phormia regina* blowflies was nurtured on a diet of sugar in an environment maintained at 25 °C and 60 % humidity. Chicken liver was provided as the egg-laying substrate. After oviposition occurred and one day was allotted for them to hatch, the first instar maggots were counted, and 100 were transferred into a Dixie cup containing 50 g chicken liver, which was in turn placed inside of a glass mason jar half-

filled with sawdust. The jar was incubated at 25 °C and 60 % relative humidity, with a 12:12 light:dark cycle. At three days after pupation, all specimens were collected and frozen at -80°C. For each experiment, pupae were given at least 30 minutes to thaw in an empty vial prior to HS-SPME sampling or liquid extraction.

5.2.2 HS-SPME Experiments

Initial experiments sought to analyze the VOC's off-gassed by pupae of the species *Phormia regina* by HS-SPME. A single pupa was placed in a 20 mL autosampler vial and extracted at 70 °C for 45 min. Following extraction, the fiber was transferred to the heated injection port of the GC-MS. The oven had an initial temperature of 40 °C held for 1 min, a ramp of 20 °C/min, and a final temperature of 300 °C held for 1 min. The same experiment was repeated using two different types of SPME fiber: a PDMS/DVB and a PDMS/CAR. Following initial attempts using this method, a wash step was added where the pupa was sonicated for 15 min in deionized water and dried prior to being placed in the autosampler vial.

5.2.3 TV-SPME Solvent Study

After the initial HS-SPME experiments proved ineffective, the research focus shifted to the development of a TV-SPME method for the analysis of fly pupa liquid extracts. A single pupa was placed into 1 mL of each of four different solvents: ethanol,

acetone, dichloromethane, and pentane. One day was provided for the lipids and other compounds of interest to partition into the liquid phase, whereafter an aliquot was taken from each extract solution corresponding to the amount required to totally saturate the interior of a 20 mL vial at a SPME extraction temperature of 90°C: 46 µL of the ethanol solution, 58 µL of the acetone solution, 195 µL of the dichloromethane solution, and 358 µL of the pentane solution. These values were calculated using Equation 1.23.

Each aliquot was analyzed by SPME-GC-MS. The SPME extraction time was 30 min. The extraction temperature was 90°C. The desorption time was 1 min. The oven had an initial temperature of 40 °C held for 1 min, a ramp of 20°C/min, and a final temperature of 300 °C held for 1 min. The same experiment was repeated using three different types of SPME fiber: a PDMS, a PDMS/DVB, and a PEG. A moderate desorption temperature of 240 °C was selected, which falls within the operating guidelines for all three fiber chemistries.

5.2.4 Liquid Injection Studies: Liquid Extraction Time, Silylation, and Sonication/Heating

Experiments continued, focusing on pentane as the choice solvent. A 50 ppm internal standard solution of decanoic acid in pentane was prepared by dissolving 50 mg of decanoic acid in 50 mL pentane and diluting by 1000 : 1. Five pupae were placed into 10 mL of the solution. Aliquots of 300 µL were taken at 1 h, 2 h, 3 h, 4 h, 1 d, 2 d, 3 d, 4 d,

5 d, 6 d, and 7 d. These aliquots were spiked with 10 μ L BSTFA w/ 1 % TMCS silylation reagent, vortexed for 10 s, and submitted to analysis by liquid injection GC-MS. The injection volume was 2 μ L. The inlet temperature was 250°C. The oven had an initial temperature of 100 °C held for 1 min, a ramp of 20°C/min, and a final temperature of 300 °C held for 1 min.

Simultaneous experiments were carried out to ascertain whether any improvement in liquid extraction efficiency could be obtained by heating or sonicating the pupa extract solutions. To that end, the above experiment was repeated two additional times: once, while keeping the extract solution under ultrasonication for the first 4 h, and a second time, while keeping the extract solution on a hot plate at a temperature of 60 °C for the first 4 h. Sampling occurred at the same intervals as mentioned previously.

5.2.5 TV-SPME vs. Liquid Injection Study

Five pupae were again placed in 10 mL pentane. At 4d, 250 μ L was sampled and transferred into an autosampler vial with a 300 μ L conical insert. Simultaneously, another 250 μ L was sampled and transferred into a 20 mL glass SPME vial. Both aliquots were silylated using 10 μ L BSTFA w/ 1 % TMCS. All samples were then analyzed by liquid injection GC-MS and SPME GC-MS, respectively. The inlet was set at 250°C. The oven had an initial temperature of 100 °C held for 1 min, a ramp of 20°C/min, and a final temperature of 300 °C held for 1 min. For all liquid injection experiments, the injection

volume was 2 μ L. For all SPME experiments, the fiber was PDMS, with an extraction time of 15 m and an extraction temperature of 90°C.

5.3 Results and Discussion

5.3.1 HS-SPME Experiments

On the surface, HS-SPME would seem to be the ideal method for analyzing pupa specimens, owing to the intrinsic simplicity and lack of sample preparation associated with the technique. However, our experiences did not bear out our initial assumptions regarding the suitability of the method. Far from delivering the sensitivity we expected, HS-SPME proved decidedly insensitive to the VOC's of the pupa. Furthermore, instead of yielding a host of cuticular hydrocarbons and other biological compounds, the principal compounds observed in the chromatograms appear to be substrate-associated, originating not with the pupa, but with the sawdust wherein pupation occurred. Figure 5.1 depicts the sort of data that HS-SPME generated, with what few relevant peaks there are overwhelmed by substrate contaminants such as alpha-terpineol and nerolidol. It should be noted that Fredericx et al. reported good results on the collection and assay of VOC's from pupae by PDMS/CAR SPME, albeit looking at *Calliphora vicina* instead of *Phormia regina*, providing the larvae with pig meat instead of chicken liver, and utilizing

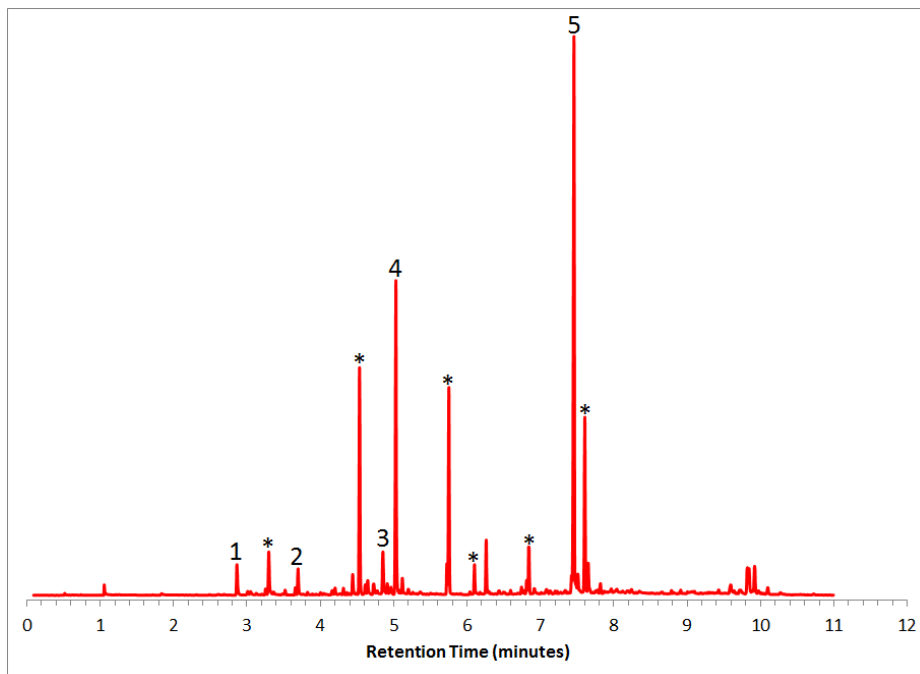


Figure 5.1. Results of HS-SPME for *Phormia regina* pupa extracted at 70 °C for 45 min. The labeled compounds are 1: alpha-pinene, 2: limonene, 3: endo-borneol, 4: alpha-terpineol, and 5: nerolidol. All are believed to originate from the pupation substrate.

vermiculite substrate instead of sawdust [82]. However, perhaps owing to differences in the experimental design, we were unable to replicate these results.

Follow-up attempts at HS-SPME sought to incorporate a wash step immediately prior to the analysis, aiming to rinse away the contaminant compounds by sonication in deionized water. However, this could not make up for the method's poor sensitivity toward the biological compounds at interest to the project. Following these difficulties, HS-SPME was dismissed as a viable option, and attention turned to the development of a new method by TV-SPME.

5.3.2 TV-SPME Solvent Study

TV-SPME work began with a series of experiments designed to establish the optimal combination of solvent and fiber for the analysis of pupa liquid extracts. Four candidate solvents were selected for evaluation: pentane, ethanol, dichloromethane, and acetone. Many of the existing methods for the extraction of biological compounds from insects utilize short-chain aliphatic solvents for the liquid phase, most notably hexane and petroleum ether [63, 73, 83-85]; pentane, whose chemical properties are similar, served as the choice non-polar solvent in this research. Dichloromethane is another solvent that has previously been cited for the extraction of biological compounds from pupae [63, 67, 69-72, 86]. Ethanol is the solvent most commonly employed for the preservation and long-term storage of entomological specimens collected from crime scenes [87], and for this reason, it was included as a solvent of interest. Acetone has not previously been published on as an insect extraction solvent, but was included in this study as a solvent of intermediate polarity between dichloromethane and ethanol.

Table 5.1 conveys the results of this study. The experiments showed pentane was the solvent best-suited to the liquid extraction of biological compounds from pupae, while PDMS proved superior to all other fiber chemistries.

Table 5.1. TV-SPME Solvent Study results. The number of detectable compounds (peaks present in each chromatogram) is listed for each combination of extraction solvent and SPME fiber.

	PDMS	PDMS/DVB	PEG
Ethanol	8 compounds	10 compounds	0 compounds
Acetone	17 compounds	18 compounds	32 compounds
DCM	14 compounds	12 compounds	5 compounds
Pentane	63 compounds	44 compounds	40 compounds

5.3.3 Liquid Injection Studies: Liquid Extraction Time, Silylation, and Sonication/Heating

With the extraction solvent and SPME fiber decided upon, additional work was carried out in liquid injection mode to further characterize and optimize the system preparatory to the final SPME vs. liquid injection comparison.

First and foremost, a liquid extraction time study was carried out to assess how thoroughly the lipids of the pupa partitioned into the pentane solvent as a function of time. This study was paired with two additional experiments, wherein identical liquid extraction solutions were either sonicated or heated for the first four hours to see whether the pupal lipids might be encouraged into the liquid phase under more rigorous conditions. Selected peak area ratios for palmitic acid, oleic acid, arachidonic acid, and cholesterol are plotted against the internal standard as a function of extraction time in

Figures 5.2–5.5. The trend was generally linear, except in the case of cholesterol, which was typified by an unexplained extraction maxima at $t=96$ hours. The data obtained did not evince any significant difference in extraction efficiency between those samples which were heated or sonicated and those which underwent simple extraction under ambient conditions; in all cases, the simple extraction was found to yield comparable results to those samples for which extra steps had been taken.

The inclusion of a derivitization step is highly desirable, as the conversion of the free fatty acids from pupae into their silylated analogues not only reduces band broadening and increases chromatographic efficiency, it also increases the overall volatility of these analytes, making them more susceptible to vaporization and collection on the surface of a SPME fiber, improving the overall sensitivity of the TV-SPME analysis. To this end, derivitization using BSTFA w/ 1 % TMCS was carried out during the course of these experiments. It was found that a complete stoichiometric conversion of all free fatty acids to their silylated counterparts could be accomplished “in-the-pot” simply by spiking a liquid aliquot with $1/30^{\text{th}}$ its volume of silylation reagent. The reaction is immediate and goes quickly to completion under ambient conditions, requiring only a brief vortex step to ensure mixing. As many of the previous published methods have generally relied on the use of concentrated acids or rigorous work-ups (as in the case of Frere et al., wherein the derivitization is accomplished by a transesterification reaction in the presence of concentrated sulfuric acid [65]; or in the manner of Folch et al., where the derivitization is achieved by lengthy sonication in a methanol : chloroform

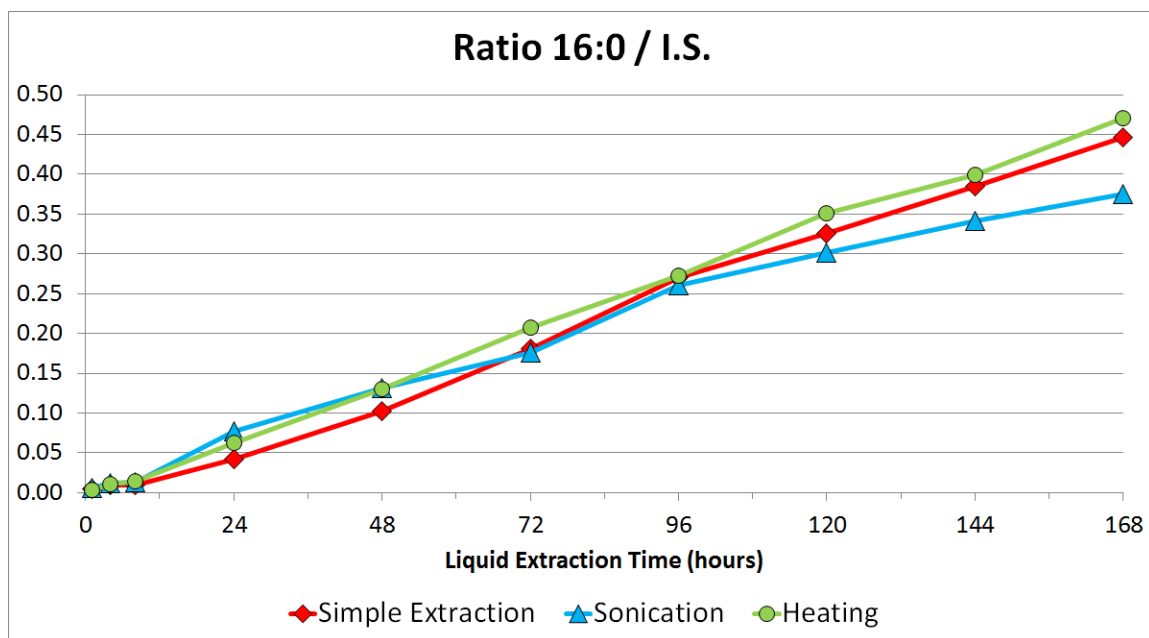


Figure 5.2. Peak area ratios for palmitic acid (16:0) divided by the undecanoic acid (11:0) internal standard.

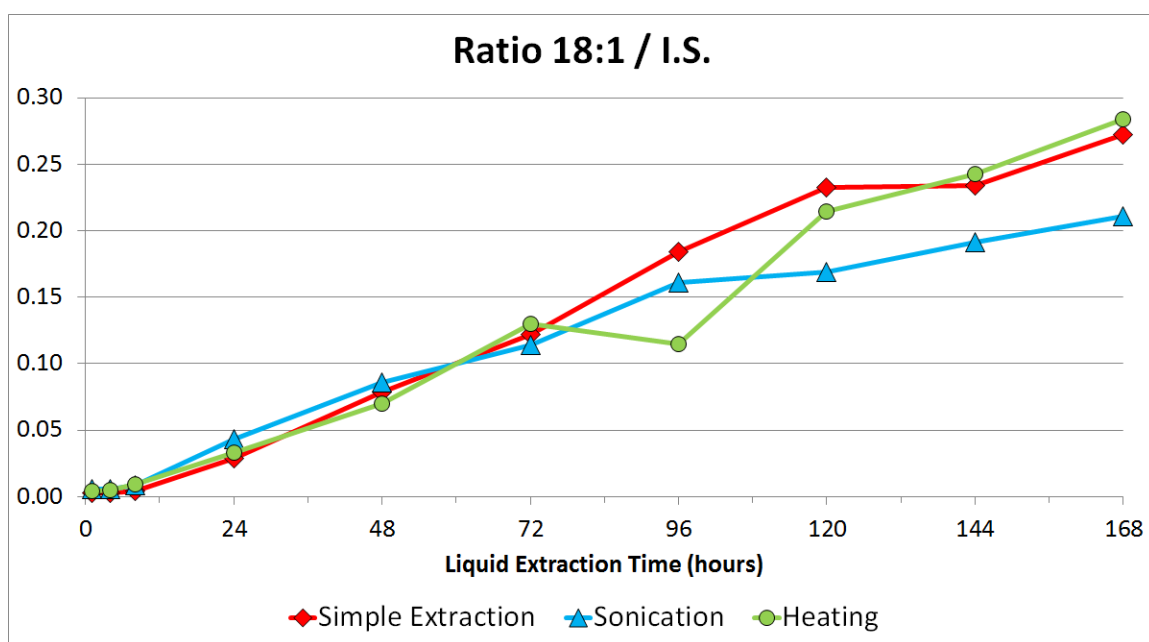


Figure 5.3. Peak area ratios for oleic acid (18:1) divided by the undecanoic acid (11:0) internal standard.

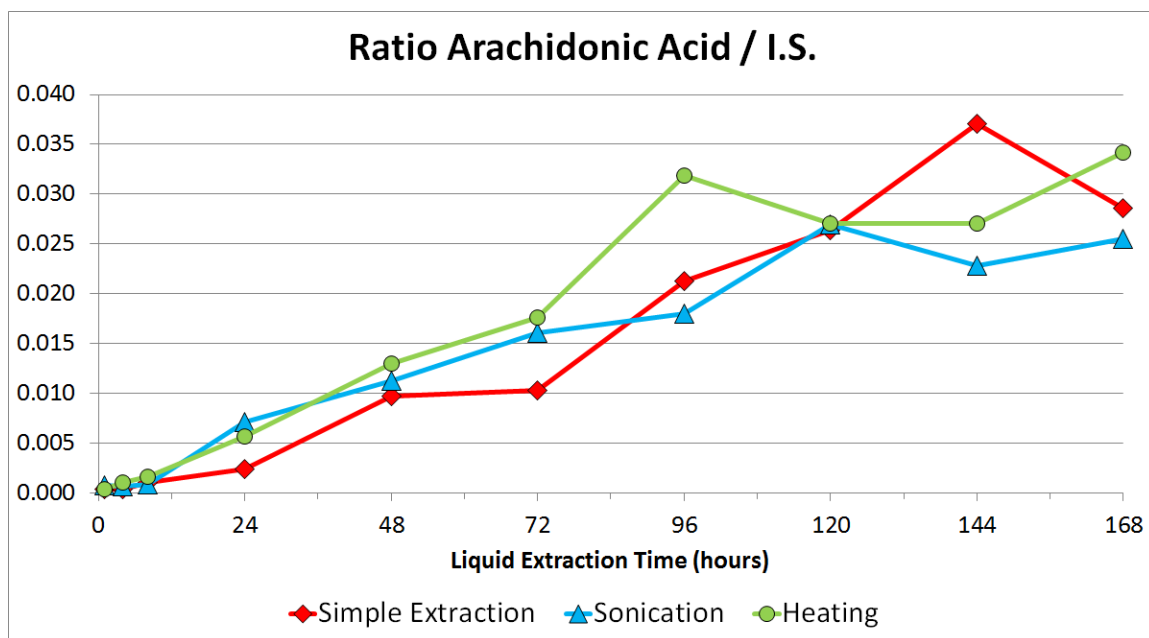


Figure 5.4. Peak area ratios for palmitic acid (16:0) divided by the undecanoic acid (11:0) internal standard.

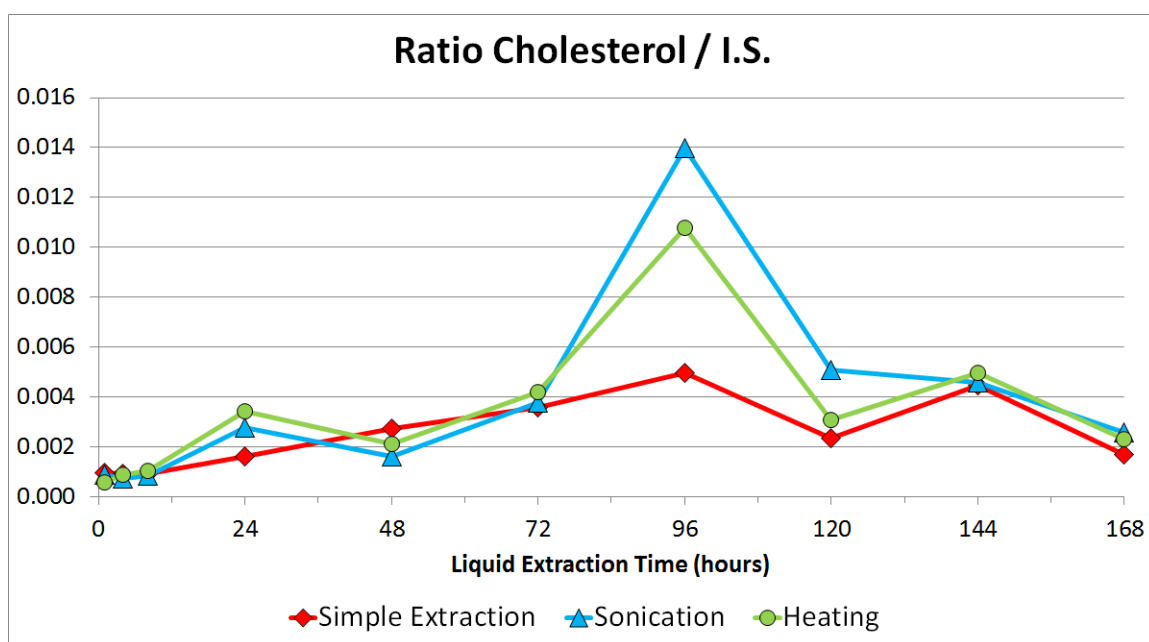


Figure 5.5. Peak area ratios for cholesterol divided by the undecanoic acid (11:0) internal standard.

solution maintained in a constant ice bath [88]), the method published here may represent a significant improvement over alternatives.

5.3.4 TV-SPME vs. Liquid Injection Study

In a separate study by Bors et al., TV-SPME was found to improve sensitivity by an order of magnitude over liquid injection [24]. The practical result of this disparity is depicted in Figure 5.6. Note that the distribution of lipids is comparable with what has been previously reported by Gołębowski et al. [63], and appears to offer an advantageous improvement in sensitivity over the method employed by Frere et al. [65].

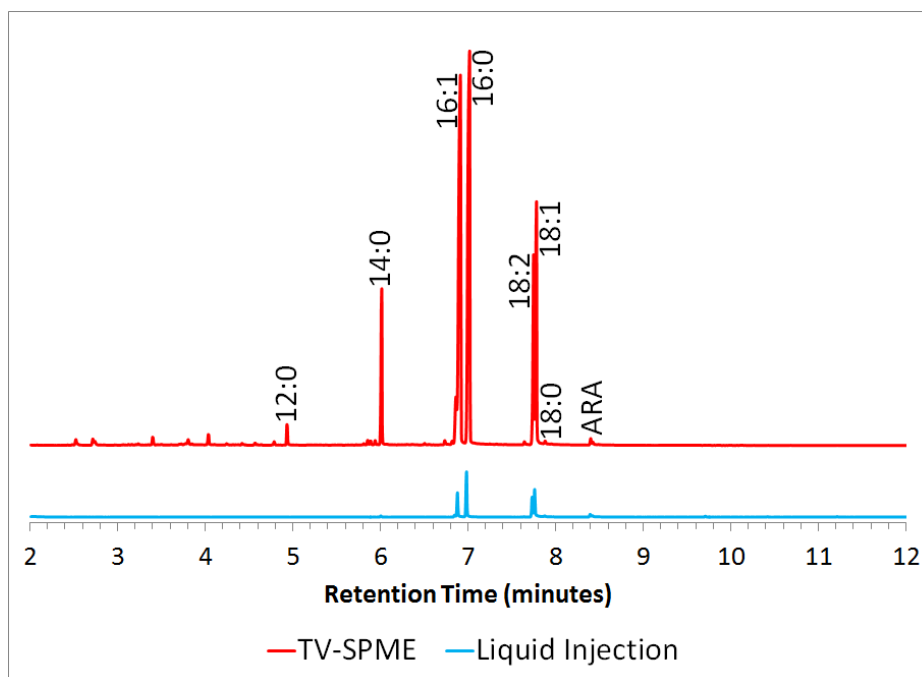


Figure 5.6. Comparison between TV-SPME and liquid injection chromatograms (non-normalized). The major fatty acids have been labeled.

Also note that these chromatograms should not be taken at face value: for both TV-SPME and the liquid injection, there are an abundance of compounds scattered amid the dominant fatty acid peaks which are simply not visible to the naked eye at this scale. The figure is provided for the sake of comparison and should not be presumed to represent the totality of the information that can be garnered from either method.

Administering the sample to the GC via TV-SPME yielded a 10-to-30-fold increase in peak area, in most cases; for myristic acid (14:0), the improvement was as high as 80-fold. The same trend was observed over multiple replicates. The limit of detection was experimentally determined to be approximately 50 ppb.

5.4 Conclusion

Attempts to analyze the VOC's of *Phormia regina* pupae via HS-SPME were unsuccessful. Not only was the method afflicted by unwanted compounds found to originate from the substrate, it was further compromised by a lack of sensitivity to any and all cuticular lipids, even at elevated temperatures. It is our experience that HS-SPME is ill-suited to the analysis of pupae without any workup. In order for any useful chemical information to be gleaned, the analysis must be preceded by some type of extraction in order to separate the fatty acids, hydrocarbons, sterols, and other compounds of interest from the matrix.

In spite of these difficulties, a new method was developed for the analysis of pupa liquid extracts by TV-SPME. The method offers at least an order of magnitude

improvement in sensitivity over traditional liquid injection techniques, which may potentially alleviate the need for rotary evaporation, reconstitution, collection of HPLC fractions, and many of the other pre-concentration steps which are commonplace in the current literature. Furthermore, the ability to derivitize the liquid extract in a single easy step carried out within the sample vial also represents a potential improvement, and an alternative to the many arduous derivitization reactions that are currently in use, serving to expedite analysis times and minimize the loss of sample due to imperfect extraction efficiencies.

