FINAL REPORT

Project Title: Species and age determination of blow fly pupae based upon headspace analysis

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ABSTRACT

The study of insects found on a body can yield valuable information about the time and circumstances of death. In forensic entomological investigations, the oldest of such insects are traditionally collected from the corpse, their species and age determined, and a postmortem interval (PMI) can be estimated. In most cases, these insects are flies of the Dipteran family *Calliphoridae*. Females will lay eggs on a body shortly after death, the eggs will hatch and larvae will feed on the decomposing tissue through three larval life stages, reach a developmental threshold and leave the body to pupate and later emerge as an adult to start the life cycle again.

The determination of the PMI assumes that the oldest specimen from the body is collected, and this is often pupae. Pupae are notoriously difficult to use as casework samples, as it is generally difficult to identify their species, and determining the age of a pupa either requires expertise not generally available. We develop several means by which the species and age of fly pupae could be identified via the analysis of chemical extracts. These compounds are contained within the cuticle—the protective "skin" of the pupa as well as the metamorphizing insect itself. The analysis of organic compounds is highly amenable to the existing infrastructure of forensic laboratories, as it relies chiefly upon the use of gas chromatography-mass spectrometry systems. We investigated the effects of several biotic and abiotic variables on the compounds generated by the pupae. The influence of diet, pupation substrate, temperature, light level, and humidity were independently evaluated, and the compounds observed for each variable were subject to rigorous statistical treatment via chemometric methods. In general, we found that a subset of organic compounds was relatively unaffected by changes in environment, and could be used for identification and aging in entomological investigations.

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EXECUTIVE SUMMARY

PURPOSE, GOALS AND OBJECTIVES

The goal of this project was to develop a chemical method to determine the species and age of forensically relevant pupae, and to determine the effects of biotic and abiotic factors on the resulting chemical profiles. More specifically, we carried out chemical analysis of fly pupae and looked for chemical markers of species and age, and studied the effect of genetic variation, temperature, humidity, light level, and diet. Chemometric techniques that were capable of discerning patterns in the data and classifying unknown samples were used for discrimination and classification of data.

RESEARCH DESIGN AND METHODS

The project was broken down into six distinct phases as follows:

Phase 1: Optimize Instrumental Conditions
Phase 2: Establish Colonies of Five Forensically Relevant Blow Fly Species
Phase 3: Assess the Effects of Genetic Variation
Phase 4: Assess the Effects of Abiotic Factors
Phase 5: Assess the Effect of Diet and Pupation Substrate
Phase 6: Apply Statistical Methods

During Phase 1 and Phase 2, we developed a method for the determination of fatty acids, sterols, and other lipids which naturally occur within pupae of the blowfly *Phormia regina*. The method relies upon liquid extraction in non-polar solvent, followed by derivatization using *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) w/ 1% trimethylchlorsilane (TMCS) carried out inside the sample vial. The collection and pre-concentration of analyte molecules is facilitated by total vaporization solid phase microextraction (TV-SPME), with gas chromatography-mass spectrometry (GC-MS) serving as the instrumentation for analysis. The TV-SPME delivery technique renders approximately an order of magnitude improvement in sensitivity over traditional liquid injection methods, which may alleviate the need for rotary evaporation, reconstitution, collection of high performance liquid chromatography fractions, and many of the other pre-concentration steps that are commonplace in the current literature. Furthermore, the ability to derivatize the liquid extract in a single easy step while increasing sensitivity represents an improvement over current derivatization methods. The most common lipids identified in fly pupae were various saturated and unsaturated fatty acids ranging from dodecanoic acid to arachinoic acid, as well as cholesterol.

During Phase 3 through Phase 6, The internal lipids of four species of blowfly pupae (*Cochliomyia macellaria, Lucilia cuprina, Lucilia sericata, and Phormia regina*) were extracted in pentane and analyzed by total-vaporization solid phase microextraction gas chromatography-mass spectrometry (TV-SPME GC-MS). Peak areas were calculated for 26 compounds of interest, and the variations tabulated over the course of six developmental timepoints. The data was analyzed by principal components analysis (PCA) and discriminant analysis (DA) to elucidate the underlying trends. It was found that statistically-significant differences can be observed in the biological compounds of pupae, thus enabling the chemotaxonomic determination of species, as well as the inference of age. In a related study, the effects of variations in temperature, humidity, diet, and substrate upon the pupa chemical profile were examined, with humidity and especially diet showing the highest susceptibility to bias an analysis.