CHAPTER 6. DIFFERENTIATING THE AGE AND SPECIES OF BLOWFLY PUPAE BY CHROMATOGRAPHIC PROFILES AND MULTIVARIATE STATISTICS

6.1 Introduction

Forensic entomology is the practice of using insects within a legal setting [89]. Frequently, in cases of homicide, suicide, and accidental death, a body is discovered, and so much time has elapsed that it is no longer possible to use physiology-based medical techniques for the determination of the time since death. Fortunately, insects recovered from the body can be used as a “clock” to determine the minimum postmortem interval (PMI_{MIN}), or the time since the body has been available to the insects, as the majority of the species in North America will only lay eggs or larvae on a resource if it is dead [90, 91].

The life cycle of the most common flies, the blowflies (Diptera: Calliphoridae), is as follows: a female fly will lay between 100–300 eggs on the remains [92], the eggs will hatch, and the larvae will begin the consumption of the soft, necrotic tissues. The larvae will molt two additional times in a temperature-dependent manner, then leave the remains in order to find a dry, safe place for pupation. This is often the soil immediately surrounding the remains. Upon the completion of metamorphosis, adults emerge from

puparia, and the cycle begins again. Typically, the remains are not present in the environment for a long enough period for a second generation of blowflies to colonize the remains, or the condition of the remains changes (e.g. there no longer is any soft tissue remaining). The species of the collected specimens is determined, and some measure of their age—usually the length of the larvae or the life cycle stage of the insects' development—is used to extrapolate PMI_{MIN} , referencing published developmental data (e.g. [93-98]).

In order to estimate the PMI_{MIN} , investigators collect the oldest insect specimens present at the crime scene, and in some cases, these are pupae. Due to their lack of morphological distinctiveness, it is often difficult, if not impossible, to identify a pupa's species, even for a trained forensic entomologist. Therefore, pupae are typically placed in an environmental chamber at known temperature and allowed to complete their development. Once they are adults, the species can be determined using published morphological keys [99]. Two shortcomings of this technique are the additional time needed for the pupae to complete development, as well as the assumption that the pupae are alive. Any damaged or dead pupae will never be identified by this method, and few crime laboratories have the resources available to rear flies. An alternative is to extract the DNA from the pupae and determine the species by genetic markers [100-106], but once again, there are considerable drawbacks. A high monetary cost and level of expertise are necessary for the analysis of the DNA sequence, and a thorough representation of all possible species is required [107].

Determining the age of a pupa is also essential for establishing the PMI_{MIN} , and once again, such estimations are often steeped in difficulties. One method for gauging a pupa's age relies on quantitative polymerase chain reaction (qPCR) of select developmental genes [108, 109]. However, this requires a well-preserved, high-quality sample with intact mRNA. Although nucleic acid-based techniques are still evolving, a viable and cost-effective means of identifying pupae remains elusive.

Publicly-employed forensic entomologists are basically nonexistent, even in the largest and most well-funded municipal, state, and federal laboratories, presenting a problem for smaller crime labs wishing to make use of insect evidence. This dilemma is compounded by the innate complexity of the insects themselves, whose life cycles can vary due to differences in genetics [110-113], sex [114], and diet [96]. Furthermore, it is important to gauge the accuracy of entomological determinations in a statistically-verifiable way, with proper attention paid to misidentification and error rates.

In light of these issues, experts in the field of forensic entomology have suggested chemical analyses to complement, or substitute for, traditional morphological examinations [90]. Such chemical analyses would be easily conducted on instruments common to all forensic laboratories, alleviating the burden on smaller labs. They would also be more objective, subject to empirical scrutiny, and able to report on their own propensity for error.

Several possible methods of analysis have been proposed in recent years, but the idea that has shown the most promise relies upon the analysis of the organic compounds that can be extracted from the insect cuticle [90]. The cuticle is the

outermost protective layer of insects, analogous to the skin in humans , and associated with the cuticle are a multitude of chemicals—hydrocarbons, free fatty acids, alcohols, aldehydes, wax esters, and fatty acid methyl esters [115]—which serve a variety of purposes with respect to the insect’s physiology and life cycle.

The production of simple alkane and alkene cuticular hydrocarbons occurs by elongation of fatty acyl-CoAs, with the number and location of double bonds determined by enzymes, such as desaturases, which are taxonomically unique [116]. Thus, while all insects have cuticular hydrocarbons, those hydrocarbons can often be individualized to species—or, even further, to the sex of a species, to a part of the life cycle, or to a caste [116]. The evolution of such compounds, which include long-chain hydrocarbons and hydrophobic moieties, appears to have originated from the need, by insects, to retain water within their bodies and prevent desiccation [85]. This knowledge is supported by the observation that species of *Drosophila* found in arid environments have a complement of hydrocarbons whose chains are longer than those in more temperate climates [117, 118]. In addition to this water retention function, the cuticular compounds serve to protect the insect in several other ways—notably, by providing a barrier to infiltration by insecticides and toxins [119], as well as fungi and bacteria [115]. The hydrocarbons of the cuticle can also convey information via chemical signaling. When one insect encounters another, the cuticular hydrocarbon profile may signal any or all of the following: (i) that the individual is a member of the same species [116]; (ii) of the same colony; (iii) male or female [120]; (iv) caste membership [121]; (v) whether the individual is a close family relation, or a dominant member of the colony attempting

to pass along an order, or an inferior member standing by to take a command [122]; and (vi) whether the other insect is fertile and ready to mate, or has already copulated [81]. Flies are able to detect cuticular hydrocarbons via the olfactory organs of the maxillary palps and antennae, or by taste, via the organs of the proboscis [118].

Likewise, a constellation of fatty acids and sterols have been isolated from flies, as described by Golebiowski et al. [63, 70, 72]. Free fatty acids ranging between hexanoic acid (6:0) and hexacosanoic acid (26:0) have been described, although many of these are at trace levels in comparison to the dominant palmitic acid, palmitoleic acid, oleic acid, linoleic acid, and stearic acid contributions [63]. Similar to the hydrocarbons, these lipids are known to provide resistance to desiccation, fungal infection, and bacterial infection. A number of sterols have also been reported, including cholesterol, campersterol, and sitosterol, although cholesterol appears to be the most consistently dominant [70].

As there appear to be qualitative and quantitative differences in the occurrence of these compounds among the various different species, the chemical profiles may be useful as a tool for identification and discrimination. Solvent extraction has been the traditional method for the analysis of insect cuticular compounds [85, 115, 123-125]. The process is straightforward: the insect is placed into a nonpolar solvent, such as methylene chloride, ether, pentane, or hexane, during which time the biological compounds leach into the liquid. Those compounds may then be concentrated and analyzed by GC-MS or by high performance liquid chromatography (HPLC).

Perhaps of greatest relevance to this research is the work of Frederickx et al., who conducted SPME experiments on fly larvae and pupae of a single species (*Calliphora vicina*) with an eye toward forensic applications. In this case, the authors utilized a PDMS/DVB/CAR SPME fiber to collect hydrocarbons, fatty acids, and sterols from larvae, and a PDMS/CAR SPME fiber to do the same for pupae. The compounds observed at time points throughout the larval and pupal stages of development were documented. The presence and absence of the compounds was noted and this qualitative data was analyzed by agglomerative hierarchical clustering (AHC). The results in this case indicated that the chemical profiles of larvae and pupae were dissimilar, as were the profiles of pupae of differing ages [82].

The purpose of this project was to interrogate the chemical profiles that could be obtained from pupae by liquid extraction followed by TV-SPME. Four species of blowfly were investigated: *Cochliomyia macellaria*, *Lucilia cuprina*, *Lucilia sericata*, and *Phormia regina*. Specimens were collected at various timepoints for the F1 and F10 generations. After these samples were analyzed by GC-MS, the peak area ratios for selected compounds of interest were scrutinized by principal components analysis (PCA) and discriminant analysis (DA). The objectives were threefold: to determine whether the chemical profiles could be used to effectively discriminate between different species; to determine the extent to which genetic variation affected the chemical profile and the integrity of these determinations; and to determine the most useful compounds for the estimation of age.

6.2 Materials and Methods

6.2.1 Rearing of Fly Colonies and Collection of Specimens

Colonies of *Phormia regina*, *Cochliomyia macellaria*, *Lucilia sericata*, and *Lucilia cuprina* were reared through ten successive generations via the protocols described in Chapter 5. At the F1 and F10 generations, 100 maggots were transferred into 19 Dixie cups containing 50 g chicken liver, which were in turn placed inside 19 separate glass mason jars half-filled with sawdust substrate. The mason jars were kept in an incubator maintained at 25 °C temperature and 60 % humidity. Seven to ten days were allotted for the maggots to develop through their early life cycle stages. At 1 d following pupation, all of the pupae were harvested from three randomly-selected jars; these pupae were stored separately in a freezer at -80 °C and classified as Timepoint 1. Twenty-four hours later, pupae were collected from three additional jars, frozen, and dubbed Timepoint 2, and twenty-four hours after that pupae were collected from three more jars, frozen, and dubbed Timepoint 3, and so on and so forth through 18 jars comprising six successive timepoints. The maggots in the 19th jar were allowed to develop to maturity, the time of emergence was recorded, and the number of adult flies was compared against the number of puparia and unhatched pupae to ensure there was no widespread incidence of mortality.

6.2.2 Instrumental Analysis

A 6890 gas chromatograph coupled to a 5975 mass spectrometer (Agilent, Santa Clara, CA, USA) served as the principal instrumentation, with autosampler functionality provided by an MPS2 (Gerstel, Mülheim an der Ruhr, Germany). The column was a J&W DB-5ms (30 m × 0.25 mm × 0.25 μm). All GC-MS analyses utilized H₂ carrier gas with a flow rate of 2.5 mL/min operated in splitless mode, with a scan range of *m/z* 40-550.

For each timepoint, three pupae from each of the three jars were thawed and extracted in 1 mL pentane. After 4 d, the extract solution was silylated and submitted to GC-MS analysis via the TV-SPME method described in Chapter 5. This method provided excellent sensitivity an order of magnitude greater than what could be achieved by liquid injection, allowing for the analysis of liquid extracts without a reconstitution step.

6.2.3 Statistical Analysis

Following instrumental analysis, a qualitative assessment of the chromatograms was undertaken, and 26 compounds of interest were identified across all four species. The mass spectra for these compounds were compiled into an AMDIS library, and automated peak integration proceeded using AMDIS mass spectral deconvolution software. The peak areas were normalized to the square root of the sum of squares for all compounds of interest in the chromatogram.

Finally, the normalized dataset was investigated by PCA and DA to illuminate the underlying trends among the different species, generations, and timepoints.

PCA is a multivariate statistical technique which serves to reduce the overall dimensionality of a complex dataset. It accomplishes this by transforming the data, restructuring it in such a way as to concentrate the total variance into a smaller number of *principal components*—linear combinations of the original variables. Each principal component (PC) is orthogonal to every other principal component, with PC1 encapsulating the highest amount of variance, PC2 encapsulating the second-highest amount of variance, and so on, with smaller and smaller amounts of variance explained by each successive PC. Therefore, a complex system of 26 variables (i.e. 26 compounds of interest) can be efficiently described by a system of three PC's, which can themselves be plotted on a Cartesian coordinate plane, thereby aiding in the visualization of the data [126].

DA is another technique whose purpose is to visualize groupings in the data, as well as to predict and ascribe group membership for new samples. Similar to PCA, it accomplishes this by finding linear combinations of the original variables. These combinations are termed *canonical variates*, and can likewise be plotted on a Cartesian coordinate plane to facilitate the visualization of a complex dataset. However, unlike PCA, which attempts to maximize variance, DA strives to maximize the discrimination among different groups. The group memberships are pre-defined by the human being conducting the test, but the integrity of these memberships can be assessed by a leave-one-out cross-validation, which is reported in a table known as a confusion matrix [126].

6.3 Results and Discussion

6.3.1 Determination of Species

The 26 compounds of interest investigated in these experiments are presented in a heat map in Figure 6.1. Wherever possible, the identity of the compound has been reported. However, due to similar retention times and indistinguishable mass spectra, the exact identities of many of the hydrocarbons remain elusive. These have been compared against the retention times of an n-alkane mix and reported with their retention indices wherever possible. Similarly, the compounds labeled 'unsaturated myristic acid' and 'unsaturated stearic acid' are believed to be mono-unsaturated 14:1 and 18:1 free fatty acids, but the precise location of the double bond is yet unknown.

There is a high degree of qualitative similarity between the four species, particularly with respect to the expression of fatty acids. Lauric acid, myristic acid, pentadecanoic acid, palmitelaidic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, and arachidonic acid are observed in all samples for all species. Substantially more variability is evident in the hydrocarbon compound class, with certain alkanes commonly seen in some species (e.g. pentadecane in *Lucilia sericata*), yet wholly absent in other species (e.g. pentadecane in *Phormia regina*). Not obvious in Figure 6.1, but still just as important to the statistical analysis, are the more nuanced quantitative differences in the expression of these compounds among the different species (i.e. differences in the relative amounts of each of the major compounds).

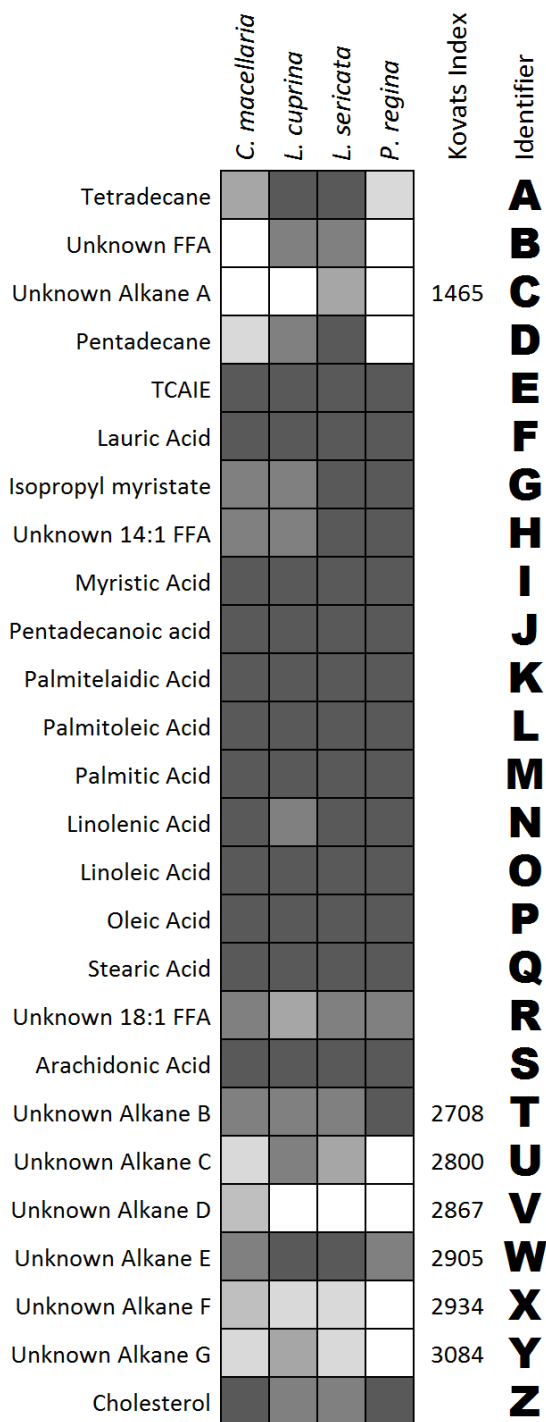


Figure 6.1. Heat map depicting the 26 compounds selected for statistical analysis. ■ denotes compounds present in 100 % of samples, ■ denotes compounds present in 75-99 % of samples, ■ denotes compounds present in 50-74 % of samples, ■ denotes compounds present in 25-49 % of samples, and ■ denotes compounds present in 1-24 % of samples. TCAIE is 2,2,4-trimethyl-3-carboxyisopropylpentanoic acid isobutyl ester.

When performing multivariate statistical analysis, it is often customary to reduce the dimensionality of the dataset first via PCA prior to attempting DA. This is typically done in order to sidestep one of the inherent limitations of the statistical test: in order for the results to be generalizable and the classifications to be accurate, DA requires that each grouping possess more observations (i.e. pupa exemplars) than variables (i.e. compounds of interest, or principal components thereof). A preliminary round of PCA therefore serves as an excellent way to reduce the number of variables whilst still capturing a sizeable amount of the variance in the data.

The DA plots for *Cochliomyia macellaria*, *Lucilia cuprina*, *Lucilia sericata*, and *Phormia regina* obtained by performing DA on the first three principal components for the F1 generation are provided in Figure 6.2. Also provided is the leave-one-out confusion matrix, which gives insight into the integrity of the statistical model as well as its reliability for future classifications. Good discrimination is observed for *Lucilia cuprina* and *Lucilia sericata*, with these sample sets achieving 94.34 % classification accuracy and 92.45 % classification accuracy, respectively. Discrimination between *Cochliomyia macellaria* and *Phormia regina*, however, is more problematic. The overlap between the two groups is extensive, resulting in substandard classification accuracies of 80.77 % for *Cochliomyia macellaria* and 47.22 % for *Phormia regina*.

Better results were achieved by performing DA on the dataset directly sans an intermediate PCA step. DA plots for the F1 and F10 generations were prepared by this method and are provided in Figure 6.3 and Figure 6.5, respectively. Although *Cochliomyia macellaria* and *Phormia regina* are still spatially close, far better

classification accuracy is observed, with *Phormia regina* classified correctly in 88.89 % of cases for the F1 generation and 94.44 % of cases for the F10 generation. Classification accuracies for the other three species are likewise improved.

Taken in total, the species could be accurately assessed based on the 26 compounds of interest in 93.40 % of cases for the F1 generation and 96.21 % of cases for the F10 generation. This is sound evidence for the theory that taxonomic information can be gleaned from the lipid profile. PCA, it appears, tends to emphasize the variance between *Lucilia cuprina* and *Lucilia sericata* at the expense of *Cochliomyia macellaria* and *Phormia regina*, and therefore obfuscates more than it aids. The results of the intermediate PCA analysis are not shown, but note that in every case, the first three principal components only managed to capture 40 to 50 % of the variance in the dataset. Based on this, it is perhaps unsurprising that *Cochliomyia macellaria* and *Phormia regina* could not be discriminated, as approximately half of the information which might be used to differentiate them could not be included in the subsequent DA analysis.

Variables plots are also provided in Figure 6.4 and Figure 6.6. These charts serve as a “roadmap” for interpreting the DA results. The reader is encouraged to directly compare each DA plot with its corresponding variables plot: the variables plot in Figure 6.4 provides tremendous insight into the DA results reported in Figure 6.3, and the variables plot in Figure 6.6 likewise provides insight into the DA results in Figure 6.5.

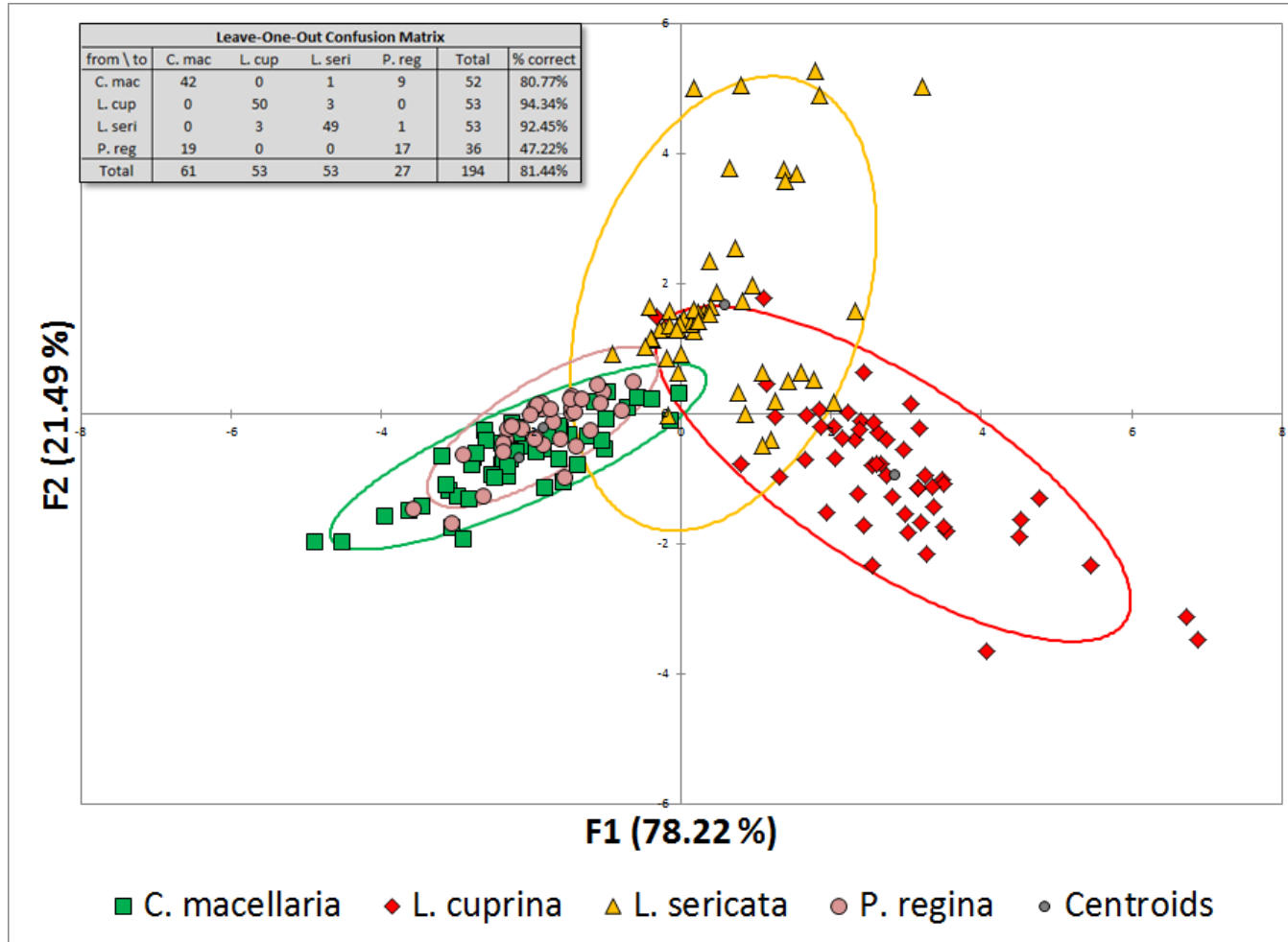


Figure 6.2. Discriminant analysis of the first three principal components for all four species, F1 generation, at all timepoints, with accompanying leave-one-out confusion matrix.

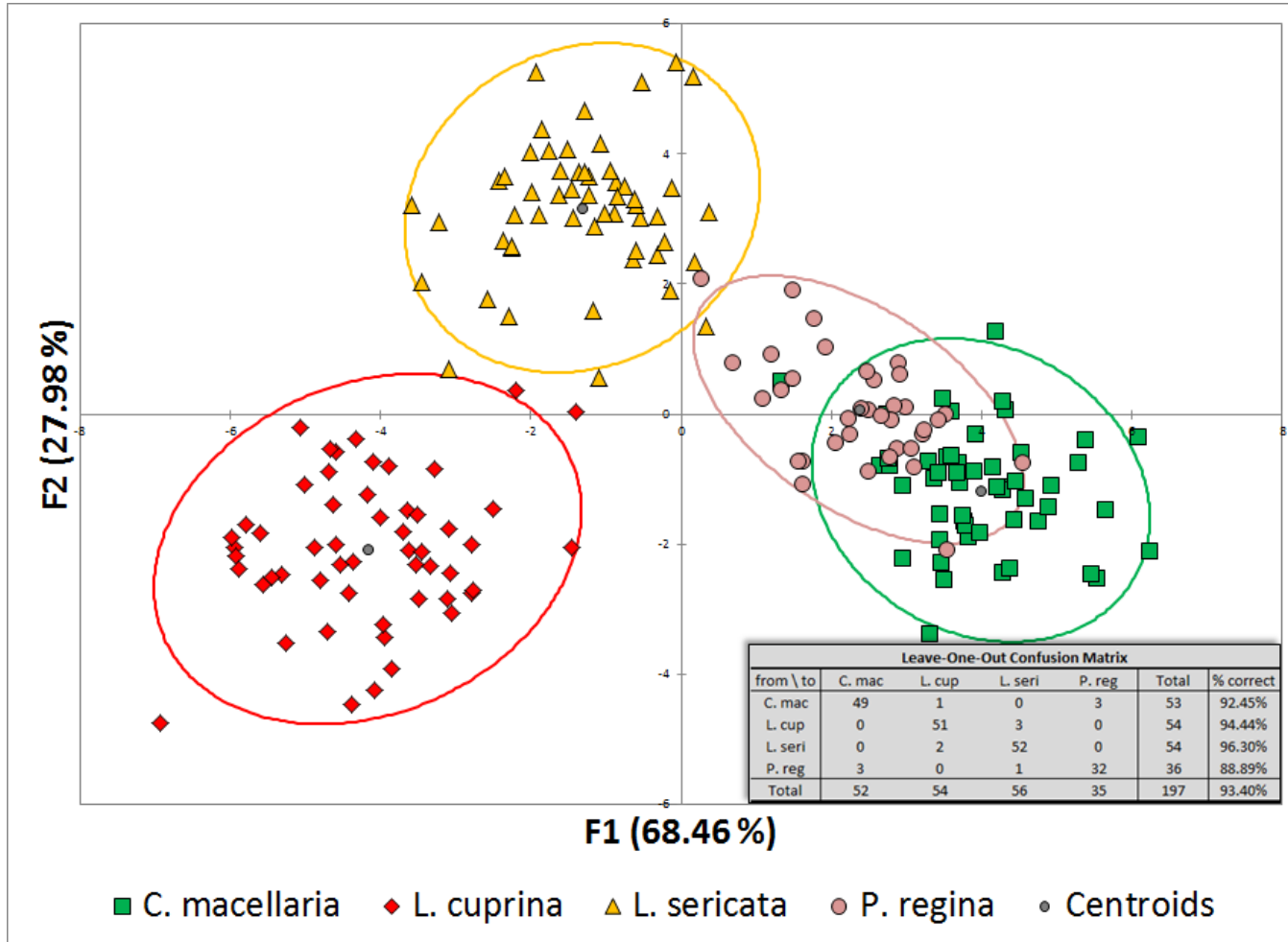


Figure 6.3. Direct discriminant analysis of all four species, F1 generation, at all timepoints, with accompanying leave-one-out confusion matrix.

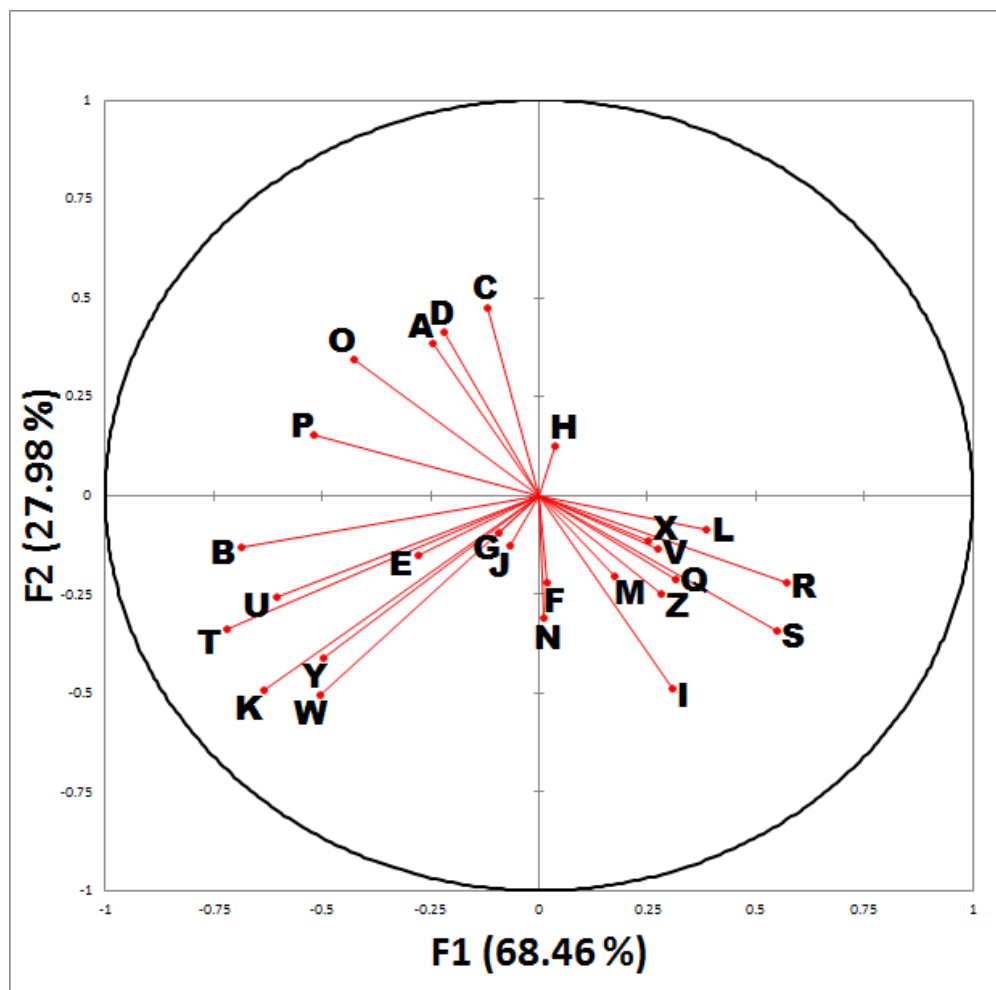


Figure 6.4. Variables plot for DA results in Figure 6.3. Identities for the rays projected from the origin correspond to the lettered compounds in Figure 6.1.

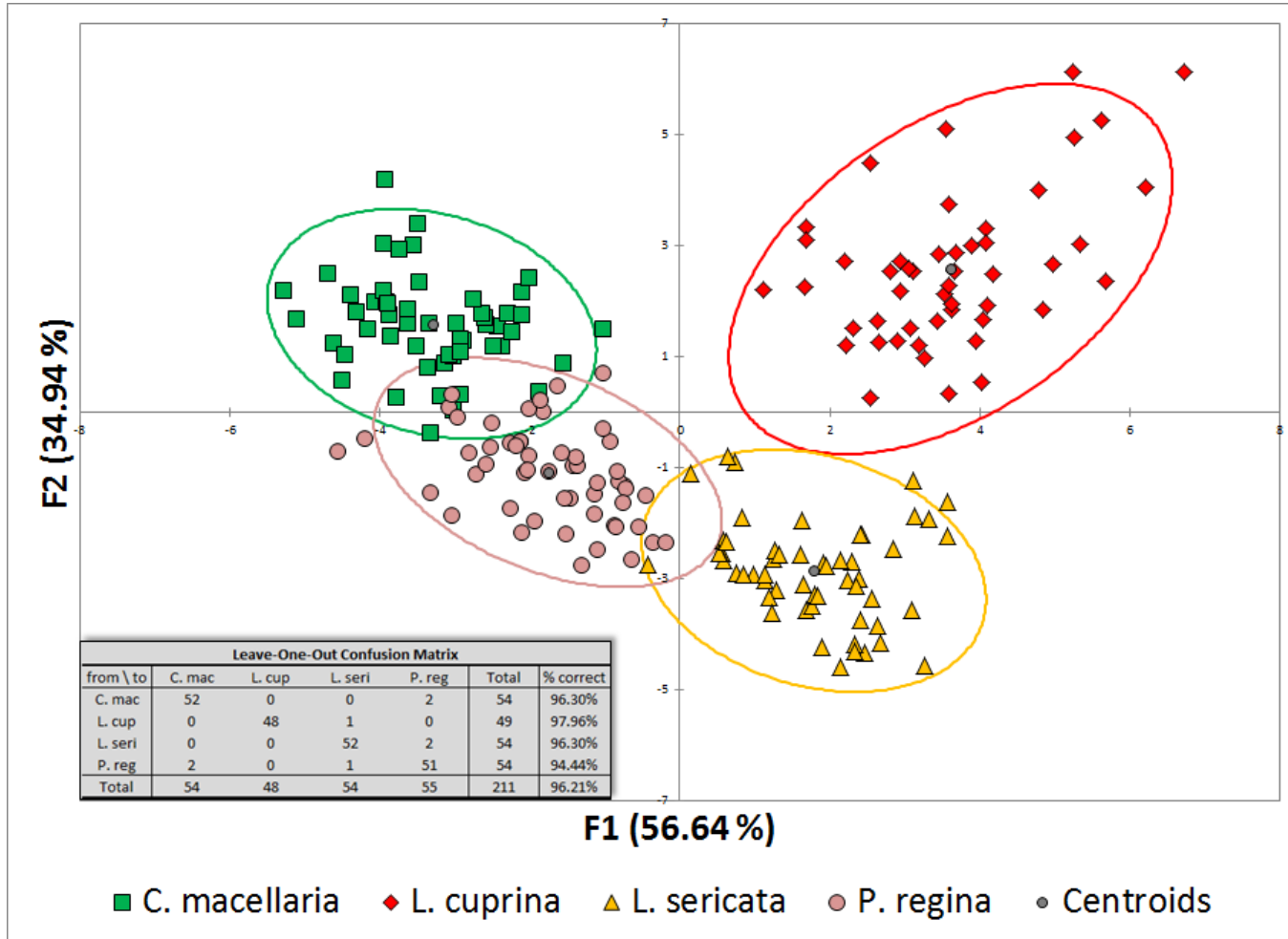


Figure 6.5. Direct discriminant analysis of all four species, F10 generation, at all timepoints, with accompanying leave-one-out confusion matrix.

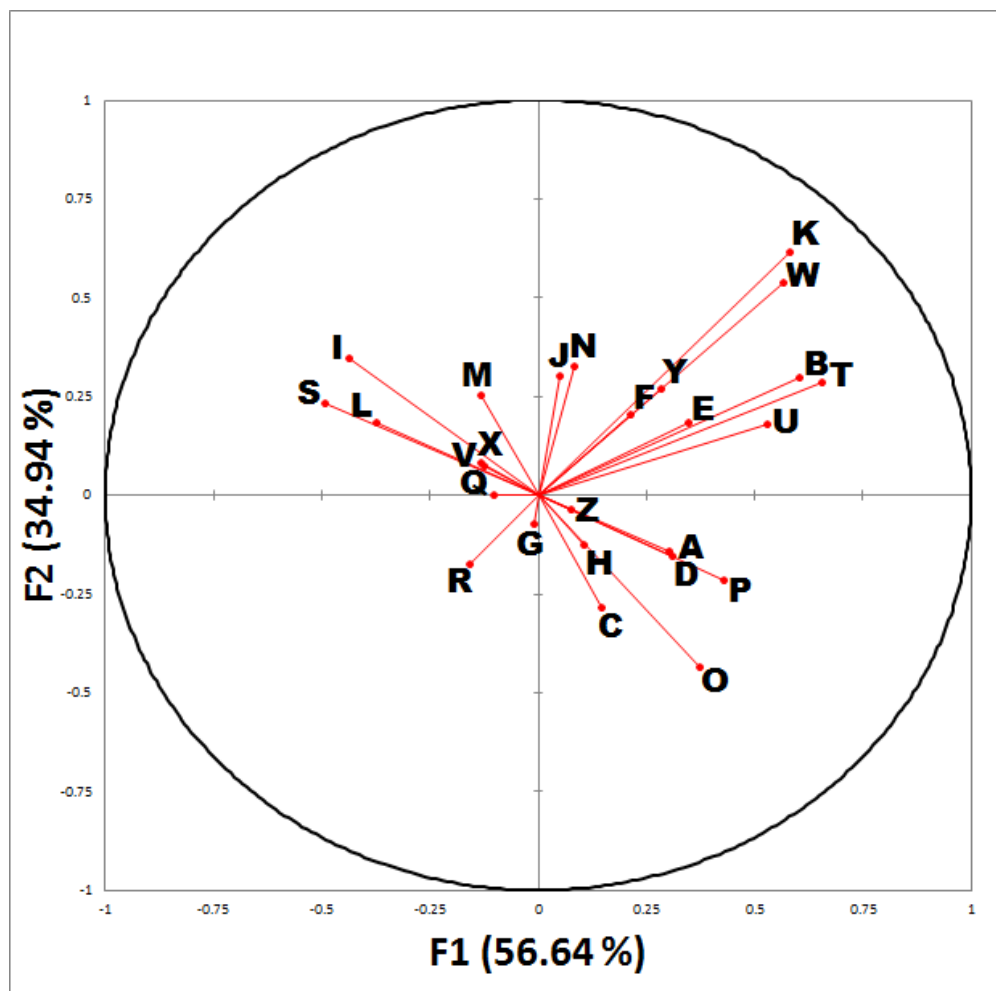


Figure 6.6. Variables plot for DA results in Figure 6.5. Identities for the rays projected from the origin correspond to the lettered compounds in Figure 6.1.

Each ray in the variables plot represents one of the 26 compounds of interest. Rays projected in the same vicinity represent compounds whose abundances are highly correlated. For example, rays I, L, and S in Figure 6.6, corresponding to myristic acid, palmitoleic acid, and arachidonic acid, all fall near to one another, and are therefore expected to rise and fall in coincidence with one another. Rays on opposite sides of the plot represent compounds that are highly anti-correlated. For example, rays A, D, and P in Figure 6.6, representing tetradecane, pentadecane, and oleic acid, are highly anti-correlated to myristic acid, palmitoleic acid, and arachidonic acid. Rays at right angles reflect compounds with little or no correlation.

The quadrants of the variables plot are also directly comparable to the quadrants of the corresponding DA chart. Myristic acid, palmitoleic acid, and arachidonic acid, which fall within the top-left quadrant in Figure 6.6, are therefore revealed to be highly-present in specimens of *Cochliomyia macellaria*, because that species likewise falls within the top-left quadrant in Figure 6.5. *Lucilia sericata*, which falls within the bottom-right quadrant in Figure 6.5, is similarly expected to provide high concentrations of tetradecane, pentadecane, and oleic acid—which, among, other compounds, all fall within the bottom-right quadrant in Figure 6.6. The length of each ray indicates the magnitude of the association. Long rays indicate strong correlations, whereas short rays that fall close to the origin embody compounds that are not strongly representative of any of the four species.

Thus, the following observations are manifest:

1. *Cochliomyia macellaria* appears to be distinguishable by high concentrations of myristic acid, palmitoleic acid, and palmitic acid.
2. *Lucilia cuprina* appears to be distinguishable by high concentrations of palmitelaidic acid and several alkanes (unknown alkane B, unknown alkane C, unknown alkane E, and unknown alkane G).
3. *Lucilia sericata* appears to be distinguishable by high concentrations of tetradecane, unknown alkane A, pentadecane, linoleic acid, and oleic acid.
4. *Phormia regina* appears to be distinguishable by high concentrations of unknown 18:1 FFA.

6.3.2 Determination of Generational Differences

A key issue this project sought to address was the effect of genetic diversity on a specimen's chemical profile. Specifically, do F1 pupae yield a markedly different suite of compounds than pupae belonging to the F10 generation, whose genetic diversity has been homogenized over successive generations of inbreeding? With the goal of answering this question, DA was used to attempt to discriminate samples hailing from the F1 and F10 generation of each species (i.e. *Cochliomyia macellaria* F1 samples were

evaluated alongside *Cochliomyia macellaria* F10 samples, *Lucilia cuprina* F1 samples alongside *Lucilia cuprina* F10 samples, etc.).

Figures 6.7–6.10 portray receiver operating characteristic (ROC) curves for each of the four generational comparisons. These plots depict the binary classification accuracy of the system. A system comprised of two indistinguishable, overlapping groups would be expected to track along the dotted line, indicating that each sample is equally as likely to be classified in one category as in another; whereas a system with flawless classification accuracy would be expected to follow up the left border and along the top border of the ROC space.

The fact that the ROC curves are well above the diagonal in each of the figures indicates there are intrinsic differences in the dataset between the F1 generation and the F10 generation. This is articulated by the area under curve values, which fall as high as 0.883 for *Phormia regina*. Thus, *Phormia regina* appears to have the greatest amount of inter-generational dissimilarity among the four species evaluated, especially in comparison to *Lucilia sericata*, whose 0.648 area under curve value implies the chemical profiles of the F1 and F10 generations bear comparatively more in common than for the other species. The confusion matrices also provide insight into these relationships, with non-negligible classification accuracies observed for *Cochliomyia macellaria* (68.87 %), *Lucilia cuprina* (73.53 %), and *Phormia regina* (82.22 %).

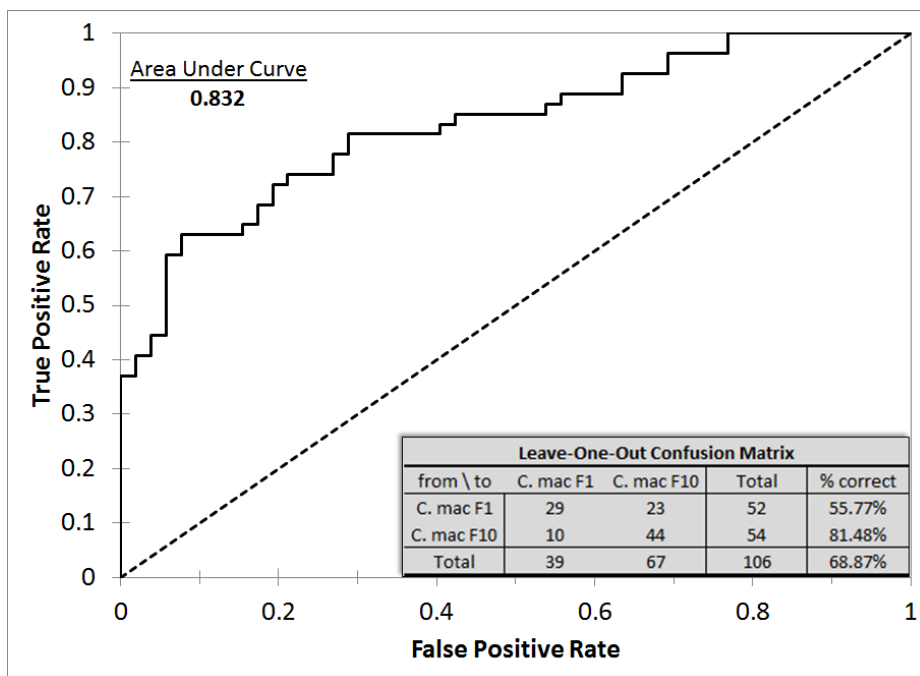


Figure 6.7. ROC curve for *Cochliomyia macellaria* with leave-one-out confusion matrix.

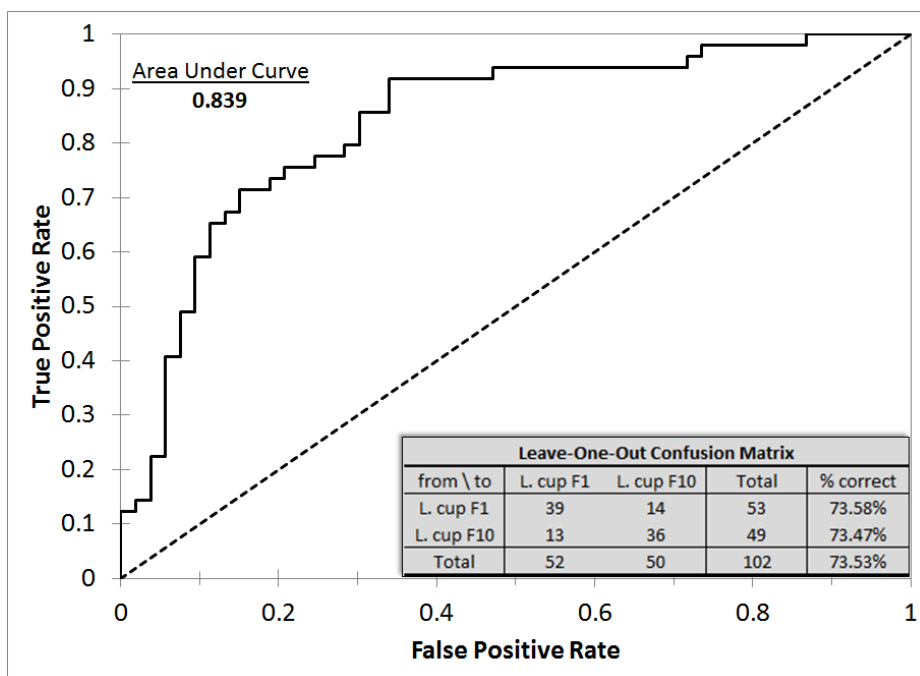


Figure 6.8. ROC curve for *Lucilia cuprina* with leave-one-out confusion matrix.

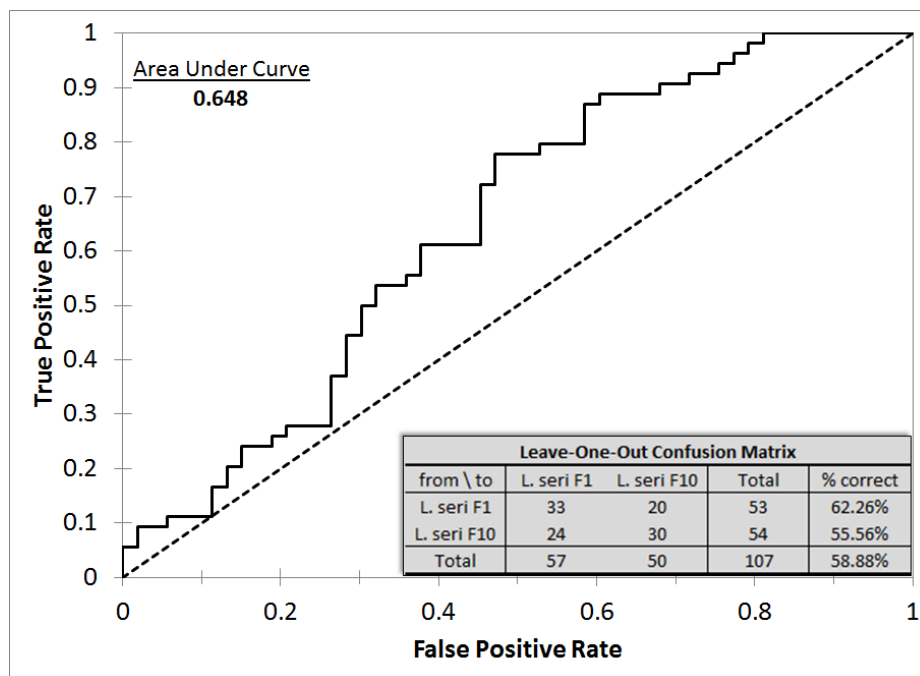


Figure 6.9. ROC curve for *Lucilia sericata* with leave-one-out confusion matrix.

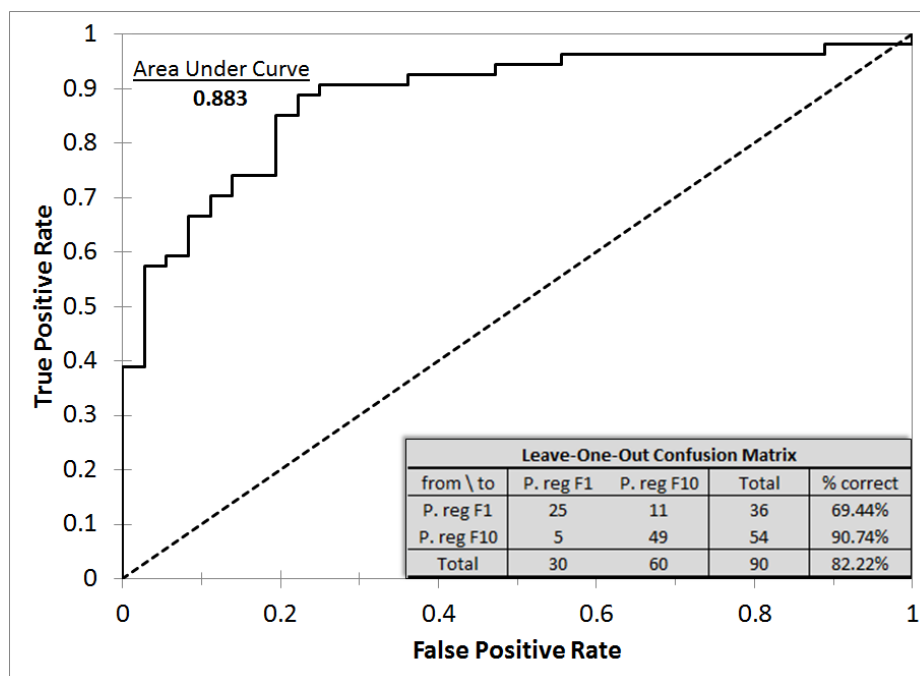


Figure 6.10. ROC curve for *Phormia regina* with leave-one-out confusion matrix.

DA was also performed by treating samples of F1 pupae as unknowns and classifying them according to the DA model constructed from F10 pupae. If the genetic differences were irrelevant, then the F1 pupae would be expected to be grouped according to their species with near 100 % accuracy, mirroring the confusion matrix for the F10 generation provided in Figure 6.5. Poor classification accuracy, conversely, would indicate that the model is indeed betrayed by genetic differences. The results are shown in Table 6.1.

Although the confusion matrix shows lower classification accuracies across the board, the accuracies for *Cochliomyia macellaria*, *Lucilia cuprina*, and *Lucilia sericata* are still quite good. *Lucilia sericata* was the least affected by inter-generational genetic change, whereas *Phormia regina* was the most affected. These observations are in agreement with the ROC curves. Based on these results, it appears that F10 pupae may yet be a suitable model for F1 pupae, although genetic

Table 6.1. Leave-one-out confusion matrix for F1 pupae grouped according to the statistical model of the F10 pupae.

from \ to	C. mac	L. cup	L. seri	P. reg	Total	% correct
C. mac	47	0	0	6	53	88.68 %
L. cup	2	49	2	1	54	90.74 %
L. seri	0	0	52	2	54	96.30 %
P. reg	7	0	2	27	36	75.00 %
Total	56	49	56	36	197	88.83 %

homogenization does indeed alter the chemical profile in a statistically-significant way. Future researchers seeking to extrapolate useful information about F1 and wild-type populations based on data gained from advanced generations are cautioned to bear this in mind.

6.3.3 Determination of Age

A correlation analysis was performed on each of the 26 compounds of interest to determine which of them exhibited statistically-significant changes over time, as measured in accumulated degree hours (ADH). The results of these measurements for the F1 and F10 generations are displayed in Figure 6.11. Special attention should be paid to the compounds most highly correlated and anti-correlated with time—highlighted in red and green on the figure—for these compounds are the most relevant to the maturity of the specimen and make the most likely candidates for the determination of age via the chemical profile.

Repeating trends in the data are also worthy of consideration. Note, for example, the positive correlation between linoleic acid and age observed across seven of the eight datasets for the F1 and F10 generations. Palmitoleic acid, linolenic acid, cholesterol, and unknown alkanes B, C, D, E and F also appear to be potentially-good markers for age determination.