FINDINGS AND CONCLUSIONS

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 all cuticular lipids and hydrocarbons, even at elevated temperatures. It was our experience that HS-SPME was ill-suited to the analysis of pupae without workup. For useful chemical information to be gleaned, the analysis needed to be preceded by an extraction to separate the fatty acids, hydrocarbons, sterols, and other compounds of interest from the matrix. Hence, a new method was developed for the analysis of pupal liquid extracts by TV-SPME. The method offers a considerable improvement in sensitivity over traditional liquid injection techniques, which may potentially alleviate the need for rotary evaporation, reconstitution, and many of the other pre-concentration steps which are commonplace in the current literature.

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 89 – 94% were obtained for the F1 generation, and classification accuracies of 93 – 98% were obtained for the F10 generation. The compounds most correlated and anti-correlated with species were identified. Data from the F10 generations were shown to be a suitable model for F1 pupae, although performance on *Phormia regina* suffered, with classification accuracy falling to 75%. 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 several unknown alkanes were found to be potentially-good markers for age determination. Unknown alkanes D and F embodied a qualitative difference between early timepoints and late timepoints in *Cochliomyia macellaria*, enabling the change over time to be visualized by PCA. Humidity and pupation substrate were found to have minimal impact on the lipid profile, whereas small chemical changes were observed with increases in temperature, and meaningful changes were observed with variations in diet.

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MAIN BODY

INTRODUCTION

STATEMENT OF THE PROBLEM

Forensic entomology is the practice of using insects within a legal setting (1). Often, in cases of homicide, suicide, and accidental death, a body is discovered, and too much time has elapsed to the point where it is not possible to use physiologically-based medical techniques for the determination of time since death. Fortunately, insects recovered from the body can be used as a "clock" to determine the minimum time since death (PMI_{MIN}), or the time since the body has been available to the insects, since the majority of the species in North America will only lay eggs or larvae on a resource if it is dead (2, 3).

The life cycle of the most common flies, the blow flies (Diptera: Calliphoridae), is as follows: a female fly will lay between 100-300 eggs on the remains (4), the eggs will hatch, and the larvae will begin the consumption of the soft 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 the 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 blow flies to colonize the remains, or the conditions of the remains change (e.g. there no longer is any soft tissue remaining). These collected specimens are then identified to species, and some measure of their age, usually length of the larvae or stage of development, is used to extrapolate age, referencing published developmental data (e.g. (5-10)).

In order to estimate the PMI_{MIN}, the investigator should collect the 'oldest' specimens, and in many cases, these are pupae. It is difficult, if not impossible, to identify the species of pupae, therefore the pupae are typically placed in an environmental chamber at known temperature and allowed to complete their development. When adults, the species determinations can be easily determined using published morphological keys (11). A drawback of this technique is the additional time needed for the completion of development, as well as the assumption that the pupae are alive. Any damaged or dead pupae will never be identified using this technique, and not many crime laboratories have the resources available to rear flies. An alternative technique exists in which the DNA can be extracted from the pupa, and its species determined (12-18). The drawbacks of such a technique are the cost and associated expertise required for the analysis of the DNA sequence data, and the reliance on a thorough representation of all the possible species and a well-defined phylogenetic tree supporting the molecular marker as a tool for species identification (19).

Estimating the age of the pupa is another requirement for which is necessary for a PMI_{MIN} estimation, and for which there are limited options. One method for which the pupal age can be estimated through the use of quantitative PCR of select developmental genes (20, 21). A major drawback of this method is the reliance on having a high quality sample that has been well preserved so the mRNA is intact. Although nucleic acid-based techniques are still evolving, a viable and cost-effective means of identifying fly 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 built-in capacity is compounded not only by the innate complexity of the insects themselves, whose life cycles can be vary due to differences in genetics (22-25), sex (26), and

diet (8); but also by the lack of any statistically-verifiable way of gauging the accuracy of entomological analyses, 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 (2). 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 volatile molecules of the insect cuticle (2).

Literature Review

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 (27)—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 (28). 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 (28).

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 (29). 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 of their cousins in more temperate climates (30, 31). 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 (32), as well as fungi and bacteria (27).

In addition to their practical functions, the hydrocarbons of the cuticle can 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 (28); (ii) of the same colony; (iii) male or female (33); (iv) caste membership (34); (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 (35); and (vi) whether the other insect is fertile and ready to mate, or has already copulated (36). 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 (31).