Figure 6.12 and Figure 6.13 showcase the change over time for palmitoleic acid and linoleic acid in *Phormia regina* F1. Palmitoleic acid steadily decreases, while

	<i>C. macellaria</i>	<i>L. cuprina</i>	<i>L. sericata</i>	<i>P. regina</i>		<i>C. macellaria</i>	<i>L. cuprina</i>	<i>L. sericata</i>	<i>P. regina</i>
Tetradecane	0.05	0.15	-0.19			0.15	-0.28	0.21	
Unknown FFA		0.08	0.14				-0.22	0.38	
Unknown Alkane A (~C14)		-0.03					-0.18		
Pentadecane		0.01	-0.31				-0.27	0.24	
TCAIE	-0.09	0.14	0.13	0.15		0.22	-0.22	0.48	0.05
Lauric Acid	-0.42	0.01	-0.04	-0.23		0.18	-0.15	0.20	-0.15
Isopropyl myristate	-0.27	0.13	0.11	-0.21		-0.03	-0.22	0.03	0.12
Unknown 14:1 FFA	-0.30	0.03	0.14	-0.59		-0.18	-0.18	-0.14	0.02
Myristic Acid	-0.44	0.10	-0.05	-0.42		-0.11	0.01	0.15	-0.15
Pentadecanoic acid	-0.07	0.09	0.01	0.01		-0.04	-0.22	-0.09	-0.04
Palmitelaidic Acid	0.08	0.21	-0.10	0.37		-0.16	0.14	0.01	0.06
Palmitoleic Acid	-0.37	-0.34	0.12	-0.56		0.02	-0.07	-0.03	-0.17
Palmitic Acid	0.01	0.05	0.19	0.06		-0.07	-0.19	0.30	0.18
Linolenic Acid	0.37	0.21	0.38	0.38		0.40	0.11	-0.04	-0.03
Linoleic Acid	0.41	0.52	0.09	0.37		0.35	0.44	0.27	0.37
Oleic Acid	0.35	-0.06	-0.36	0.26		-0.10	-0.03	-0.39	-0.21
Stearic Acid	0.44	0.14	0.10	0.16		-0.12	-0.01	-0.15	-0.27
Unknown 18:1 FFA	-0.19	-0.20	-0.01	-0.02		-0.01	0.23	-0.20	0.17
Arachidonic Acid	-0.33	0.05	0.16	-0.12		-0.15	0.14	-0.17	-0.13
Unknown Alkane B (~C26)	0.34	0.26	0.12	0.41		0.46	0.31	0.45	0.43
Unknown Alkane C (~C26)		0.20	-0.05				0.30	0.26	
Unknown Alkane D (~C27)	0.55					0.28			
Unknown Alkane E (~C27)	0.47	0.14	0.06	0.32		0.57	0.17	0.31	0.49
Unknown Alkane F (~C27)	0.48					0.29			
Unknown Alkane G (~C28)			0.14					0.25	
Cholesterol	0.51	0.11	0.35	0.29		0.22	-0.16	0.15	0.15

Figure 6.11. Correlations between selected compounds and ADH. Compounds in green increase markedly with time; compounds in red decrease markedly with time. Compounds highlighted in bold are ascribed a statistically-significant p-value < 0.05.

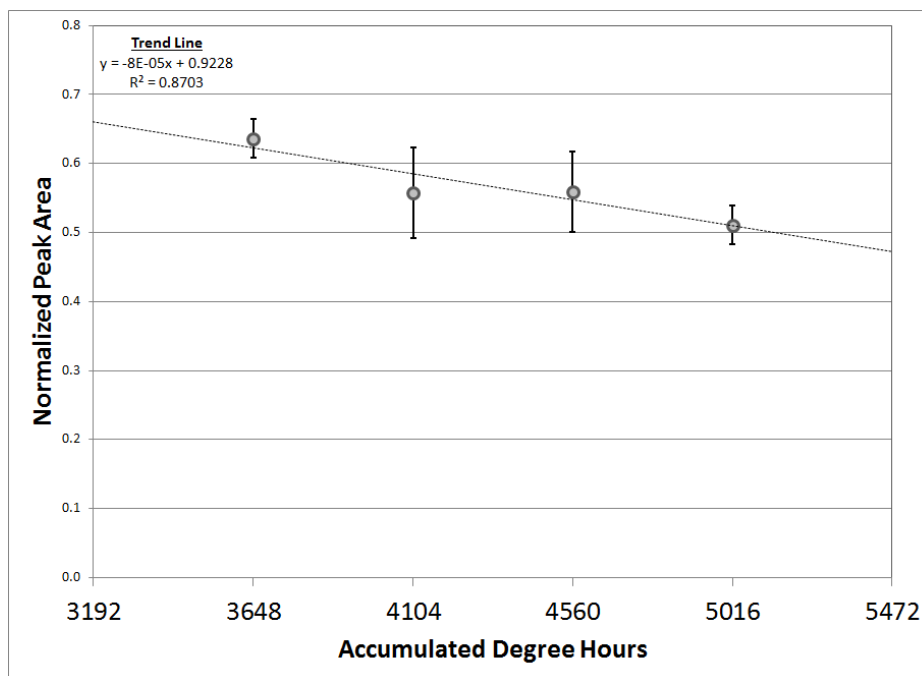


Figure 6.12. Normalized peak areas for palmitoleic acid in *Phormia regina* F1 as a function of ADH.

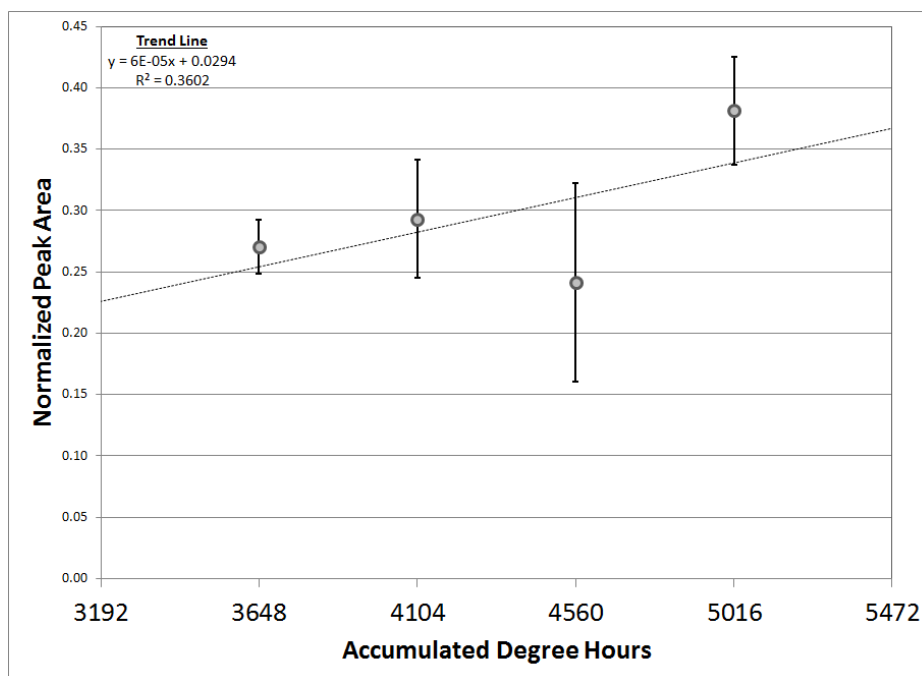


Figure 6.13. Normalized peak areas for linoleic acid in *Phormia regina* F1 as a function of ADH.

linoleic acid increases. Similar trends are observed in *Cochliomyia macellaria* F1 and *Lucilia cuprina* F1. A possible avenue for estimating the age of the specimen might lie in taking the ratio of one compound to the other. For example, the ratio of palmitoleic acid to linoleic acid in *Phormia regina* F1 at ADH 3648 is 2.3 +/- 0.2, whereas the ratio is 1.3 +/- 0.2 at ADH 5016. The error rates of these computations may vary wildly, however, and additional experimentation is required to verify the usefulness and practicality of such a comparison.

Also noteworthy are unknown alkane D and unknown alkane F, which—while not predominating in *Lucilia cuprina*, *Lucilia sericata*, or *Phormia regina*—do appear in *Cochliomyia macellaria*, but only at timepoints 5 and 6 (ADH 5016 and ADH 5472, respectively). Qualitative differences in the chemical profile such as these are a tremendous boon to age determination. In this case, the effect of these two compounds was so pronounced that it was possible to distinguish between the earlier timepoints and the latter timepoints of *Cochliomyia macellaria* in a 3D-projected PCA plot, presented in Figure 6.14. Note how samples at the latter timepoints fall perceptibly to the right of the vertical axis, a phenomenon which appears to be wholly attributable to the statistical nuance of these alkanes. Unfortunately, no other qualitative differences were discovered in *Lucilia cuprina*, *Lucilia sericata*, and *Phormia regina*, and similar PCA projections for those species proved inadequate at visualizing any chemical changes due to age.

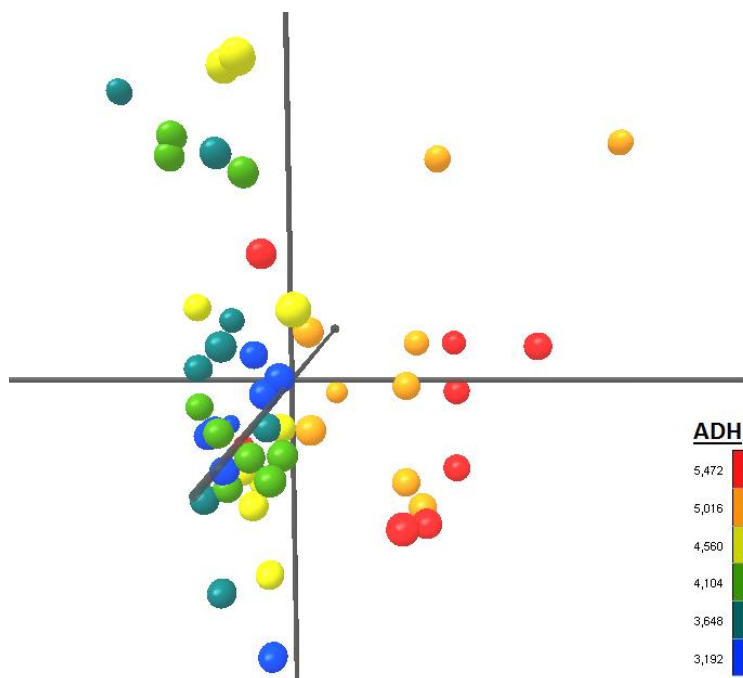


Figure 6.14. 3D PCA plot of *Cochliomyia macellaria* samples, categorized according to ADH.

6.4 Conclusion

Considerable chemical differences were documented among *Cochliomyia macellaria*, *Lucilia cuprina*, *Lucilia sericata*, and *Phormia regina* as a function of species, genetics, and age. Classification accuracies of 92.45 %, 94.44 %, 96.30 %, and 88.89% were obtained for the F1 generation, and classification accuracies of 96.30 %, 97.96 %, 96.30 %, and 94.44 % were obtained for the F10 generation. The compounds most correlated and anti-correlated with species were identified. The F10 groupings were shown to be a suitable model for F1 pupae, although performance on *Phormia regina* suffered, with classification accuracy falling to 75.00 %. However, genetic

homogenization was found to have an impact on the observed chemical profiles, which should serve as a cautionary note for future researchers hoping to extract data with real world relevance from colonies at advanced generations. Compounds with high correlation and anti-correlation to age were determined and assessed for statistical significance. Palmitoleic acid, linolenic acid, cholesterol, and unknown alkanes B, C, D, E and F were found to be potentially-good markers for age determination. Unknown alkanes D and F, in particular, embodied a qualitative difference between early timepoints and late timepoints in *Cochliomyia macellaria*, enabling the change over time to be visualized by PCA.

CHAPTER 7. FUTURE DIRECTIONS

7.1 Determination of TV-SPME Distribution Coefficients

In order for TV-SPME to become established as a viable alternative to HS-SPME and other means of sample introduction, the underlying physical and mathematical relationships must be fully understood. To that end, there is an opportunity for further exploration of the multiphase equilibria of the SPME system, most notably with regard to the determination of the distribution coefficient, K_{fs} .

It so happens that the partitioning that occurs between the headspace and the fiber in a closed TV-SPME system is comparable to the partitioning that occurs between the mobile phase and the stationary phase within a chromatography column. Therefore, the well-understood physiochemical principles of chromatography can be utilized to estimate K_{fs} based on the isothermal retention times of known standards. A useful equation for this purpose is:

$$K_{fh} = (t_r - t_0) \left[F \left(\frac{T}{T_m} \right) \left(\frac{p_m - p_w}{p_m} \right) \right] \frac{3}{2} \left[\frac{\left(\frac{p_i}{p_o} \right)^2 - 1}{\left(\frac{p_i}{p_o} \right)^3 - 1} \right] \frac{1}{V_L} \quad (\text{Equation 7.1})$$

where t_r is the retention time of the target standard; t_0 is the column dead time; F is the column flow, as measured by a flow meter; T and T_m are the temperatures of the column and the flow meter, respectively; p_m and p_w are the flow meter vapor pressure and the saturated water vapor pressure, respectively; p_i and p_o are the inlet and outlet pressures of the column; and V_L is the volume of the column stationary phase. Note that p_m and p_o are almost always equal to the atmospheric pressure. Also recall that in TV-SPME, there is no K_{hs} partitioning that occurs between the sample and the headspace, as all of the sample is brought to the vapor state. Therefore, for all intents and purposes, $K_{hs} = K_{fs}$ [4].

Thus, if the retention time of a given standard, t_r and the retention time of the mobile phase, t_0 , are known, then the distribution coefficient of that standard in a SPME fiber can be estimated—provided, of course, that the chemistry of the chromatography column is a good approximation for the chemistry of the SPME fiber under scrutiny. A good starting point, then, would be to attempt to estimate K_{fs} for a PDMS fiber using the retention times attained using a DB-1 capillary column. The DB-1 capillary column is 100 % dimethylsiloxane, and it is therefore speculated to be a good analogue for the polymer of a PDMS SPME fiber [4].

An experimental method for determining K_{fs} uses the equation:

$$K_{fh} = \frac{C_f^\infty}{C_h^\infty} = \frac{n_f V_h}{V_f n_o} \quad \text{(Equation 7.2)}$$

where C_f^∞ and C_h^∞ are the equilibrium concentrations of the solute in the fiber and in the solute in the headspace, respectively; n_f and n_0 are the mass extracted by the fiber and the mass spiked into the sample vial; and V_f and V_h are the volumes of the fiber and the headspace, respectively. An experiment to verify the estimates obtained using Equation 7.1 would therefore involve quantifying n_f by GC-MS analysis and using Equation 7.2 to back-calculate K_{fh} , which is also equal to K_{fs} for TV-SPME [4].

An excellent future project would be to estimate the distribution coefficients for a series of known standards using Equation 7.1, then proceed to verify these estimates experimentally by carrying out GC-MS analysis on standards subjected to TV-SPME as well as HS-SPME conditions. By demonstrating the value of the distribution coefficient, the mathematical inner workings of the TV-SPME methodology will be better understood, and any advantages TV-SPME offers over HS-SPME will become manifest by direct comparison of their K_{fs} values.

7.2 Pseudo-Explosives and Odor Theory

To date, the development of odor mimics has generally relied on a substrate to facilitate the protection, transport, and delivery of odor molecules to the canine nose. During the fabrication of these training aids, the substrate may be placed in a contained environment with a sample of the base explosive material, during which time the vapor profile of the contraband transfers to the substrate via the sorption of off-gassed VOC's

[127]. Alternatively, the substrate may be exposed to an artificial cocktail of VOC's believed, based on current research, to be responsible for canine positive alerts; or, these elements may be introduced to the substrate by chemical deposition, coating, or extraction from a liquid solution [33].

In all of the above cases, the development of training aids has proceeded with the expectation that the original odor of the target explosive will be accurately encapsulated within the substrate, and once there, accurately reproduced for the canine by passive diffusion back into the air. However, these assumptions generally neglect the physiochemical reality of the substrate's own interaction with the odor compounds. Even assuming equivalent distribution coefficients, different compounds will have different substrate interactions: due to a wide variety of underlying causes (e.g. pi-pi interactions or differences in polarity), certain species will be "held" more or less tightly by the substrate relative to other species, resulting in disparate release kinetics. The unfortunate consequence of this is that, on a quantitative level, an intrinsically-different odor profile will be rendered available to the canine than what was originally put into the training aid. In essence, the substrate acts as an unwelcome third party—a biasing element which alters the relative abundances of the odor compounds, hindering the reliable re-creation of the odor in its original form. Hence, it is our belief that in order for dogs imprinted on odor mimics to achieve their greatest potential, they should be exposed to an odor profile representing the correct combination of all odor compounds in the proper proportions. To help allay these concerns and pave the way to

the development of better training aids, the distribution coefficients of explosive-related volatiles as well as the release kinetics should be studied.

Both values can be experimentally determined for a PDMS-based training aid using a SPME fiber as a model system. PDMS SPME fibers can be easily acquired in bulk from commercial vendors, and their surface chemistry is carefully controlled to ensure the replicability of analytical assays. This makes them ideal for the purposes of this project, as the release kinetics can therefore be assumed to be equivalent from fiber to fiber. Furthermore, the liquid polymer of a PDMS SPME fiber is chemically-indistinguishable from the substrate matrix of a PDMS-based training aid. Barring differences in film thickness, a SPME fiber is as accurate and controllable a model as is likely to be found.

The release of compounds from a SPME fiber is a first order process and can be modeled with the integrated first order rate equation:

$$\ln[A] = -kt + \ln[A]_0 \quad (\text{Equation 7.3})$$

Therefore, a graph of $\ln[A]$ plotted against time will return a straight line of slope $-k$.

In order to determine the value of k for 2-ethyl-1-hexanol, a microliter quantity of the compound should be transferred to a vial and capped. Four PDMS SPME fibers inserted through the septum will sample the vial headspace simultaneously, resulting in an equal loading of 2E1H between all fibers. After 6 h has been provided for the system

to safely come to equilibrium, the fibers will be removed and placed in a temperature-controlled fume hood at 25 °C with their surfaces exposed to the air, allowing the 2E1H to desorb naturally over time. At 0 d, 1 d, 2 d, and 3 d, one of the fibers will be removed from this environment and extracted in 500 μ L pentane, which shall strip all of the remaining odor compound from the fiber and isolate it in the liquid solvent. A reconstitution step may be added at this point to aid in concentrating the analyte, and an internal standard included to facilitate the subsequent analysis. Afterwards, the extract solutions will be submitted to analysis by liquid injection GC-MS. Comparison of the 2E1H integrated peak area to a calibration curve will enable the concentration of 2E1H to be determined at each timepoint, allowing $\ln[2E1H]$ to be plotted and k to be determined graphically. The distribution coefficient can be found by determining the concentration of 2E1H at $t = 0$ d. The same experiment can be repeated to determine the distribution coefficients and dissociation rate constants for any number of other purported odor compounds, including DMNB, cyclohexanone, and DNT.

7.3 Commercial Tire Treatments

The largest outstanding question pertaining to the tire treatment project is a question of cross-contamination: if two cars race on the same racetrack, and one of the cars has had a treatment applied to its wheels, then what is the risk of that treatment

transferring onto the surface of track, and from there onto the wheels of an innocent third party?

To answer this question, a collaborative effort should be undertaken with local organizations to make arrangements for the use of an official racetrack. At the start of the day, a number of cars should be raced on the track, and their tires should be collected to form a control group. Next, the racetrack should be subjected to deliberate contamination by applying a tire treatment to the wheels of a “contaminator” car and driving it along the tarmac, being sure to follow a pre-defined course. Finally, cars with untreated tires should drive the same course, and their tires should summarily be collected for laboratory analysis. If cross-contamination of the questioned tires is apparent, then the same experiment can be repeated at various intervals in order to gauge the potential degradation of the tire treatments over time. It may also be useful to “paint” the racetrack with various diluted concentrations of the tire treatment and sample from cars driven through the puddles in order to construct a rough calibration curve of signal-to-concentration.

An analyst’s ability to conclusively determine whether a sample has come into contact with an illicit treatment is contingent on whether or not that treatment has been previously analyzed and catalogued. Therefore, in addition to a study to ascertain the risk of cross-contamination, future work should focus primarily on developing a more complete and comprehensive database of known tire treatments. Continued communication with race administrators and officials is essential in order to guide these

efforts. With shrewd observation by eyes on the ground at race events, even the most problematic tire treatments can be discovered and indexed.

7.4 Forensic Entomology

Work on the forensic entomology project will continue, with the objective of determining the effects of various biotic and abiotic factors of the chemical profiles of *Cochliomyia macellaria*, *Lucilia cuprina*, *Lucilia sericata*, and *Phormia regina*. These factors include the ambient temperature and humidity of the pupation environment, the pupation substrate, and the diet of the maggots in the days leading up to the commencement of pupation.

Another logical continuation of the project would be to attempt to characterize flies that have been brought up on actual carrion. Hence, the services of a body farm could be employed, and flies collected from a bovine, porcine, or human corpse. The uncontrollable nature of temperature and humidity conditions in an outdoor environment would almost certainly give rise to new difficulties, but nothing could come closer to approximating the challenges faced by forensic investigators responding to the discovery of a body in the field.

Although HS-SPME was ineffective at capturing informative chemical data from whole pupa in the absence of an extraction step, the same goal might be realized by

more advanced methods of sample introduction. In particular, Direct Analysis in Real Time (DART) could prove viable where traditional GC-MS failed.

DART is one of the first ambient ionization techniques not to require sample preparation, allowing solid and liquid samples to be analyzed directly in their native state [128]. In brief, and without delving into the reaction chain, the technique works by holding the sample in front of a stream of excited or metastable gas—typically helium, nitrogen, or neon [128]. Collision between the gas molecules and the sample surface causes energy to be transferred to the embedded analyte, resulting in the creation of a radical cation or an anion [128]. The ionization event imparts enough energy to eject this newly-formed molecular ion from the sample wholesale, rendering it unto the mass analyzer via the gas stream [128].

Past studies have seen DART utilized for the analysis of fatty acids [129] as well as sterols [130], two compound classes that are inherent to entomological specimens. An interesting project would be to attempt to profile insect pupae via DART. If successful, this technology could offer a dramatic improvement over existing GC-MS methods, enabling investigators to make chemotaxonomic determinations about species and age all but immediately after collecting entomological evidence from the scene of a crime. Although DART is still a nascent technology and not at all widespread in existing crime labs, the promptness and convenience of such an analysis should not be ignored, and may go a long way in answering the needs of the forensic community.

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APPENDIX

APPENDIX. ADDITIONAL FIGURES FOR FLY AGE/SPECIES PROFILING

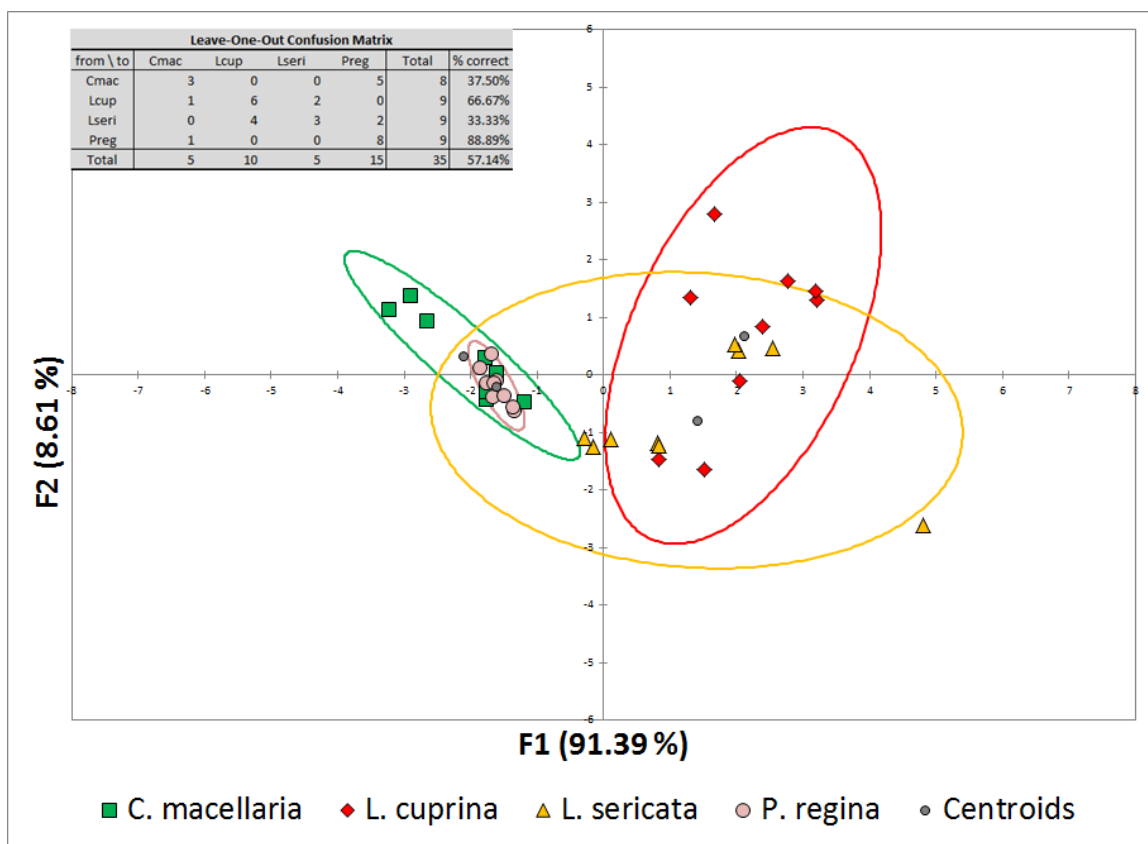


Figure A1. Discriminant analysis of the first three principal components for all four species at ADH 3648 with accompanying leave-one-out confusion matrix.

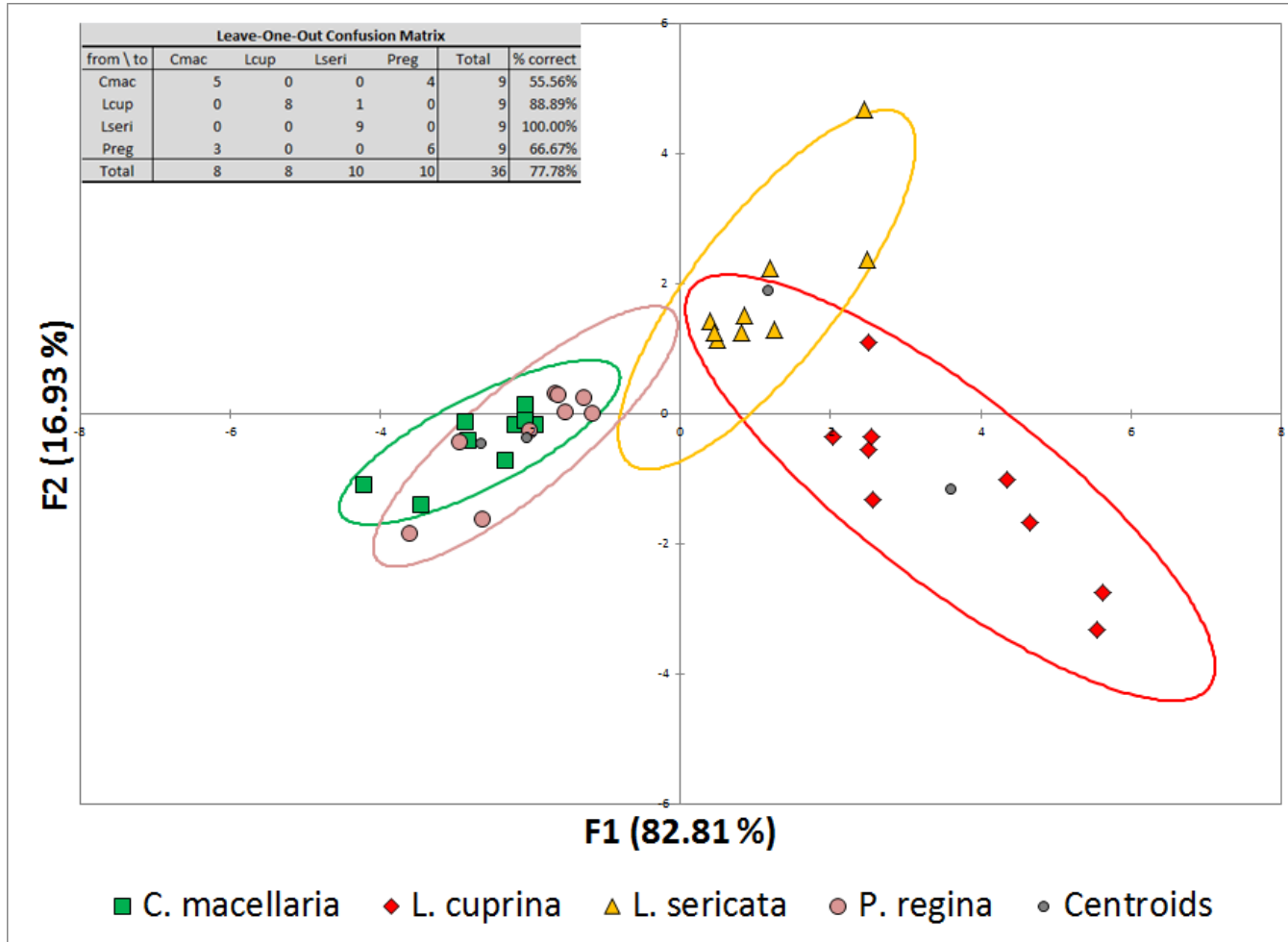


Figure A2. Discriminant analysis of the first three principal components for all four species at ADH 4104 with accompanying leave-one-out confusion matrix.

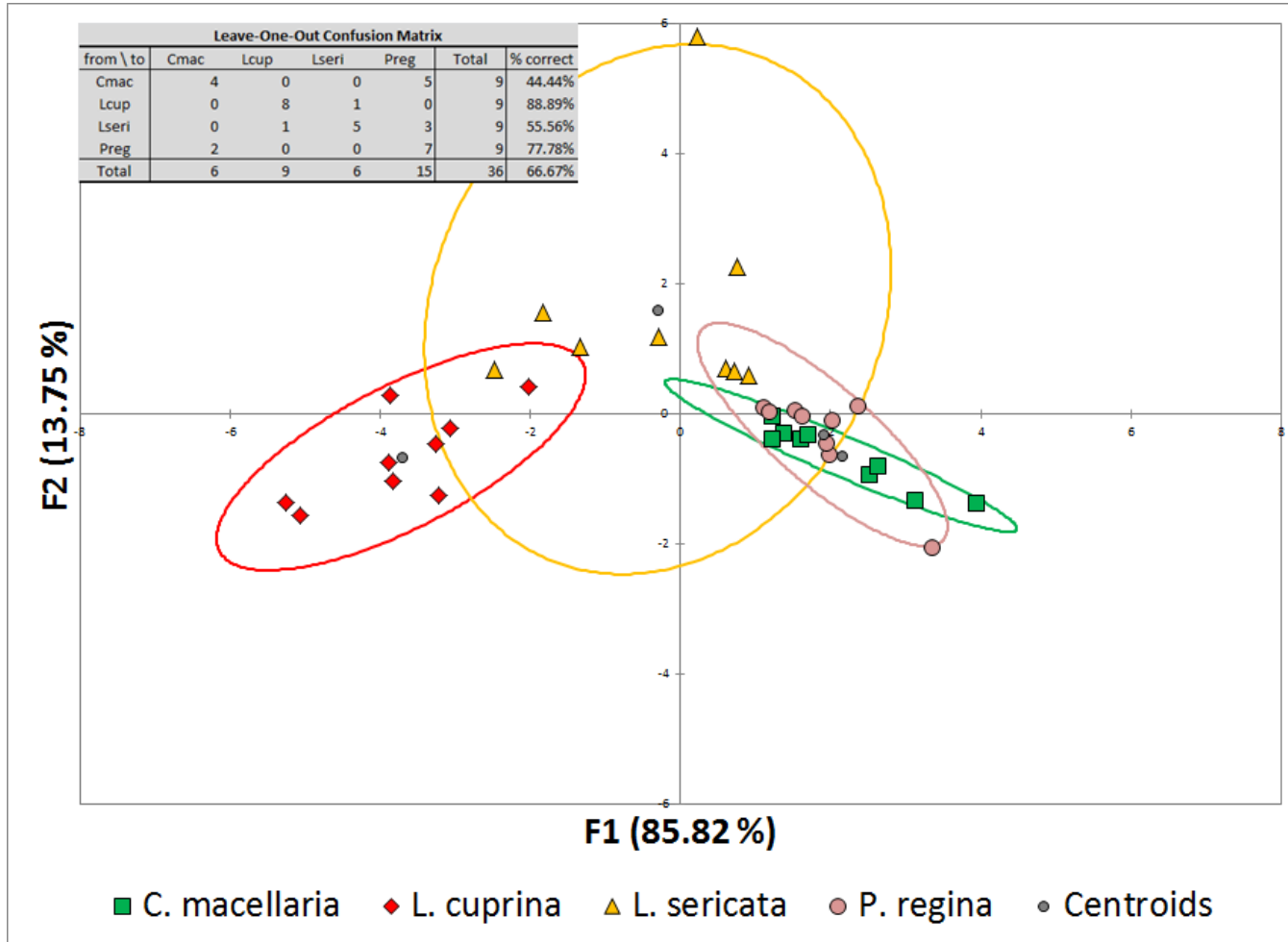


Figure A3. Discriminant analysis of the first three principal components for all four species at ADH 4560 with accompanying leave-one-out confusion matrix.

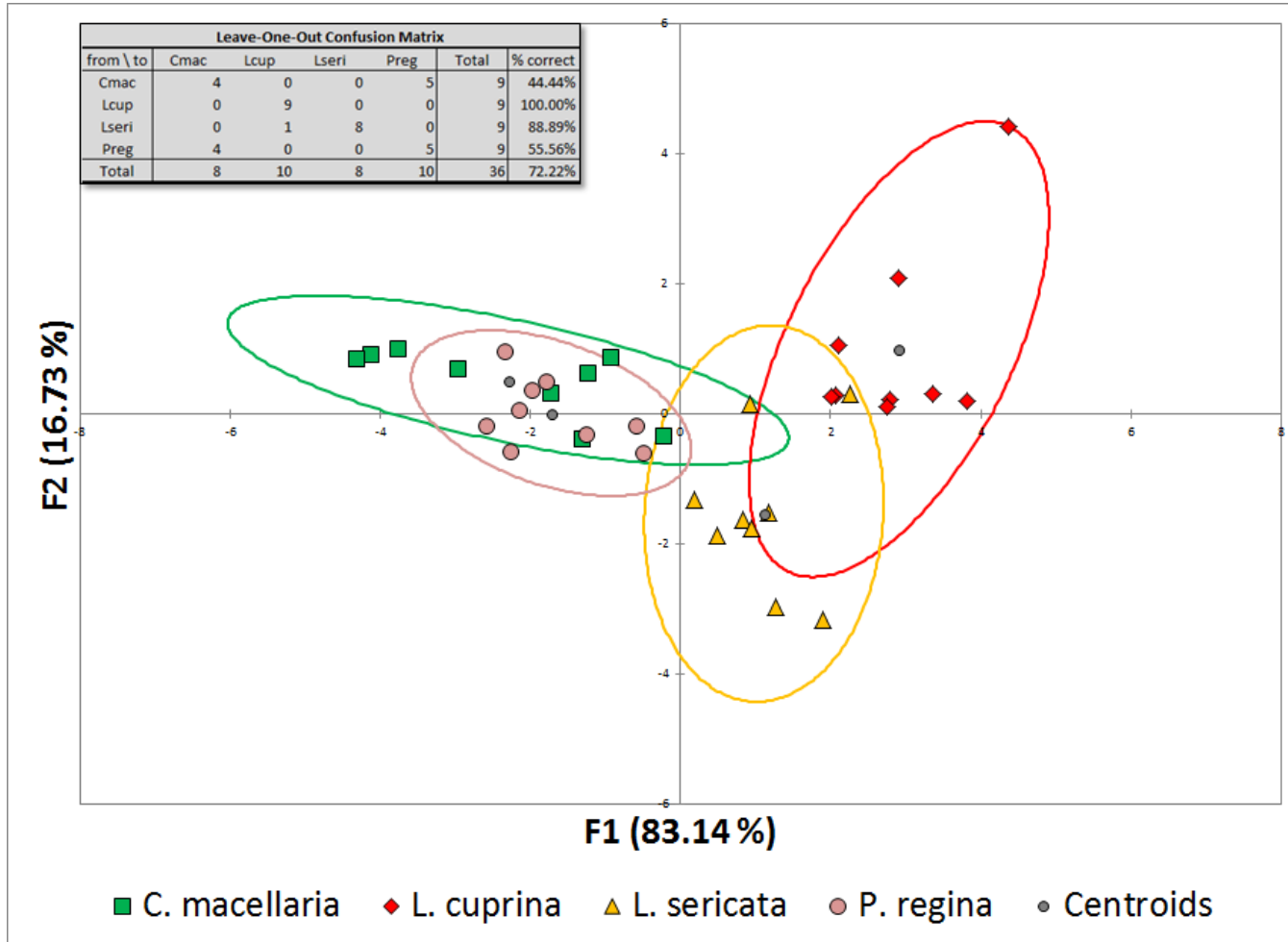


Figure A4. Discriminant analysis of the first three principal components for all four species at ADH 5016 with accompanying leave-one-out confusion matrix.

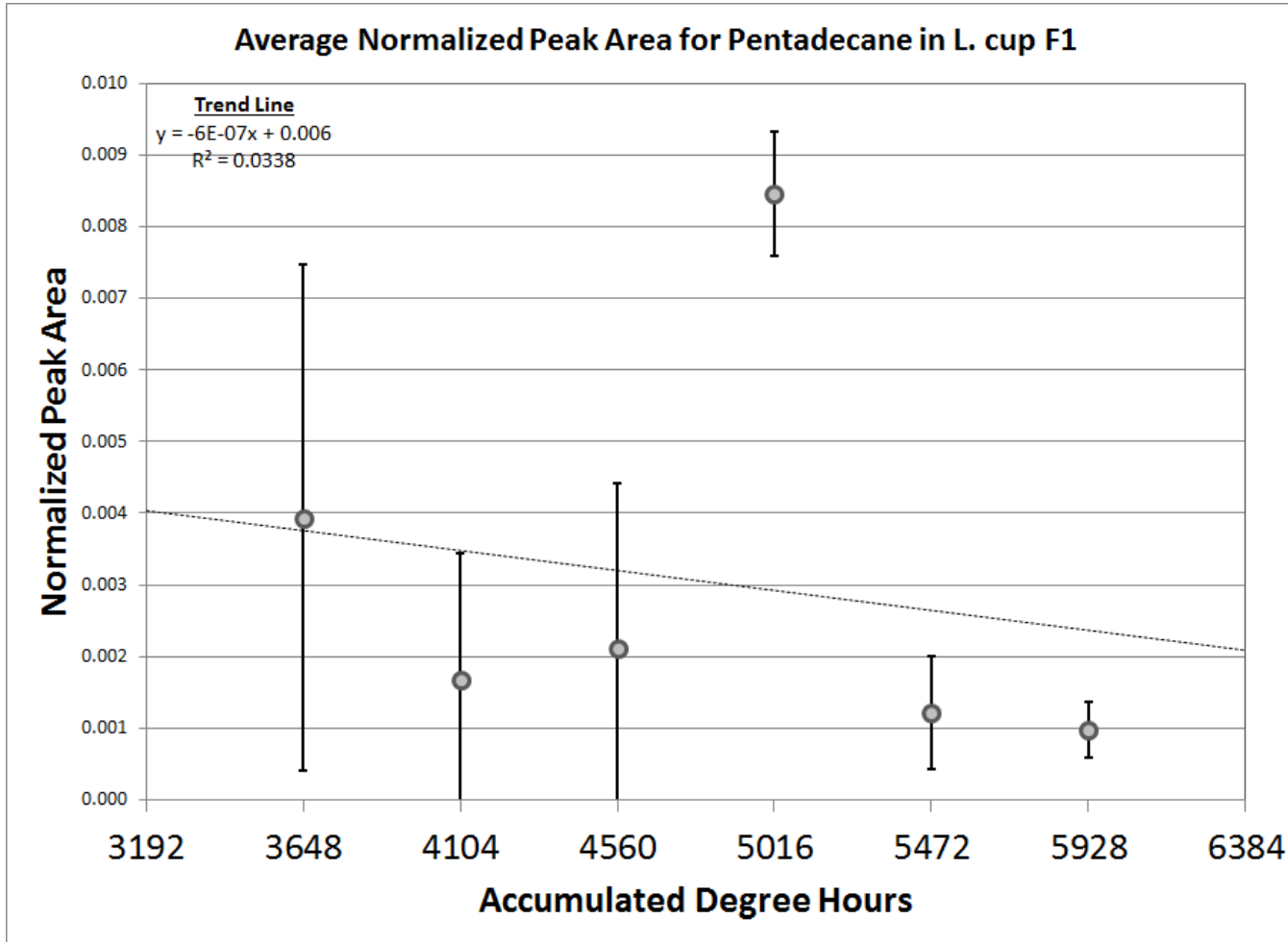


Figure A5. Change in normalized peak area of pentadecane over time for *Lucilia cuprina* F1.

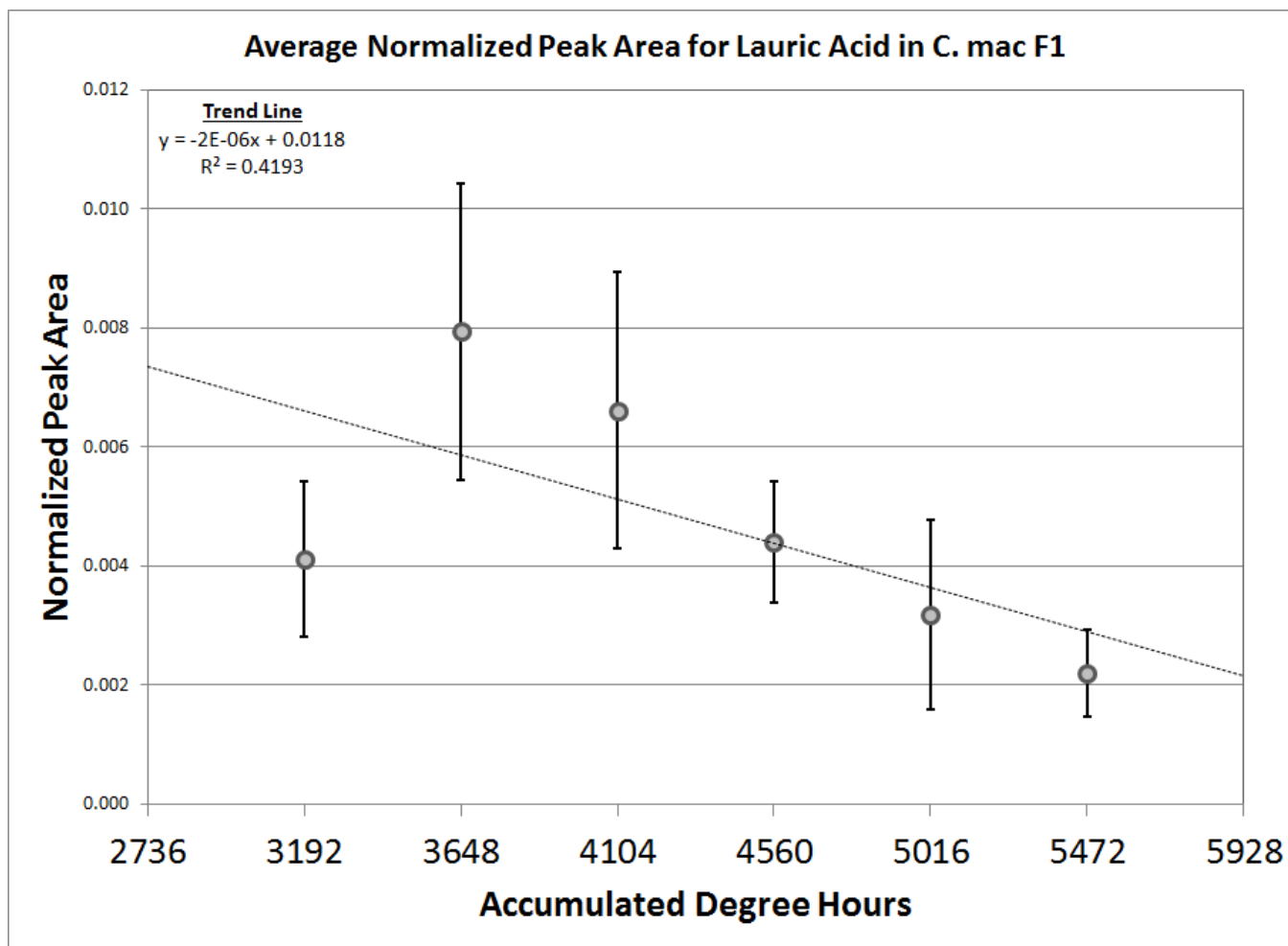


Figure A6. Change in normalized peak area of lauric acid over time for *Cochliomyia macellaria* F1.

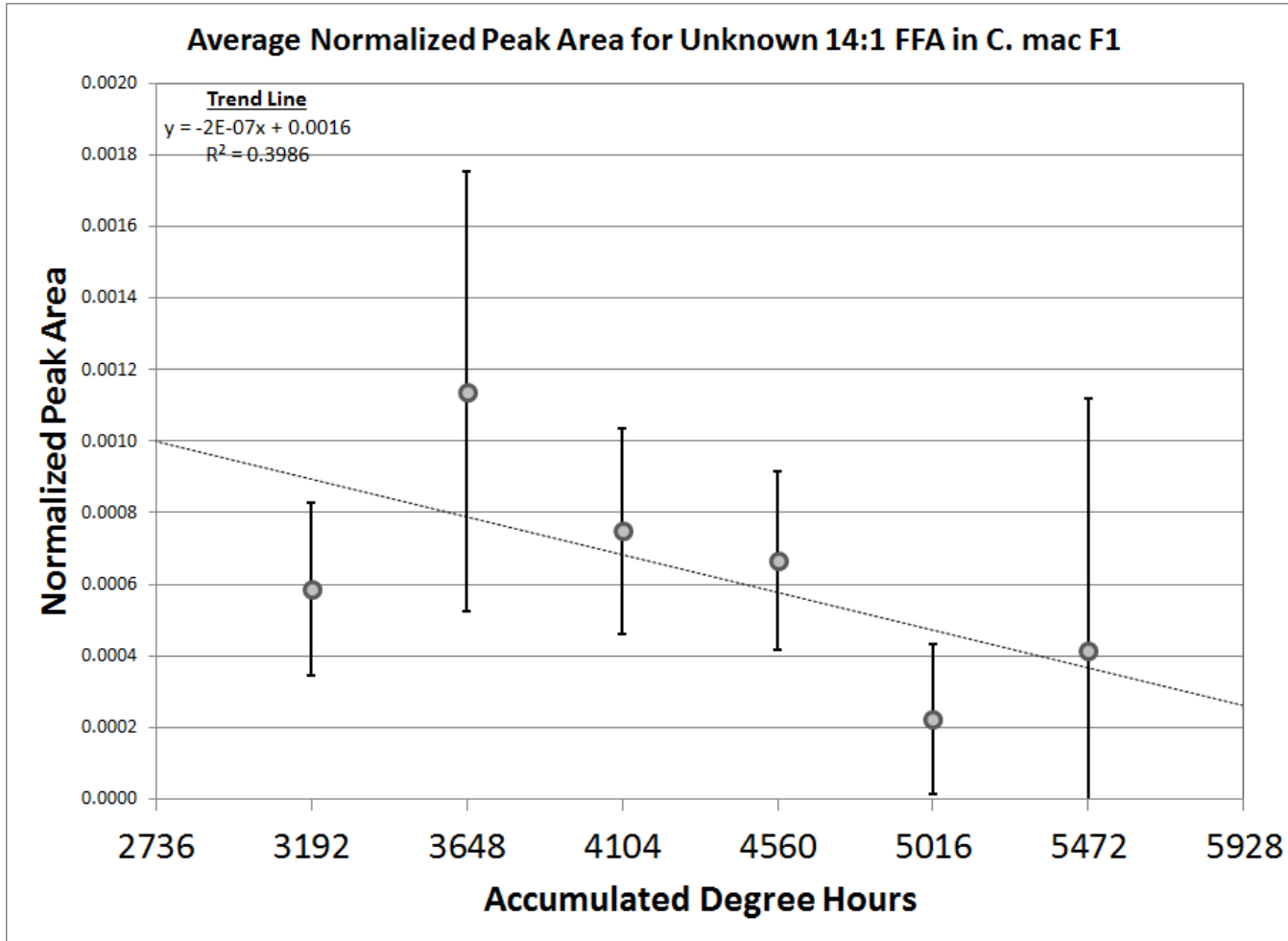


Figure A7. Change in normalized peak area of unknown 14:1 FFA over time for *Cochliomyia macellaria* F1.

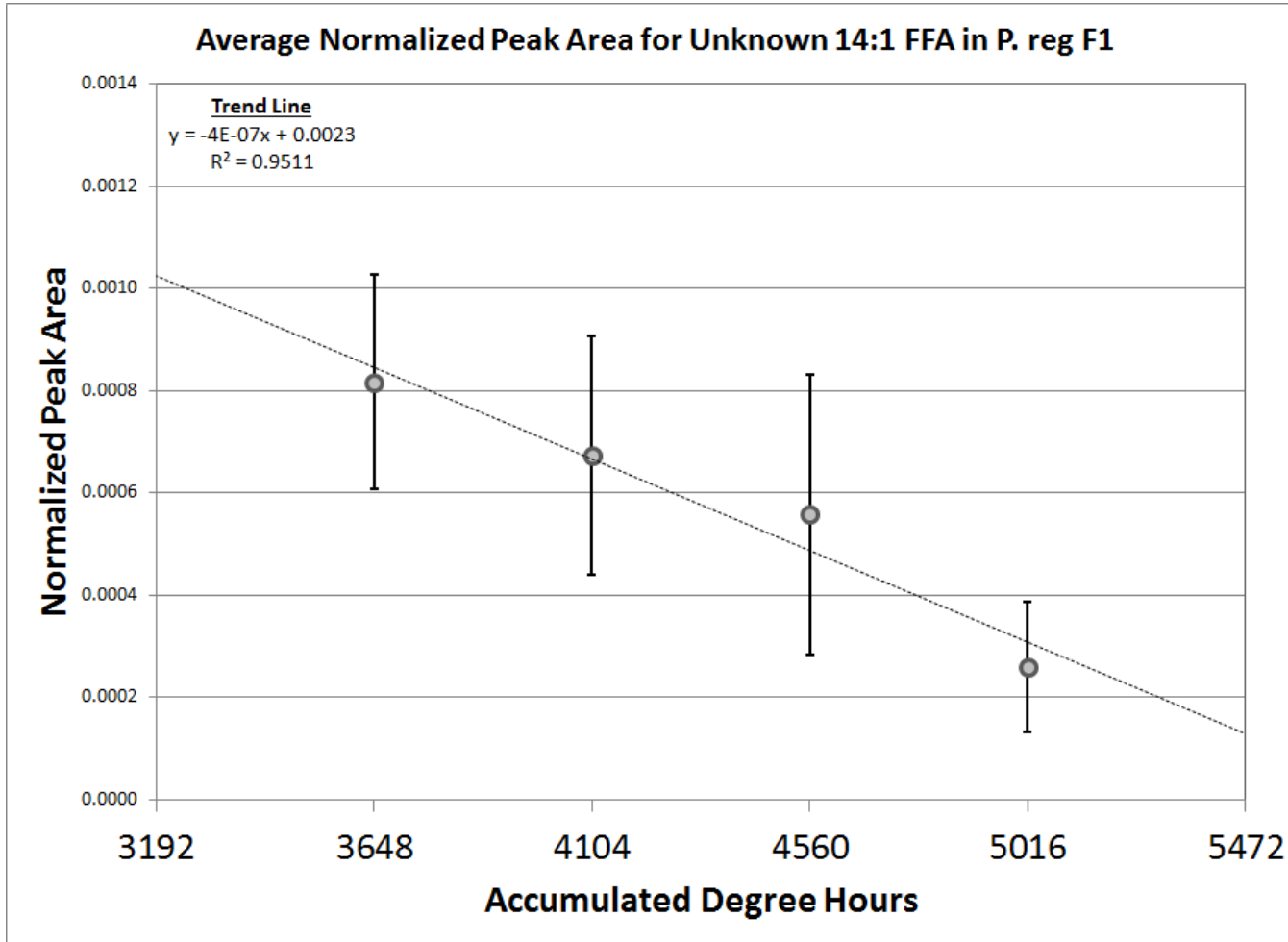


Figure A8. Change in normalized peak area of unknown 14:1 FFA over time for *Phormia regina* F1.