The role of the cuticular compounds in mating cannot be understated. It has been noted that volatile organic compounds (VOCs) released by female members of *Drosophila* play an integral part in the mating rituals of that species, directly contributing to courtship and inciting pre-copulatory behavior in mates (28). Such compounds, predominantly straight-chain hydrocarbons 23 to 29 carbon atoms in length, are predominant in the female cuticle. Similar straight-chain volatile hydrocarbons are emitted from the cuticles of males, but with a few notable variations: they are often shorter by as much as five carbons; and females release a suite of compounds rich in dienes, whereas male odor compounds are typified by an abundance of monoenes (36). Some such monoenes produced by males are thought to be sexual inhibitors. Vaccenyl acetate, for example, observed in *Drosophila simulans* and *D. melanogaster*, is thought to be an inhibitor of male copulation, which—when detected by fellow males—discourages homosexual behavior. Dienes, on the other hand, as well as the compound 7-pentacosene, act as

aphrodisiacs, encouraging mating (37). The compounds of the cuticle are very likely to be relevant in sexual selection, to the extent that a pleasant-smelling aroma of cuticular hydrocarbons can overrule other considerations, such as mate viability and physical traits (38). The exact palette of cuticular hydrocarbons, then, is something that has arisen as much through sexual selection as natural selection (38).

Since VOC profiles are unique to the species (and even more specific categories), then they can be useful as a tool for identification. Solvent extraction has been the traditional method for the analysis of insect cuticular compounds (27, 29, 38-40). The process is straightforward: the insect is placed into a nonpolar solvent, such as methylene chloride, ether, pentane, or hexane, during which time the compounds of the cuticle leach into the liquid. Those compounds may then be concentrated and analyzed by gas chromatography-mass spectrometry (GC-MS) or high performance liquid chromatography (HPLC). This method is not without its drawbacks, however, foremost of which compounds originating from the insects body complicate the interpretation of the resulting data (28).

One alternative to solvent extraction is solid phase microextraction (SPME), an off-column pre-concentration technique. SPME utilizes a fiber housed within a protective needle to facilitate analysis (41). After the needle penetrates the septum of a sample container, the fiber is exposed to the headspace and volatile organic compounds adsorb to the coating. The fiber is then transferred to the heated injection port of a gas chromatograph, where desorption of VOCs takes place (41). SPME is a popular technique that has seen considerable utility in gathering volatile profiles for many different types of insects, including termites (42, 43), shield bugs (44), ants (35, 45), cockroaches (46), and beetles (47). Theoretically, SPME provides an ideal method for the analysis of the cuticular compounds of flies. The lack of any significant sample preparation makes it well-suited to forensic laboratories, and the absence of any solvent eliminates the possibility of internal compounds diffusing through the insect's cuticle and confounding the analysis. To date, however, there have been few SPME experiments on flies . Farine et al. utilized SPME to probe the volatile cuticular hydrocarbons believed to be involved in courtship and sexual interaction in *Drosophila melanogaster* (36).

Of greatest relevance to this proposal is the publication by Frederickx et al., who conducted SPME experiments on fly larvae and pupae of a single species (*Calliphora vicina Robineau-Desvoidy (Diptera: Calliphoridae)*) with an eye toward forensic applications. In this case, the authors utilized a divinylbenzene–carboxen–polydimethylosiloxane (DVB–CAR–PDMS) SPME fiber for larva and a carboxen–polydimethylosiloxane (CAR–PDMS) SPME fiber for pupae. The compounds observed at time points throughout the larval and pupal stages of development were documented. These compounds included cyclic and non-cyclic hydrocarbons, alcohols, esters, acids, aldehydes, nitrogen compounds, ketones and sulfur compounds. The presence/absence of the compounds was noted and this qualitative data was analyzed by agglomerative hierarchical clustering (AHC). AHC is an unsupervised chemometric technique that attempts to display any underlying structure in a data set as a dendrogram, whereby each sample is linked to all other samples at varying levels of similarity/dissimilarity. The AHC results in this case indicated that the VOC profiles of larvae and pupae were dissimilar, as were the profiles of pupae of differing ages (48).

Statement of hypothesis or rationale for the research

A crucial question that remains unanswered is whether the VOC profiles of different species of blow fly can be differentiated. In addition, the effect of environmental variables (e.g., genetic variation, temperature, light level, and diet) on VOC profiles is not known. Finally, several additional chemometric techniques are available that, unlike AHC, can determine the relative impact of different compounds on a classification scheme as well as allow for unknown samples to be analyzed and then classified.

METHODS

INSTRUMENTATION

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 (30m × 0.25mm × 0.25µm). All GC-MS analyses utilized H_2 carrier gas with a flow rate of 2.5mL/min operated in splitless mode, with a scan range of *m/z* 40-550. All data was analyzed using Agilent Chemstation and Thermo Excaliber software.