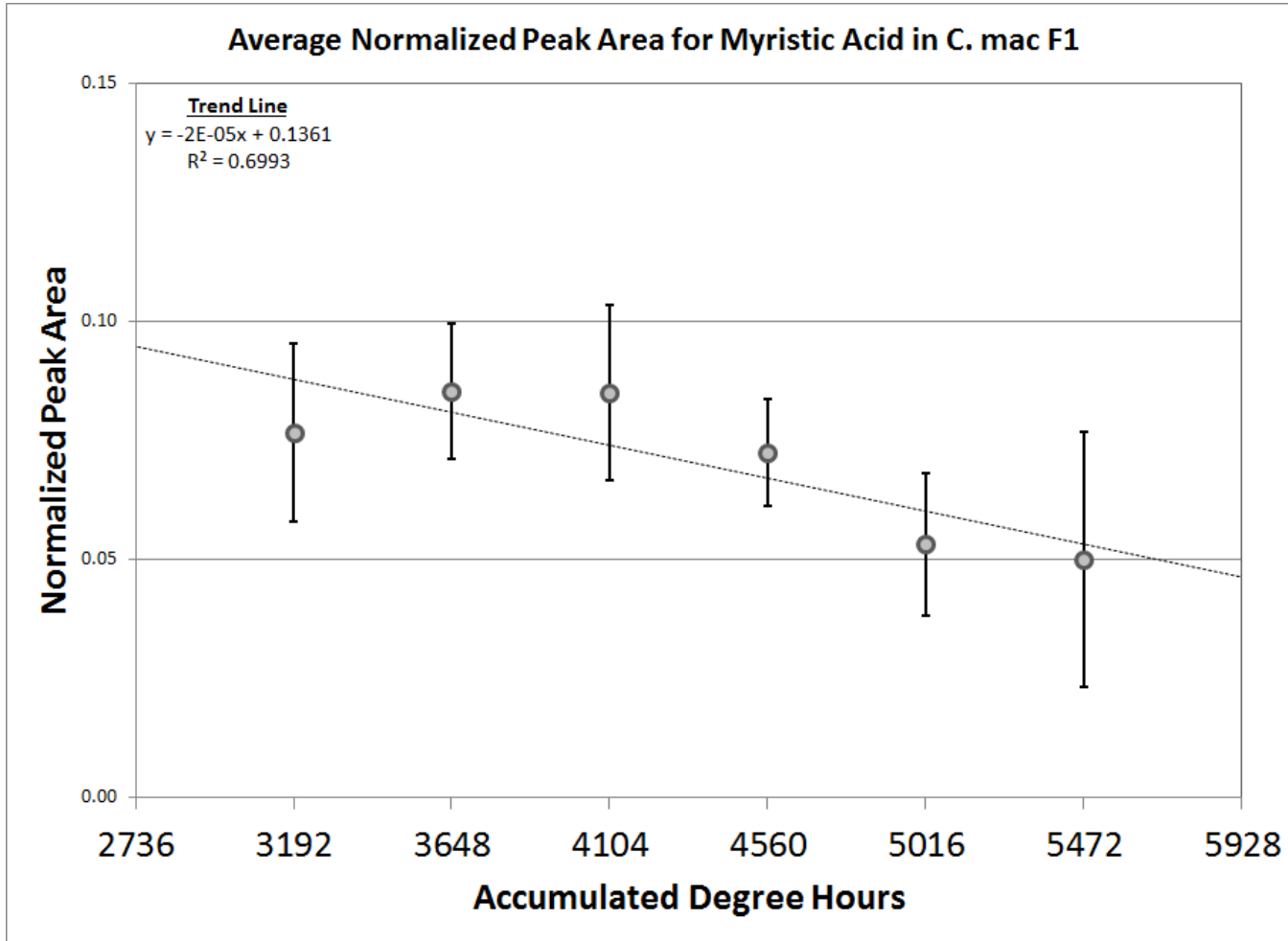


Figure A9. Change in normalized peak area of myristic acid over time for *Cochliomyia macellaria* F1.

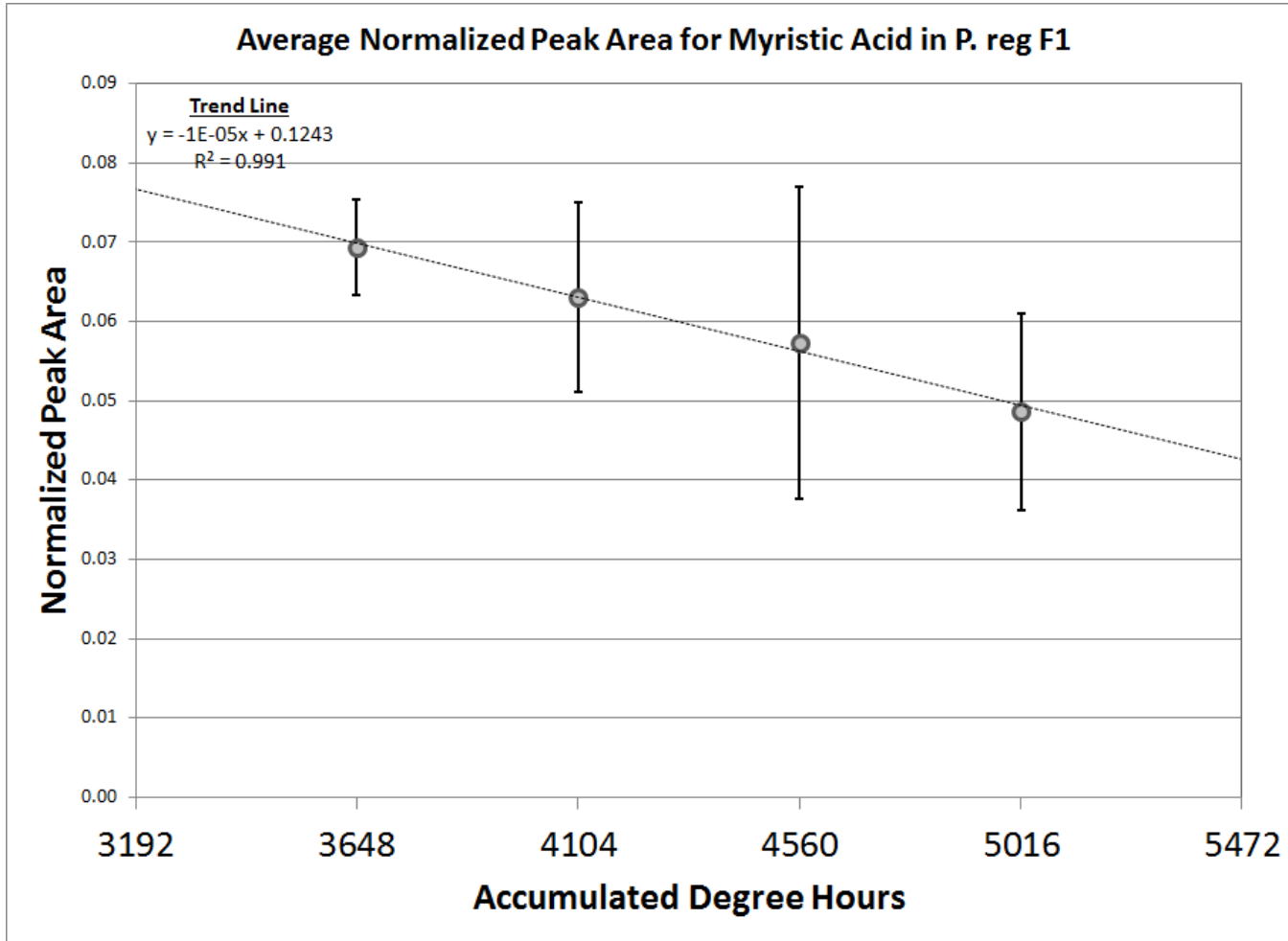


Figure A10. Change in normalized peak area of myristic acid over time for *Phormia regina* F1.

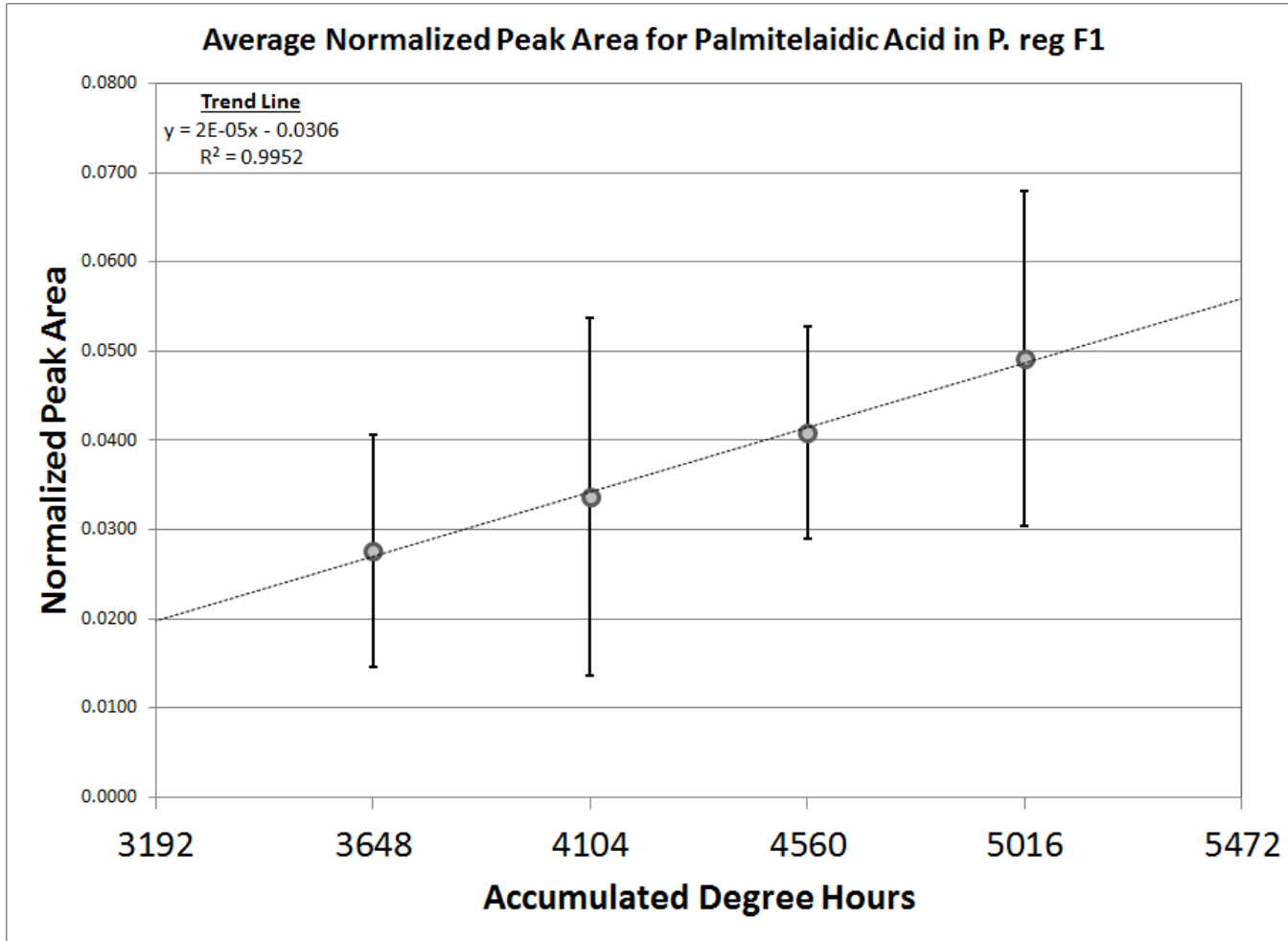


Figure A11. Change in normalized peak area of palmitelaidic acid over time for *Phormia regina* F1.

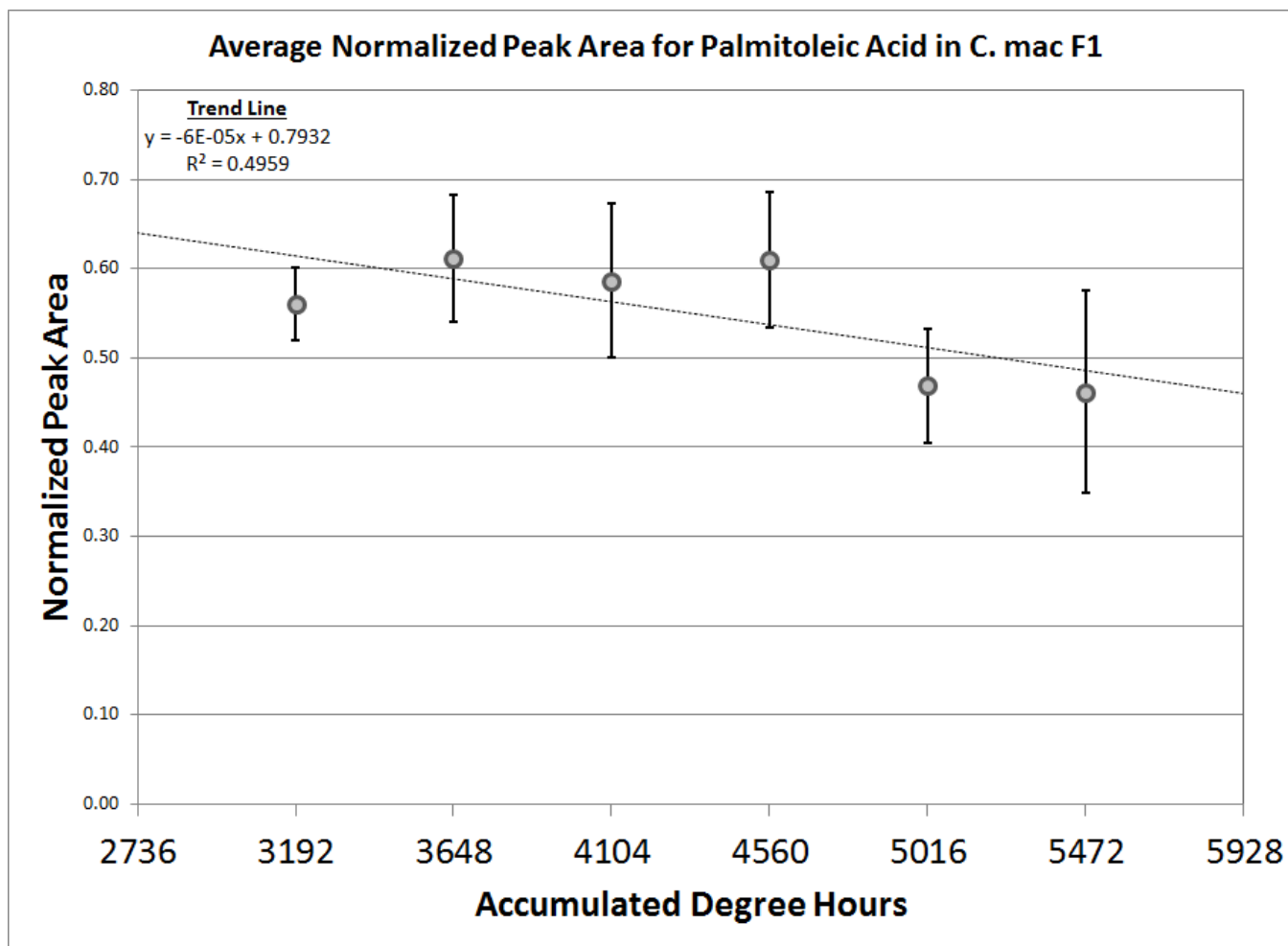


Figure A12. Change in normalized peak area of palmitoleic acid over time for *Cochliomyia macellaria* F1.

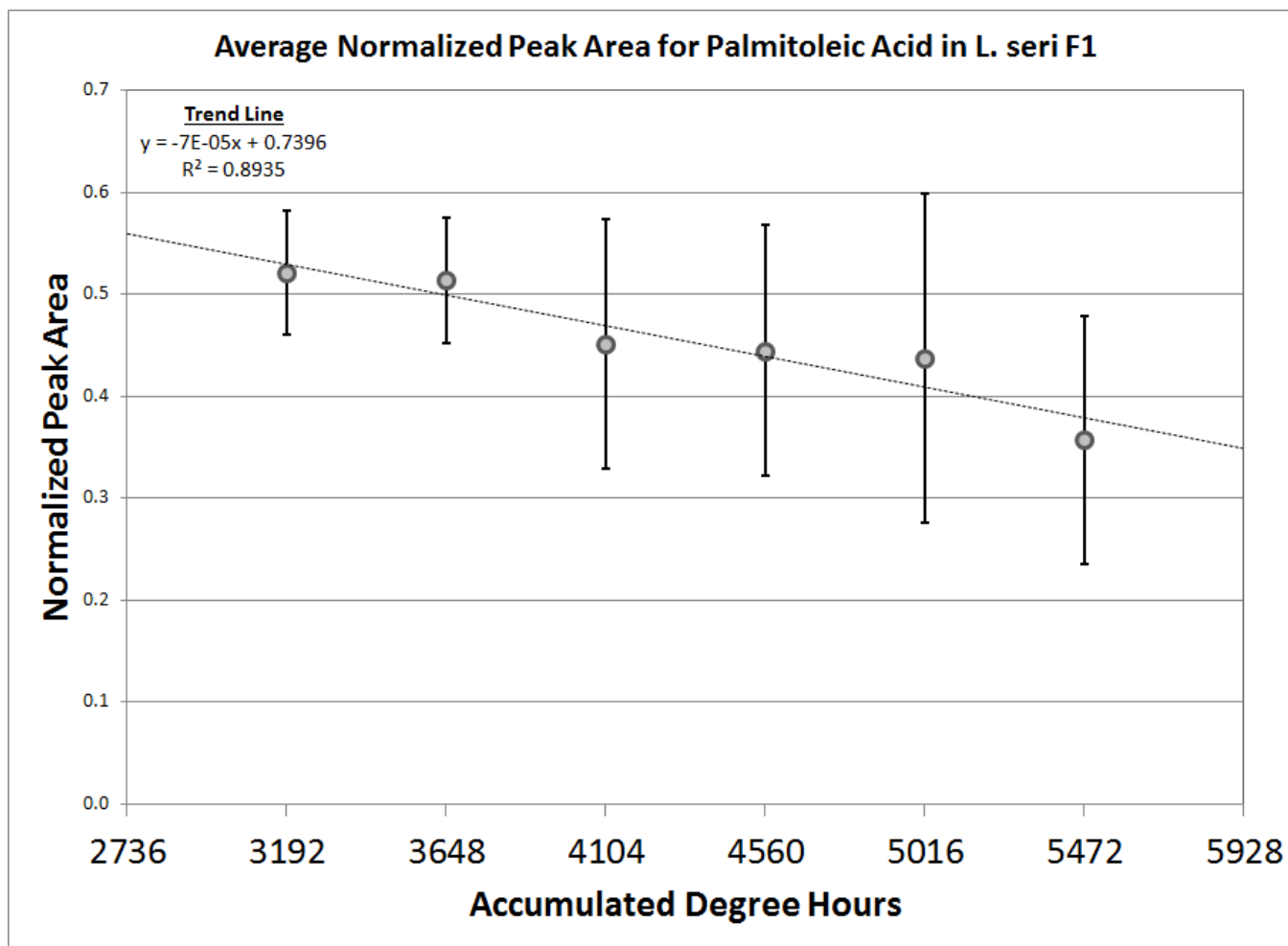


Figure A13. Change in normalized peak area of palmitoleic acid over time for *Lucilia sericata* F1.

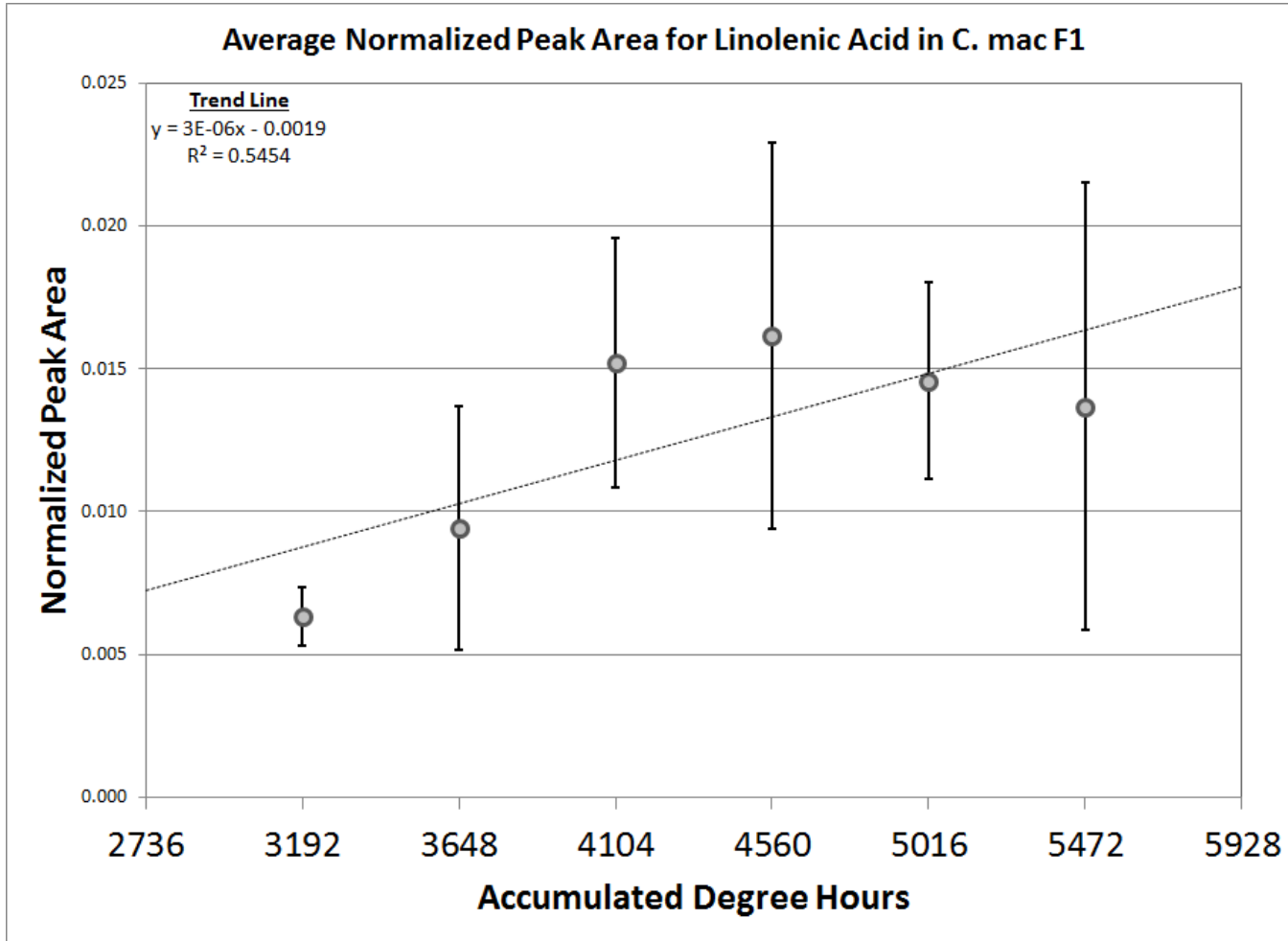


Figure A14. Change in normalized peak area of linolenic acid over time for *Cochliomyia macellaria* F1.

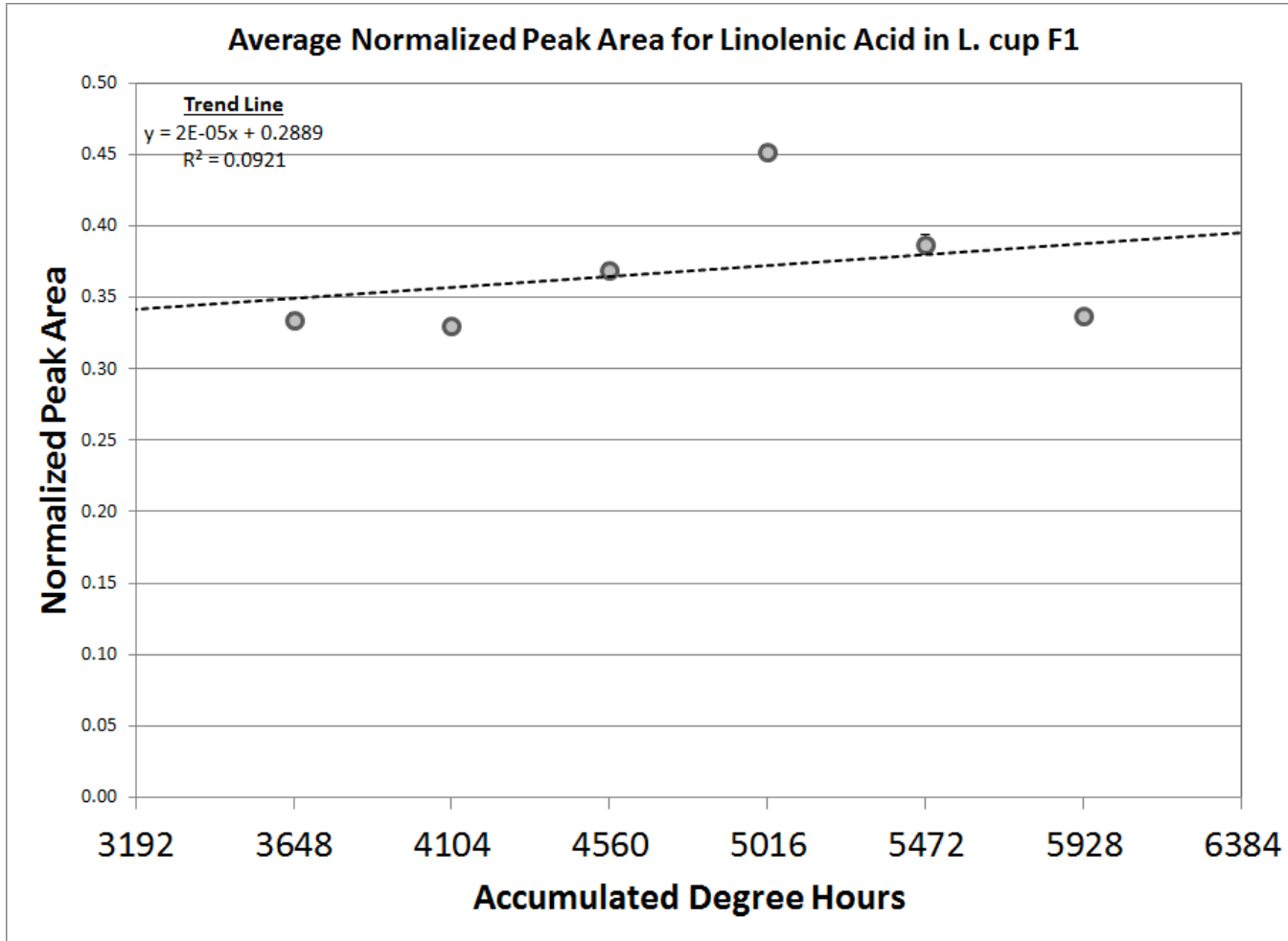


Figure A15. Change in normalized peak area of linolenic acid over time for *Lucilia cuprina* F1.