REARING OF FLY COLONIES (METHOD DEVELOPMENT)

A colony of *Phormia regina* blowflies (following ten generations in colony) was provided sugar and water ad libitum at 25 °C ambient temperature and 60 % ambient humidity in a 30 x 30 x 30 cm cage (Bioquip, Rancho Dominguez, CA). Approximately one week post-eclosion, chicken liver was provided to the colony for ovary maturation. Chicken liver (25 g) was provided as an oviposition substrate for a period of 2-4 hours. Twenty-four hours post-oviposition, approximately 100 first instar larvae were transferred to a 100 mL plastic cup containing 50 g fresh chicken liver, which was placed within a quart-sized glass jar half-filled with fine pine shavings (Lanjay Inc., Montreal, QC). The glass jar was incubated at 25 °C and 60 % relative humidity with a 12:12 light:dark cycle in an environmental chamber (Percival, Perry, IA). After pupation, all specimens were collected and frozen at –80 °C. For each experiment, pupae were given at least 30 minutes to thaw prior to HS-SPME sampling or liquid extraction.

REARING OF FLY COLONIES (SPECIES AND AGE DETERMINATION)

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.

In order to gauge the chemical effects wrought by changes in temperature, humidity, pupation substrate, and diet, the same experimental design was carried out using pupae of F10+ *Phormia regina*. During this set of experiments, pupae were likewise collected over six timepoints.

- Pupae were collected after having been incubated at 15 °C, 20 °C, and 30 °C, holding humidity at 55 %, utilizing chicken liver for diet and sawdust as the pupation substrate.
- Pupae were collected after having been incubated at 40 %, 70 %, and 85 % humidity, holding temperature at 25 °C, utilizing chicken liver for diet and sawdust as the pupation substrate.
- Pupae were collected utilizing vermiculite and sand as the pupation substate, holding temperature at 25 °C and humidity at 55 %.
- Pupae were collected using high-fat ground beef, low-fat ground beef, and feeder rats for diet, holding temperature at 25 °C and humidity at 55 %, and utilizing sawdust as the pupation substrate.

Each of these experiments was compared against the baseline data provided by *Phormia regina* pupae collected at 25 °C, 55 % humidity, using sawdust as the pupation substrate and chicken liver for diet.

Initial 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 thawed pupa was placed in a 20 mL autosampler vial and extracted at 70 °C for 45 minutes. The fiber was then 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.

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 thawed 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 the total vaporization equation:

$$V_s = \left(\frac{\left(10^{A-\frac{B}{T+C}}\right)V}{RT}\right)\left(\frac{M}{\rho}\right) \qquad \text{(Equation 1)}$$

where V_s is the volume of liquid sample that will saturate the headspace of the vial (mL), V is the vial volume (mL), 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, and A, B, and C are the Antoine constants for the solvent (49, 50).

Each aliquot was analyzed by TV-SPME GC-MS. The SPME extraction time was 30 min with an extraction temperature of 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.

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

Experiments continued, focusing on pentane as the choice solvent. A 50 ppm undecanoic acid internal standard solution was prepared by transferring 50 µL of undecanoic acid into a 1000 mL volumetric flask and diluting to the mark with pentane. Ten milliliters of the internal standard solution was transferred to a glass vial, whereafter five pupae were placed into the solution for passive lipid extraction. 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 µL BSTFA w/ 1 % TMCS silylation reagent, vortexed for 10 s, and submitted to analysis by liquid injection GC-MS. The injection volume was 2 µL. 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 pupal extract solutions. 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.

TV-SPME vs. Liquid Injection Study

Five pupae were placed in 10 mL pentane. At 4d, 250 µL was sampled and transferred into an autosampler vial with a 300 µL conical insert. Simultaneously, another 250 µL was sampled and transferred into a 20 mL glass SPME vial. Both aliquots were silylated using 10 µL 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 µL. For all SPME experiments, the fiber was PDMS, with an extraction time of 15 m and an extraction temperature of 90°C.

Calibrant solutions of known concentration were also prepared and analyzed by the TV-SPME method and the liquid injection method to determine how the analytical figures of merit compared. A 1,000 ppm stock solution of palmitic acid-TMS in pentane was first prepared by dissolving 25 mg palmitic acid in a 25 mL volumetric flask, diluting to the mark, and derivitizing with BSTFA w/ 1 % TMCS. From this, standards of approximately 4.3 ppm, 2.2 ppm, 1.1 ppm, 0.5 ppm, and 0.2 ppm were prepared by transferring 100 µL of the stock solution into volumetric flasks of 25 mL, 50 mL, 100 mL, 200 mL, and 500 mL, respectively. An appropriate amount of 1,000 ppm undecanoic acid-TMS was added to each of the flasks to provide a static concentration of internal standard. Peak area and peak area ratio were plotted to construct a five-point calibration curve, from which the figures of merit could be determined.

Statistical Analysis (Discrimination of Age and Species)

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 (51).

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. Like 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 (51).

RESULTS

HEADSPACE SPME

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 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

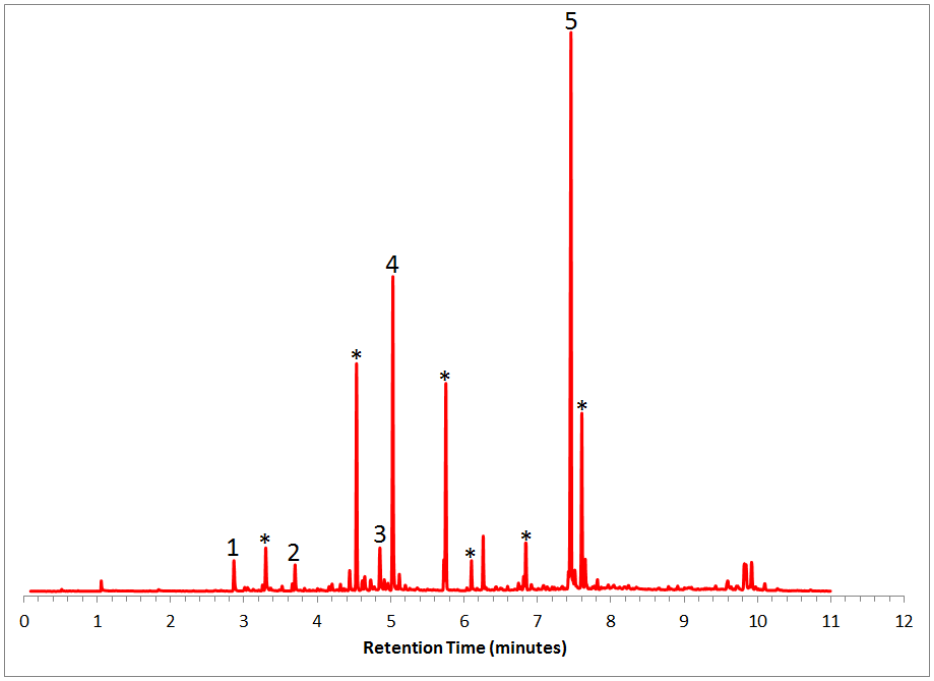

Figure 1. Results of HS-SPME for *Phormia regina* pupa extracted at 70 °C for 45 min. The labeled compounds are *1:* alpha-pinene, *2:* limonene, *3:* endo-borneol, *4:* alpha-terpineol, and *5:* nerolidol. All are believed to originate from the pupation substrate.

vermiculite substrate instead of sawdust (48). 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.

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 (29, 52-55); 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 (52, 56-61). Ethanol is the solvent most commonly employed for the preservation and long-term storage of entomological specimens collected from crime scenes (62), 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 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 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

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 2–5. 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 1/30th 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 (63); or in the manner of Folch et al., where the derivitization is achieved by lengthy sonication in a methanol : chloroform.

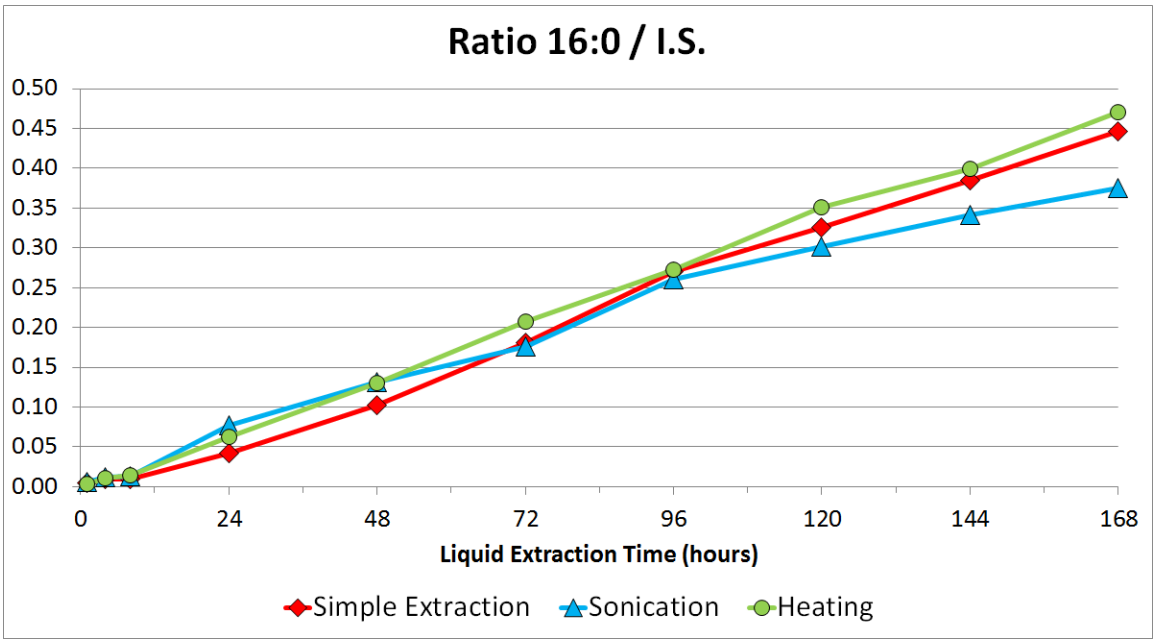

Figure 2. Peak area ratios for palmitic acid (16:0) divided by the undecanoic acid (11:0) internal standard.