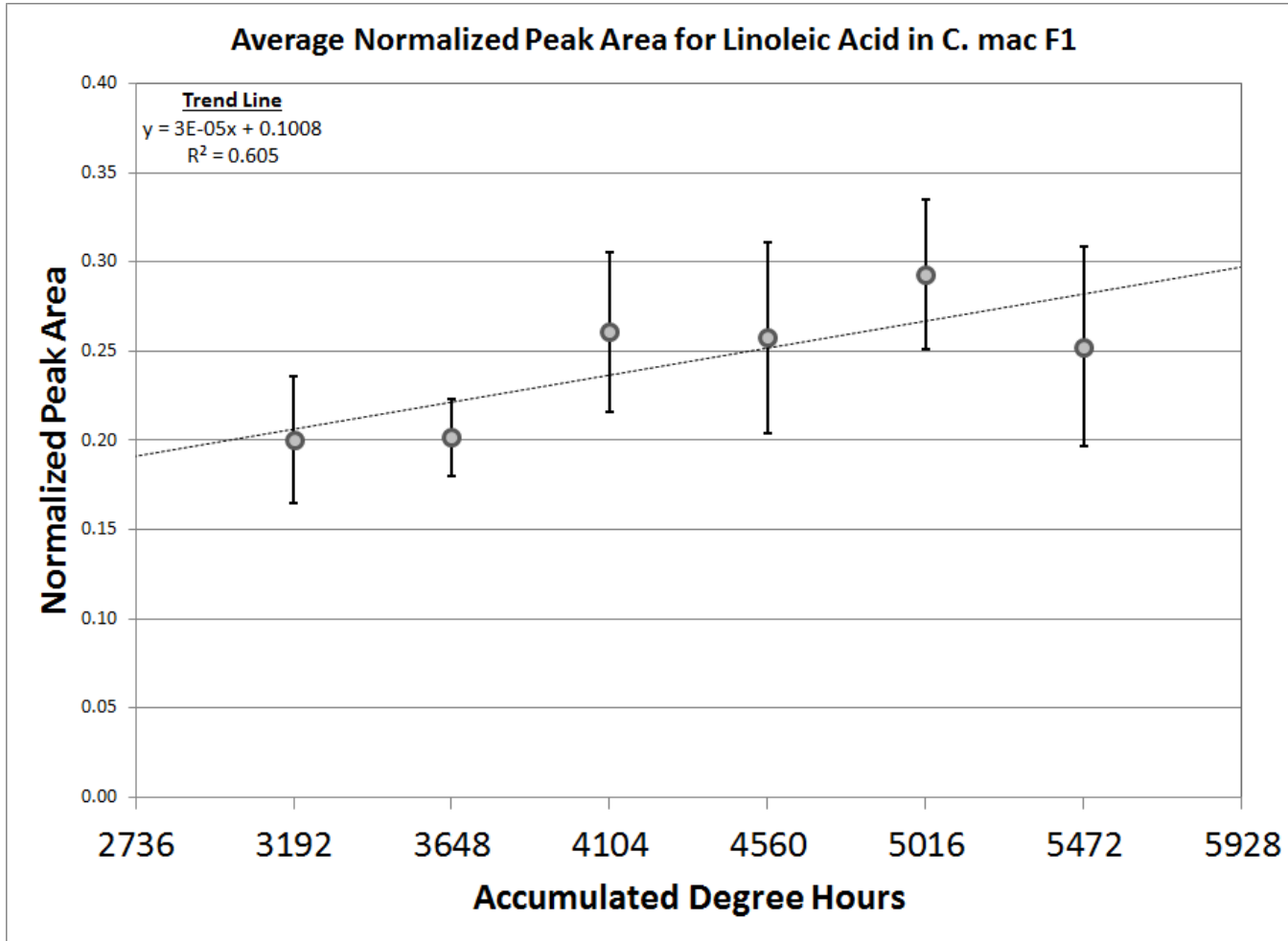


Figure A16. Change in normalized peak area of linoleic acid over time for *Cochliomyia macellaria* F1.

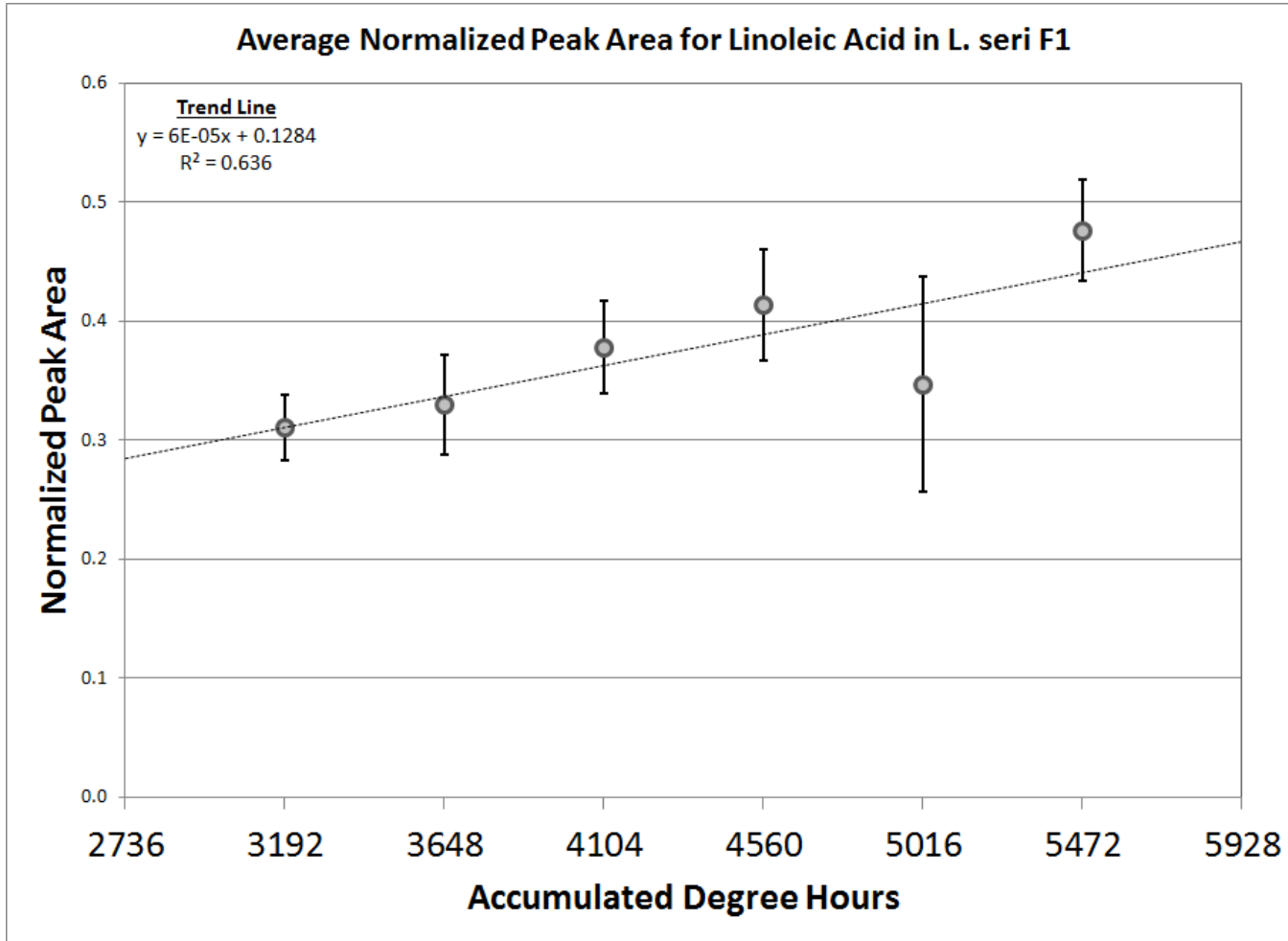


Figure A17. Change in normalized peak area of linoleic acid over time for *Lucilia sericata* F1.

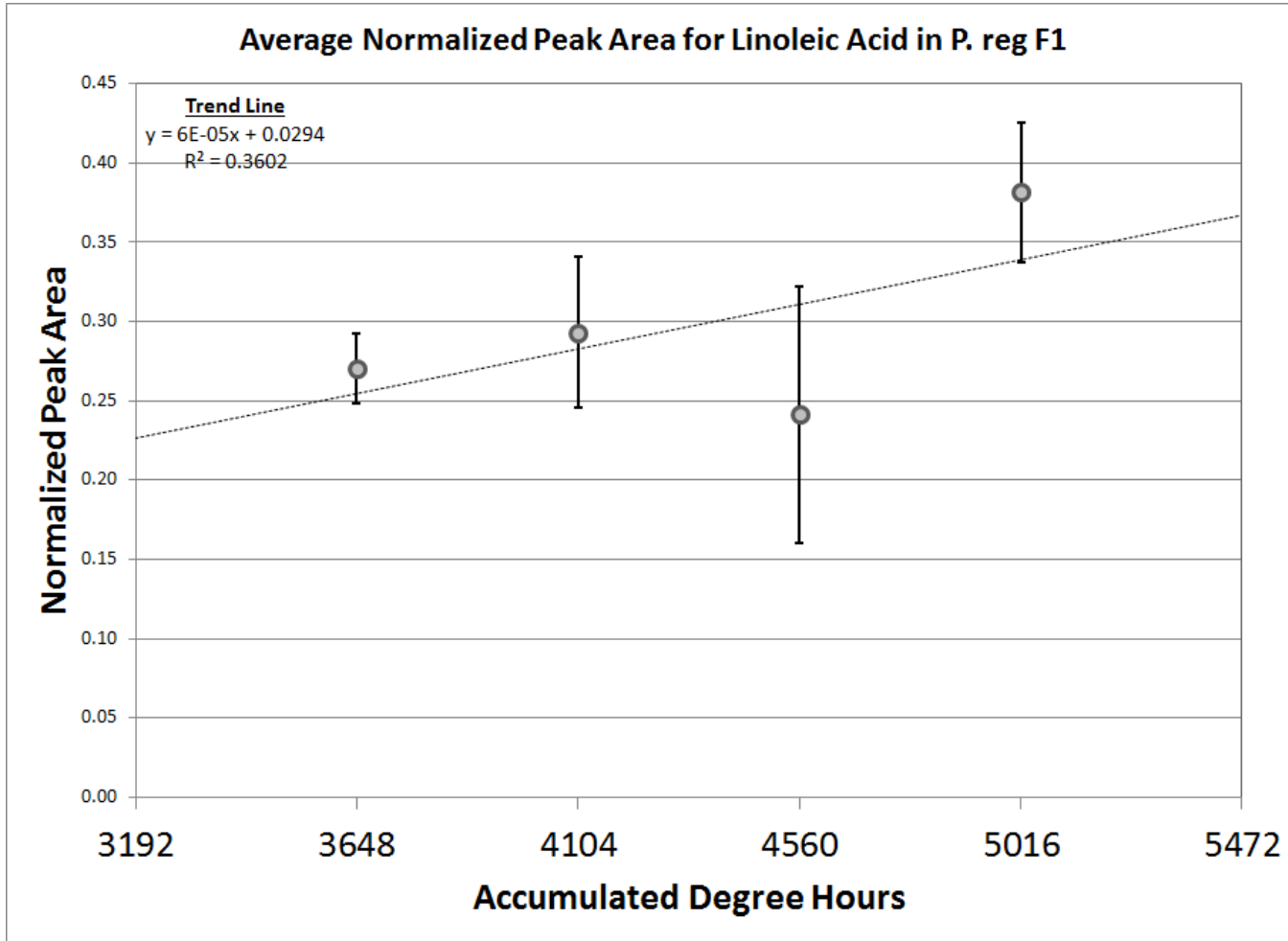


Figure A18. Change in normalized peak area of linoleic acid over time for *Phormia regina* F1.

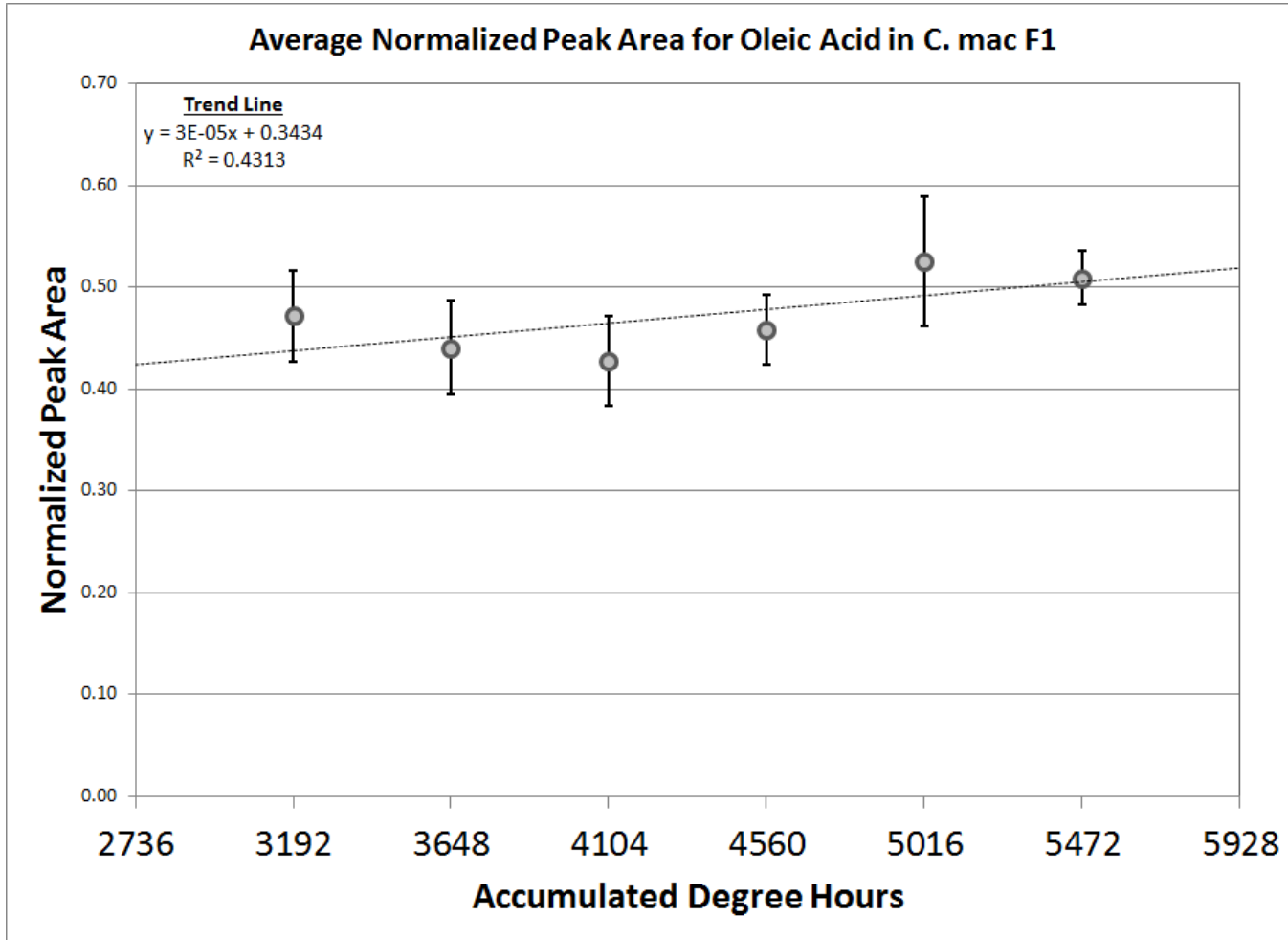


Figure A19. Change in normalized peak area of oleic acid over time for *Cochliomyia macellaria* F1.

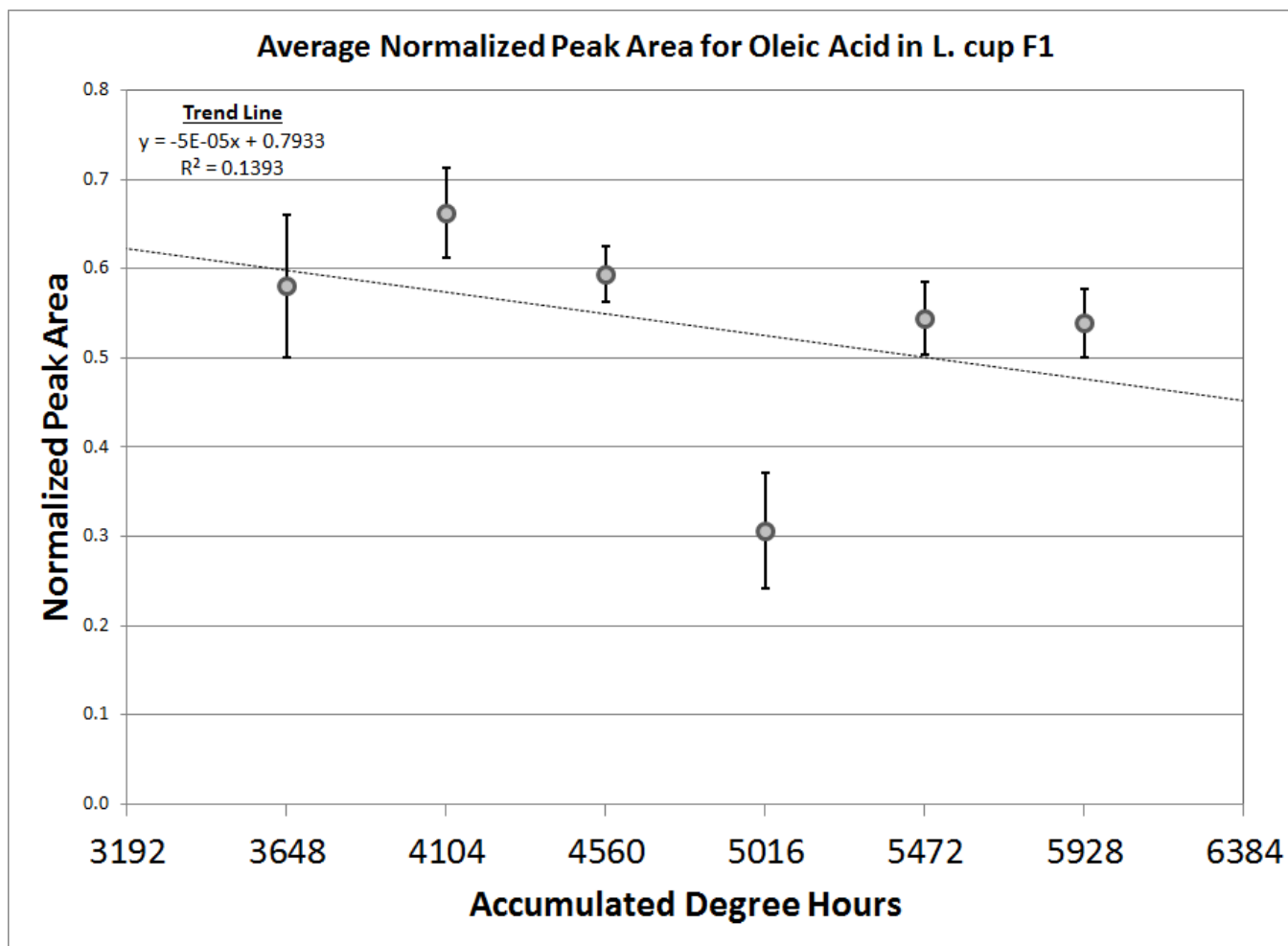


Figure A20. Change in normalized peak area of oleic acid over time for *Lucilia cuprina* F1.

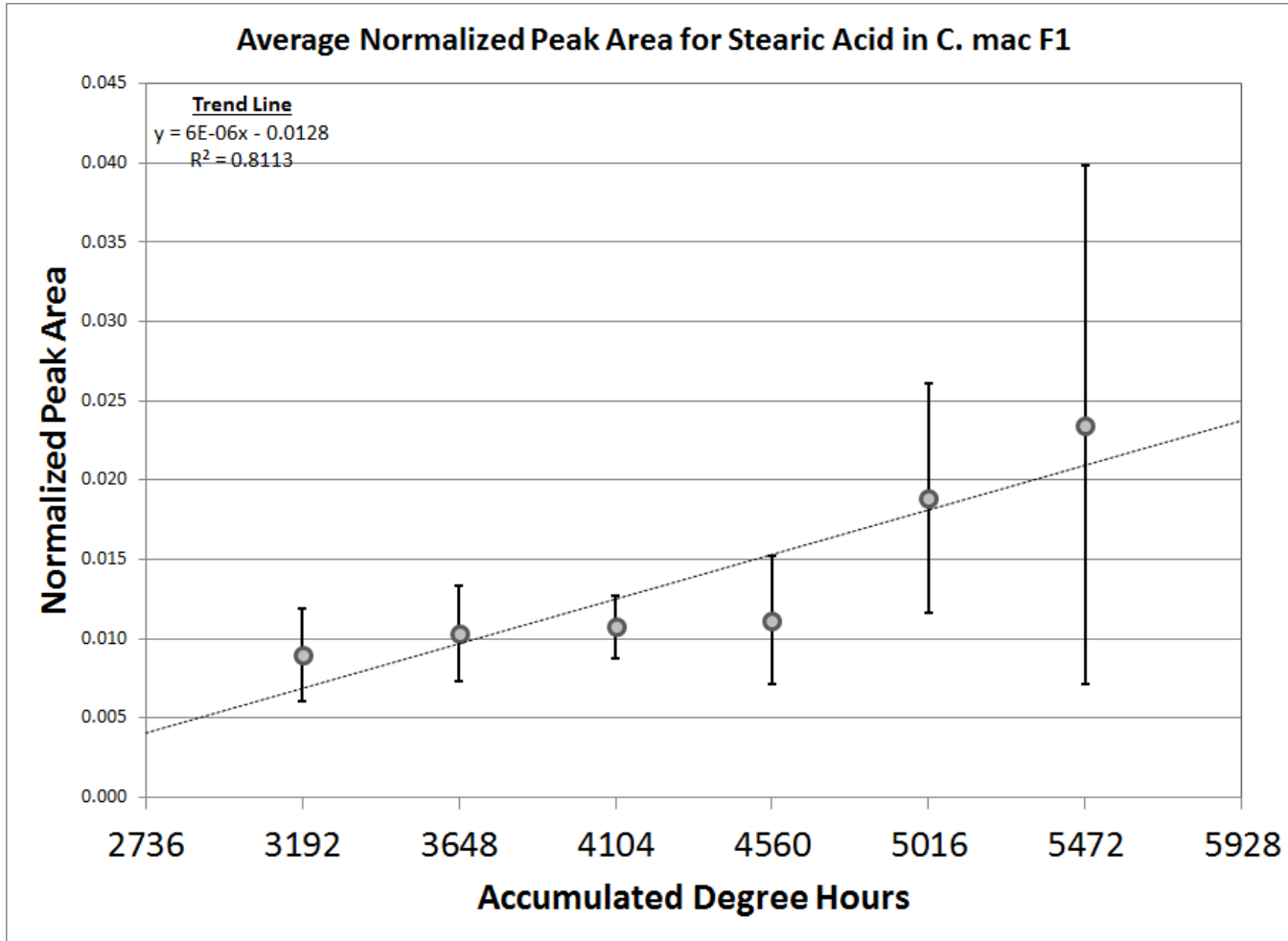


Figure A21. Change in normalized peak area of stearic acid over time for *Cochliomyia macellaria* F1.