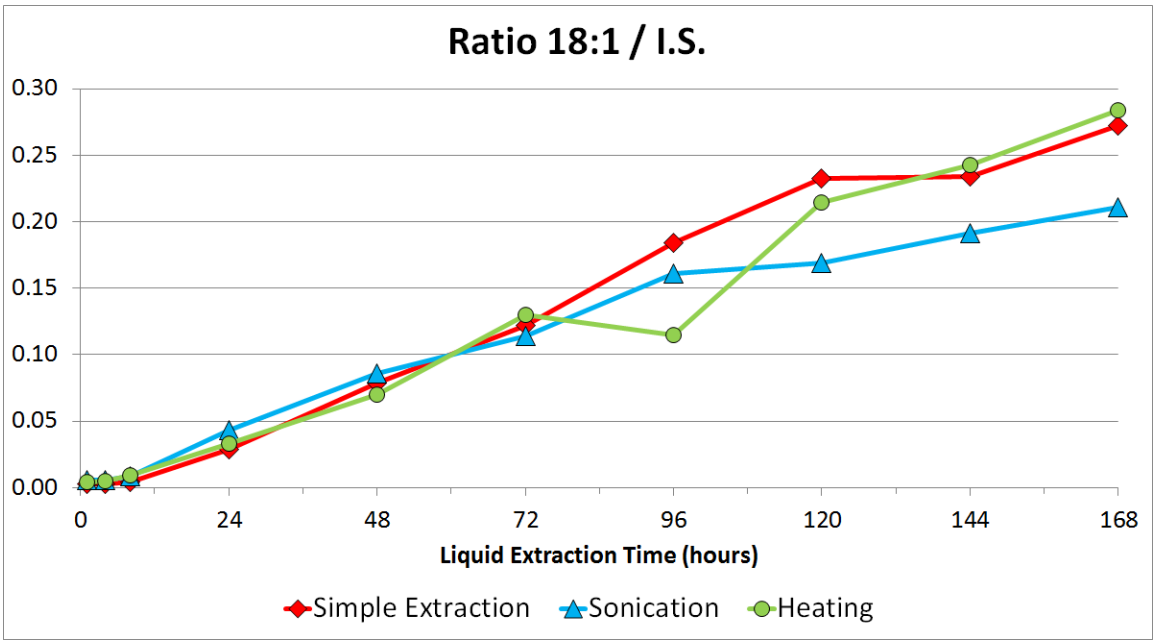

Figure 3. Peak area ratios for oleic acid (18:1) divided by the undecanoic acid (11:0) internal standard.

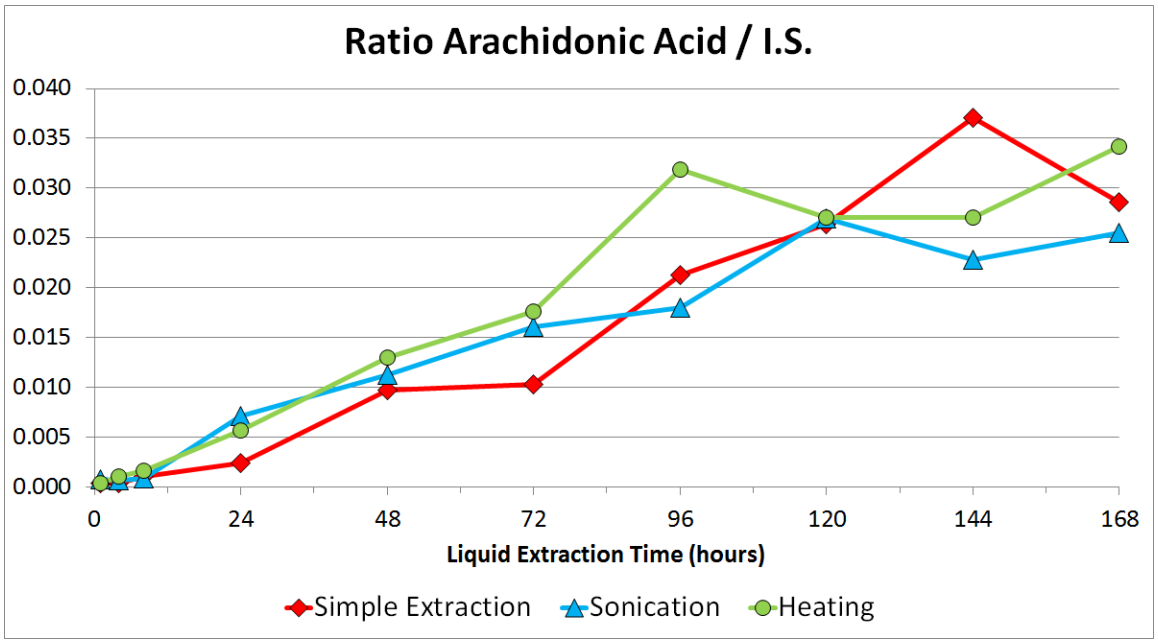

Figure 4. Peak area ratios for palmitic acid (16:0) divided by the undecanoic acid (11:0) internal standard.

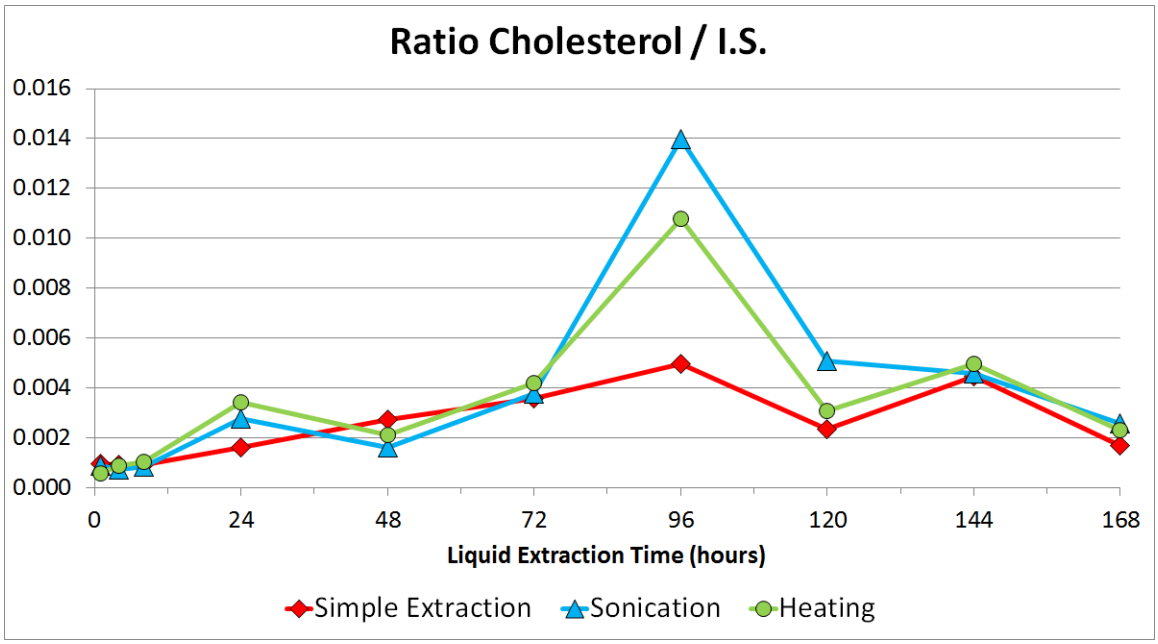

Figure 5. Peak area ratios for cholesterol divided by the undecanoic acid (11:0) internal standard.

solution maintained in a constant ice bath (64)), the method published here may represent a significant improvement over alternatives.

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 (50). The practical result of this disparity is depicted in Figure 6. Note that the distribution of lipids is comparable with what has been previously reported by Gołębiowski et al. (52), and appears to offer an advantageous improvement in sensitivity over the method employed by Frere et al. (63).