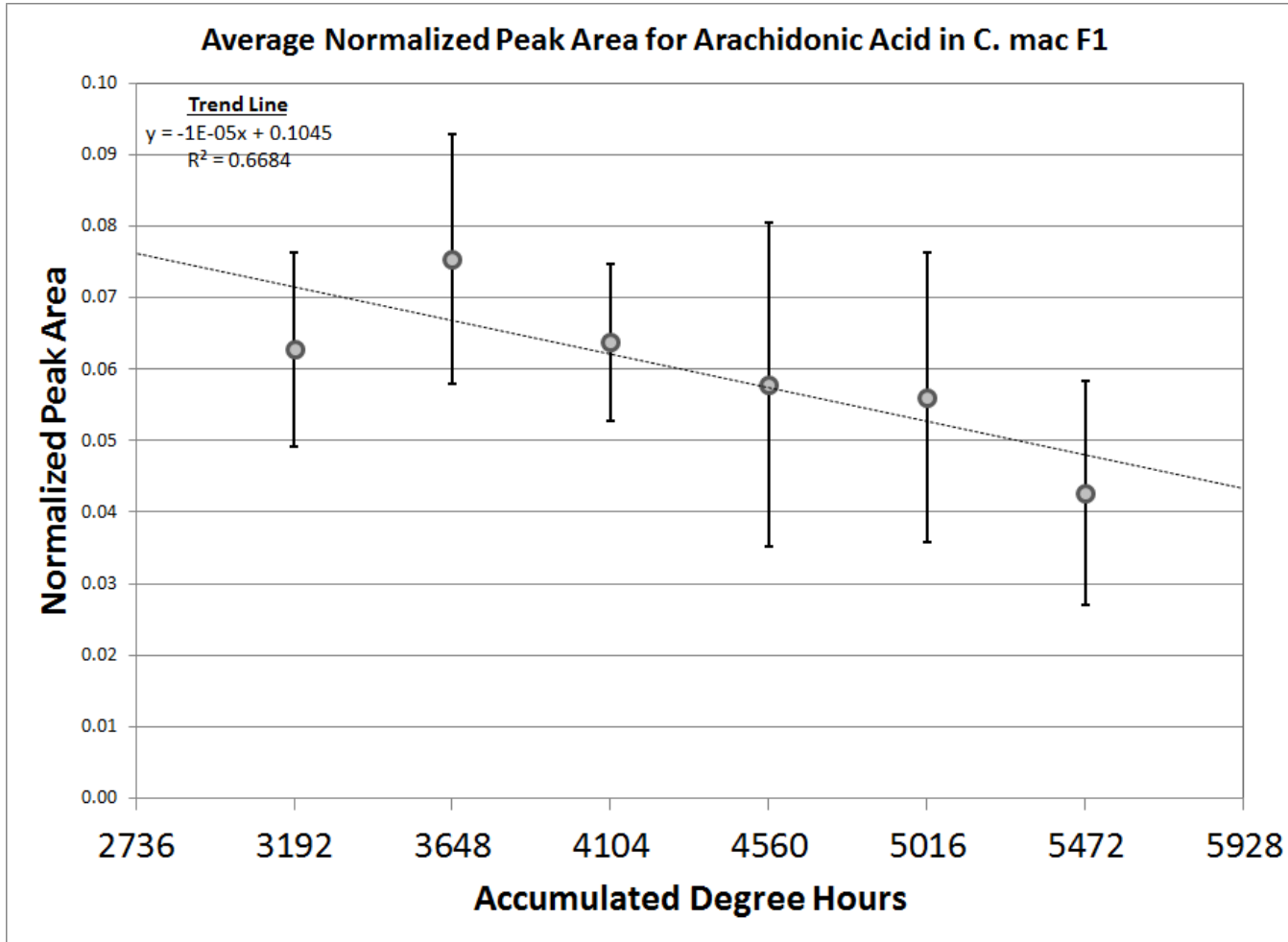


Figure A22. Change in normalized peak area of arachidonic acid over time for *Cochliomyia macellaria* F1.

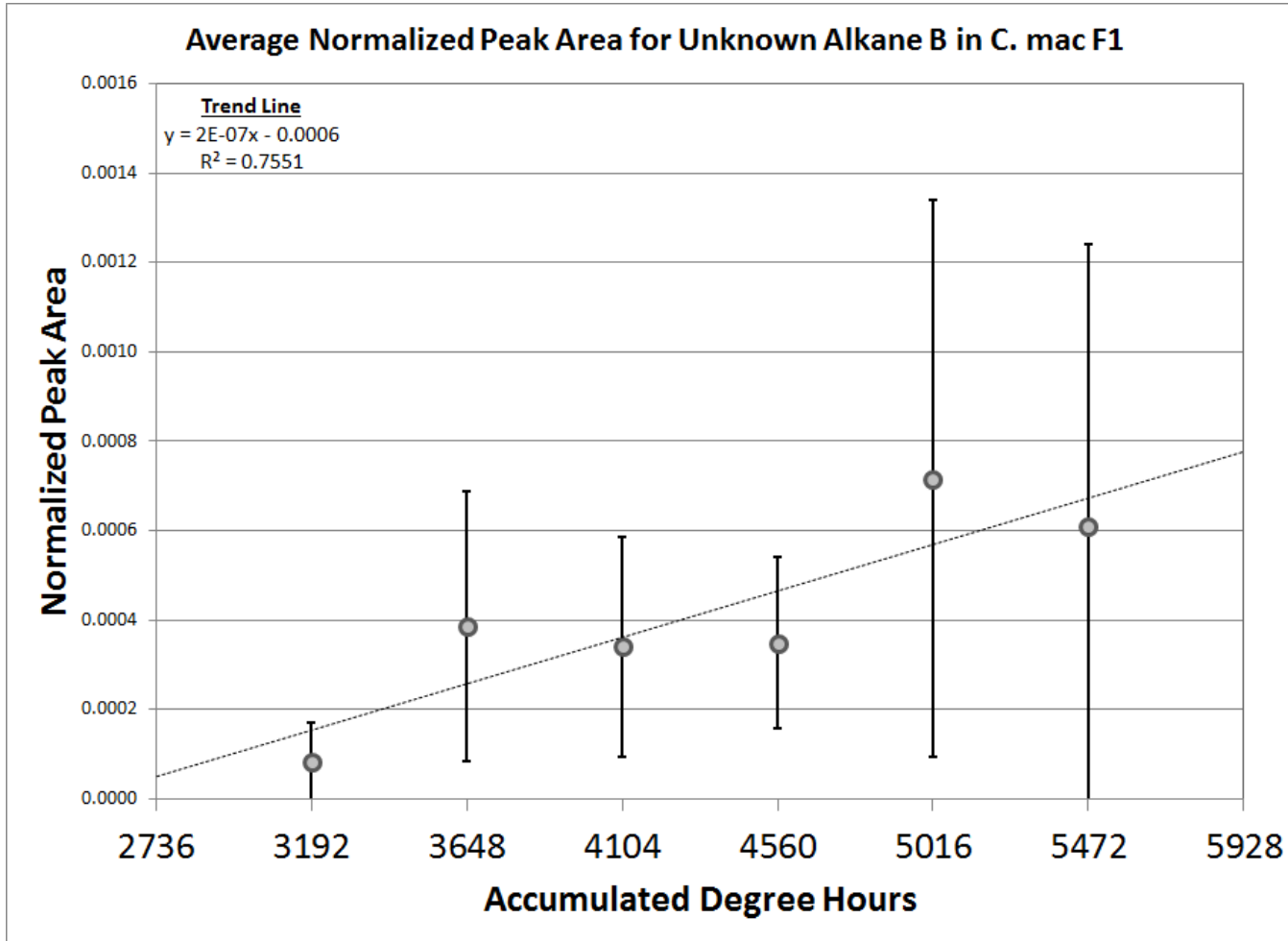


Figure A23. Change in normalized peak area of unknown alkane B time for *Cochliomyia macellaria* F1.

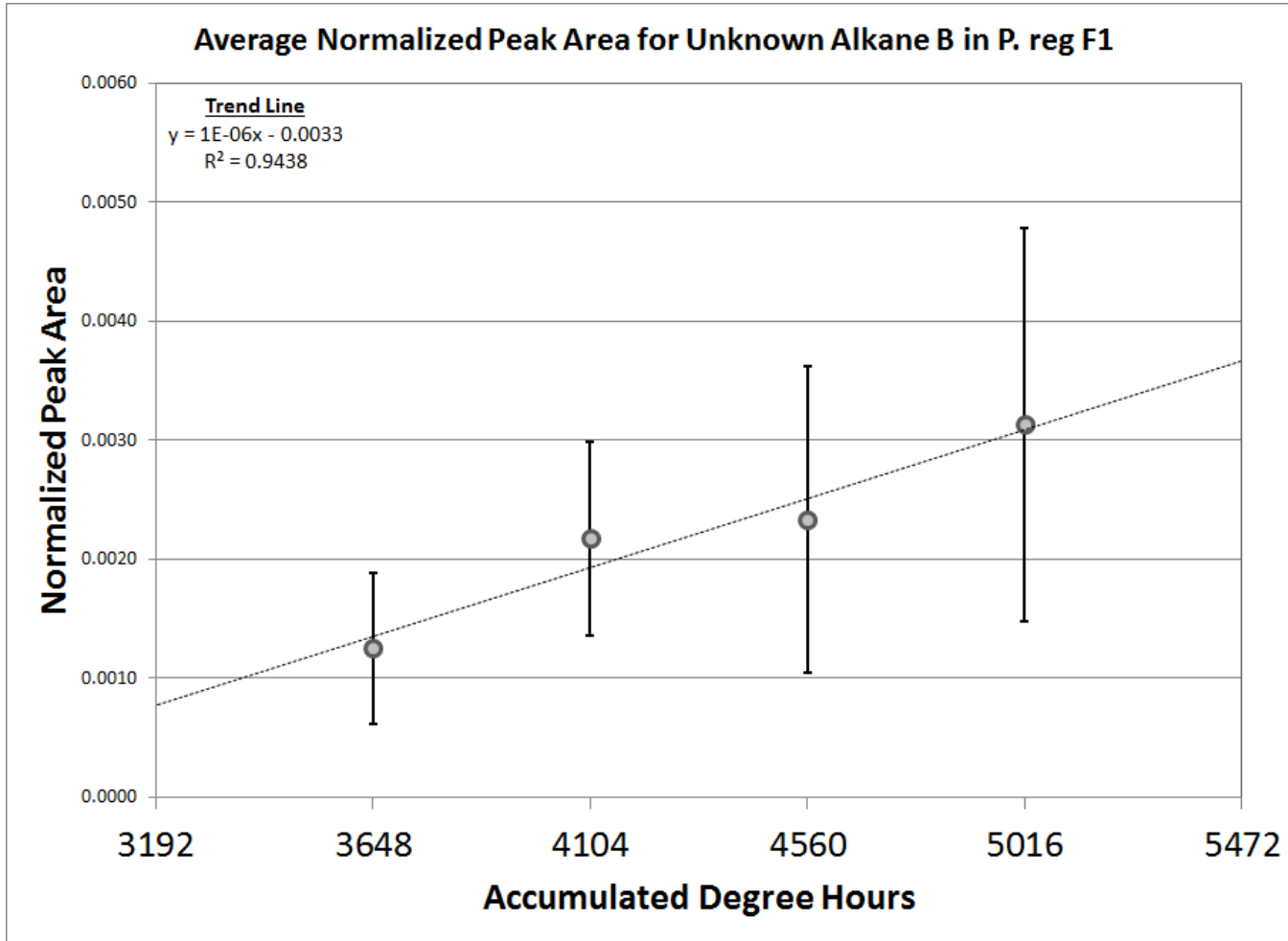


Figure A24. Change in normalized peak area of unknown alkane B over time for *Phormia regina* F1.

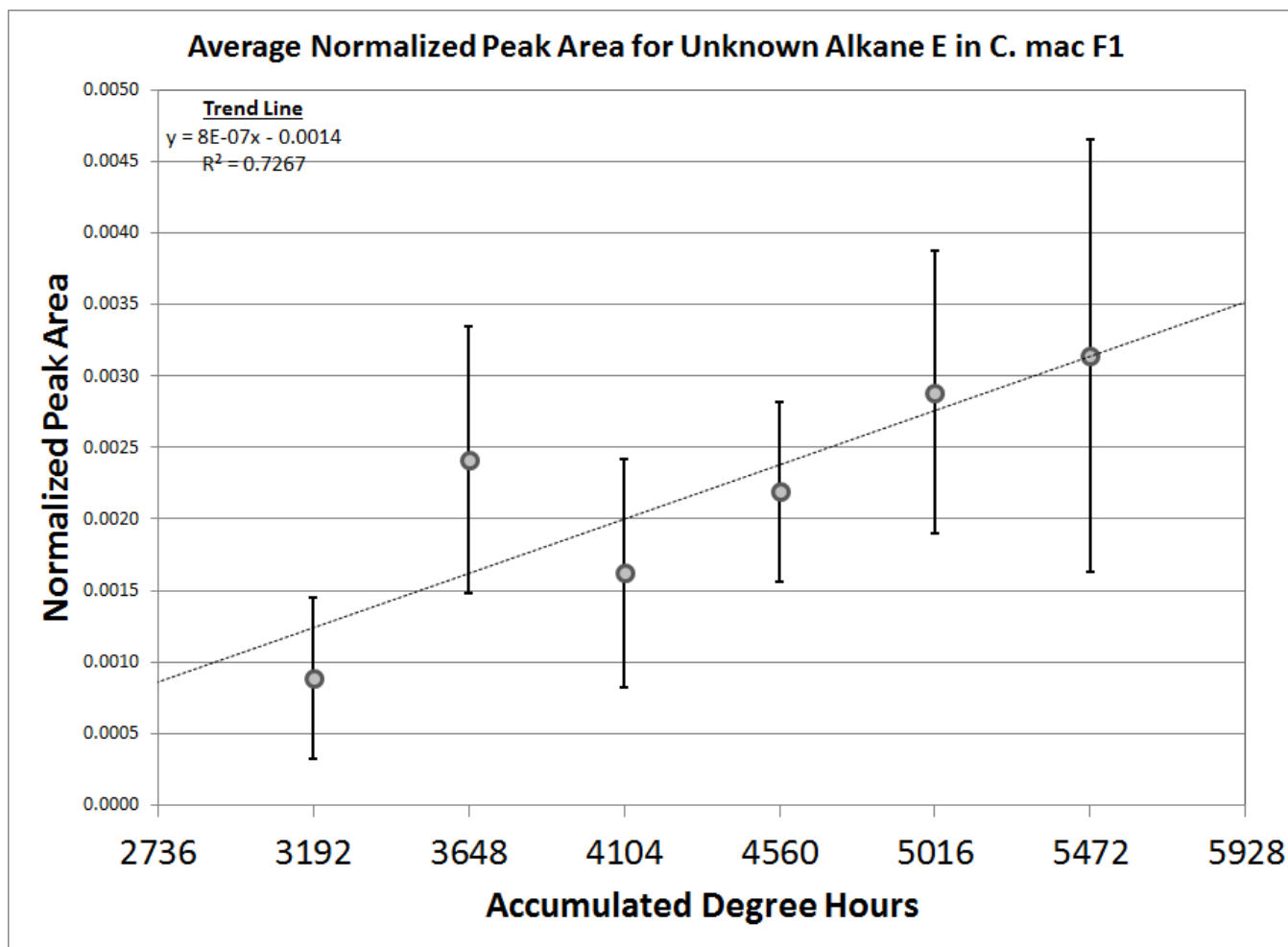


Figure A25. Change in normalized peak area of unknown alkane E over time for *Cochliomyia macellaria* F1.

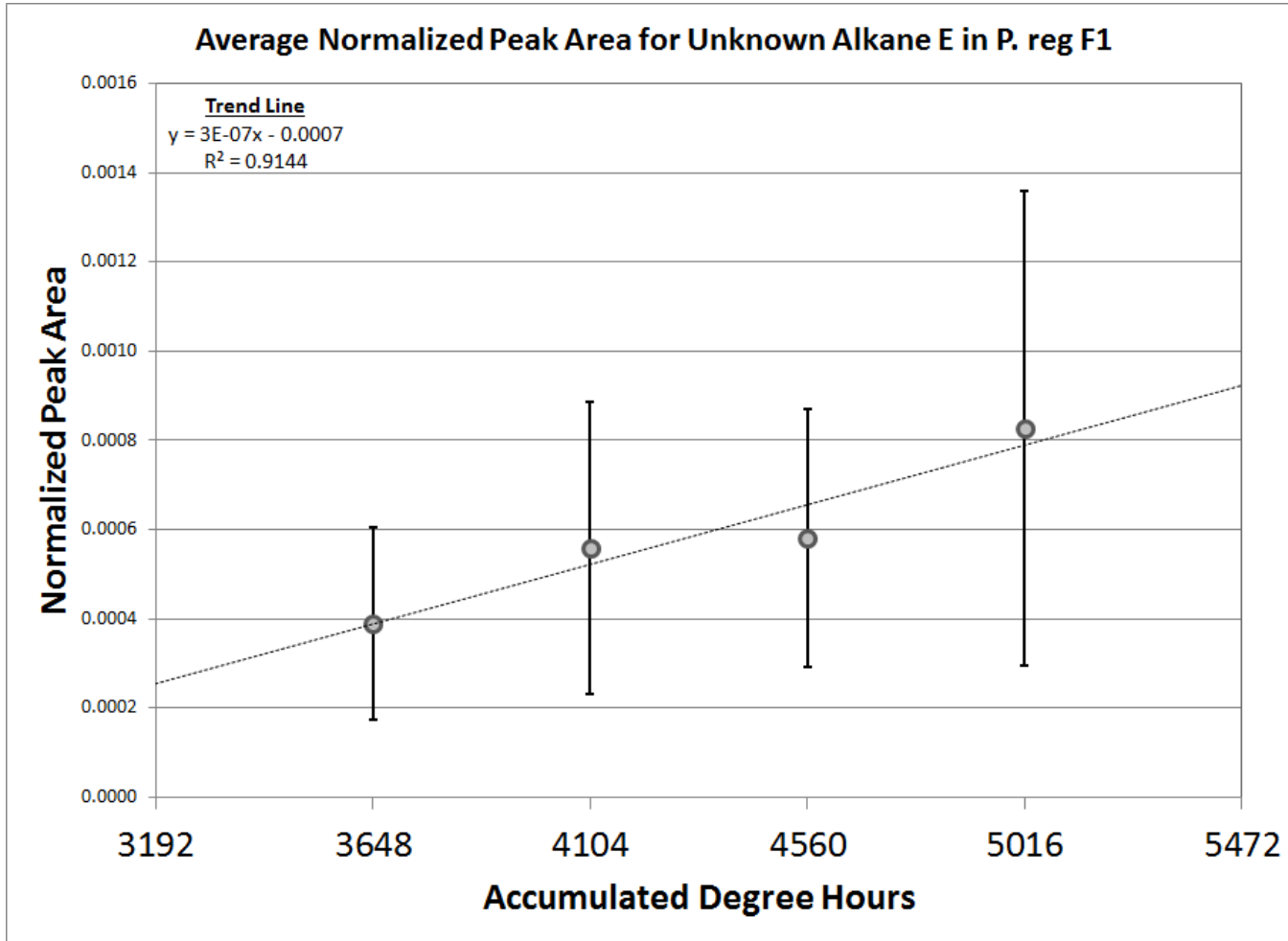


Figure A26. Change in normalized peak area of unknown alkane E over time for *Phormia regina* F1.

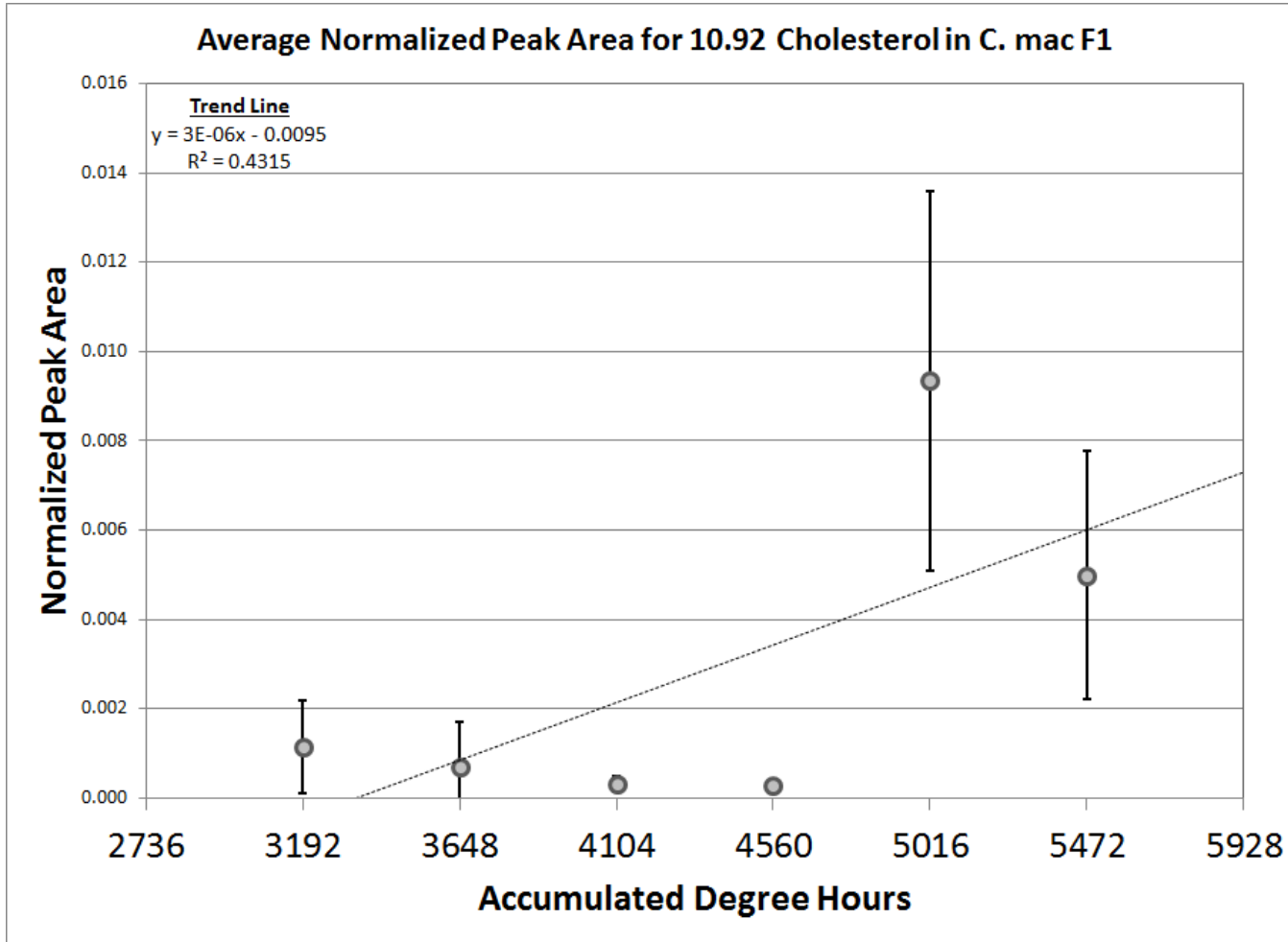


Figure A27. Change in normalized peak area of cholesterol over time for *Cochliomyia macellaria* F1.

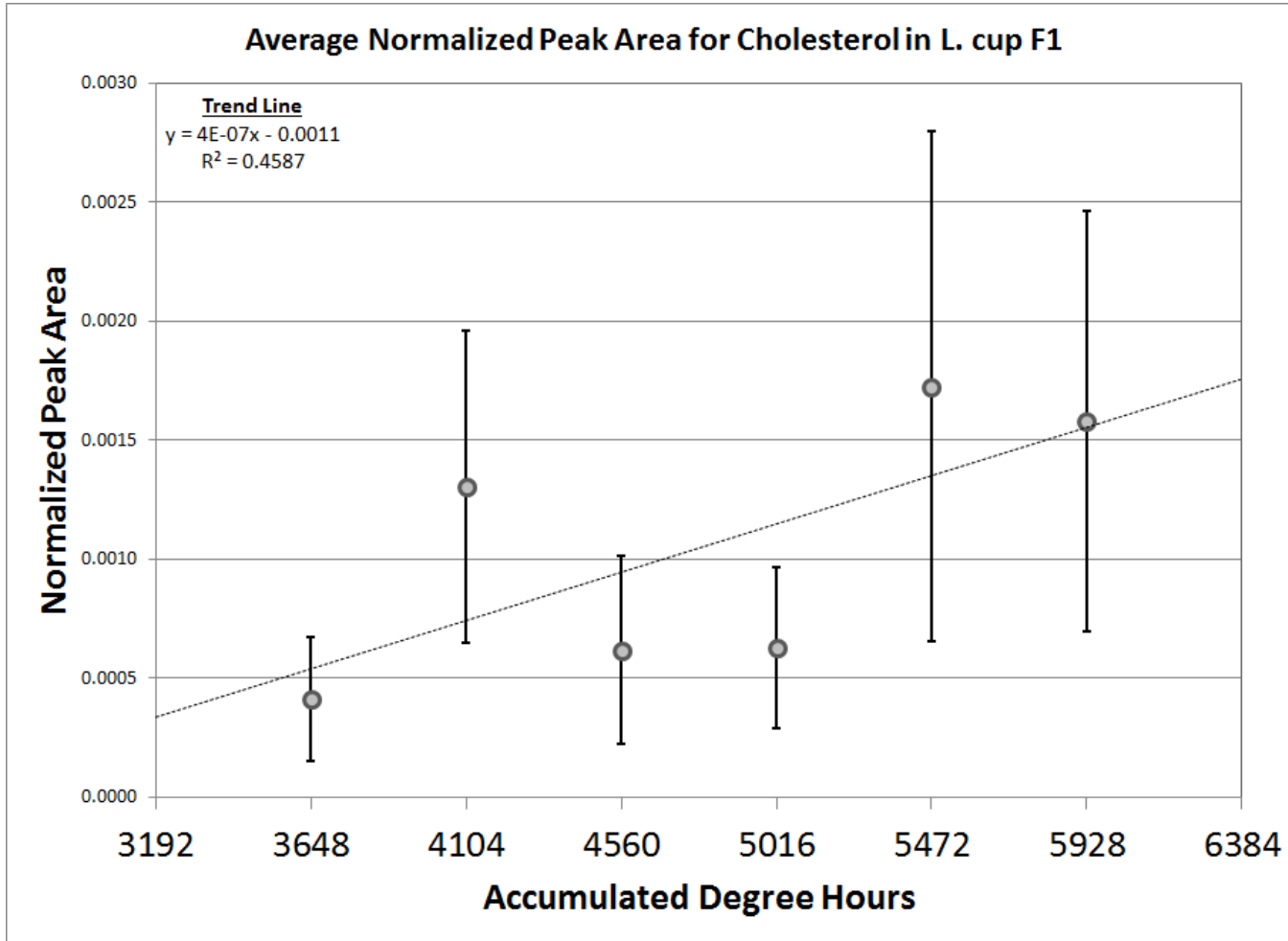


Figure A28. Change in normalized peak area of cholesterol over time for *Lucilia cuprina* F1.

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William D. Kranz was born in Sacramento, California in 1987. After graduating from Hopkins High School in Minnetonka, Minnesota in 2006, he attended college at the University of Central Florida in Orlando, where he received his B.S. in forensic science in 2011. In 2016, he received his Ph.D. from Purdue University for work done at Indiana University-Purdue University Indianapolis under Dr. John Goodpaster.