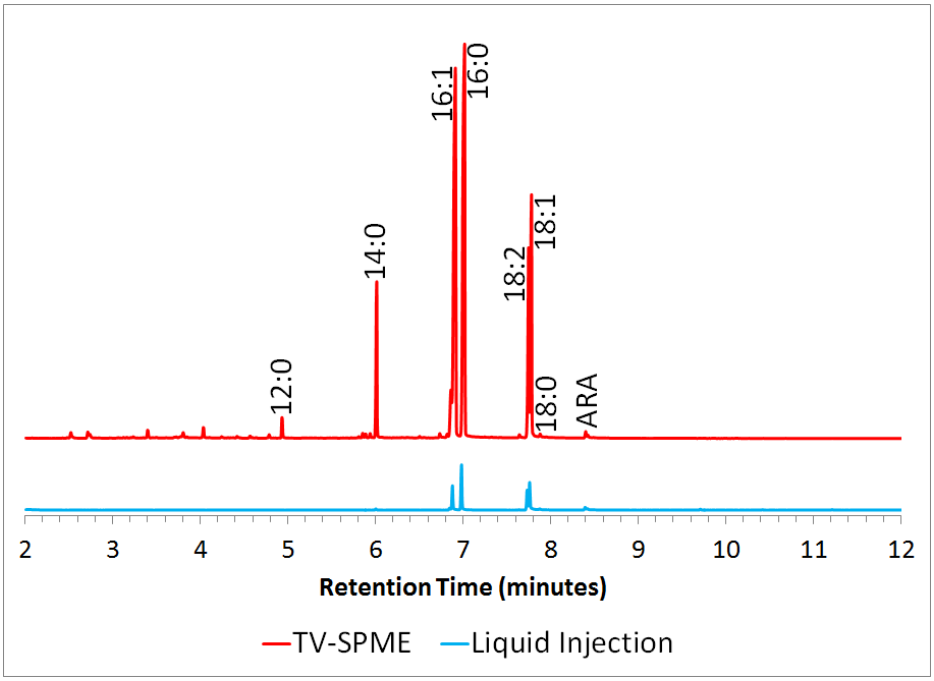

Figure 6. Comparison between TV-SPME and liquid injection chromatograms (non-normalized). The major fatty acids have been labeled.

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.

Determination of Species

The 26 compounds of interest investigated in these experiments are presented in a heat map in Figure 7. 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).

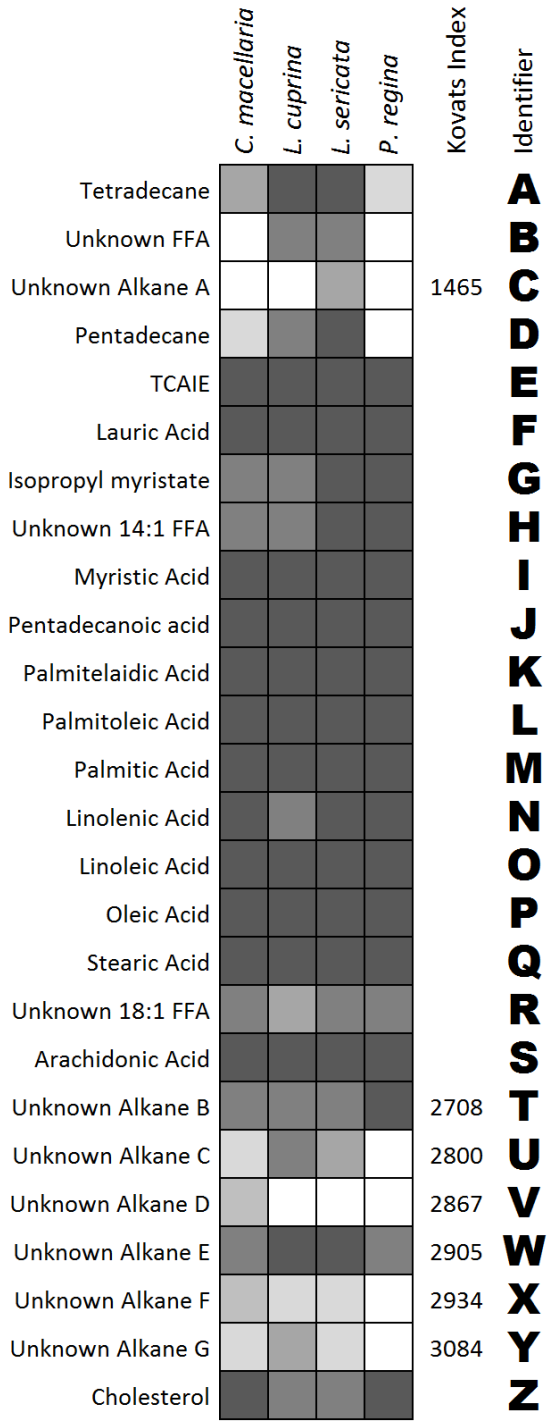

Figure 7. Heat map depicting the 26 compounds selected for statistical analysis. ■ denotes compounds present in 100 % of samples, ■ denotes compounds present in 75-99 % of samples, ■ denotes compounds present in 50-74 % of samples, ■ denotes compounds present in 25-49 % of samples, and ■ denotes compounds present in 1-24 % of samples. TCAIE is 2,2,4-trimethyl-3-carboxyisopropylpentanoic acid isobutyl ester.

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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 8. 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 9 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 10 and Figure 11. 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 provides tremendous insight into the DA results and the variables plot likewise provides insight into the DA results

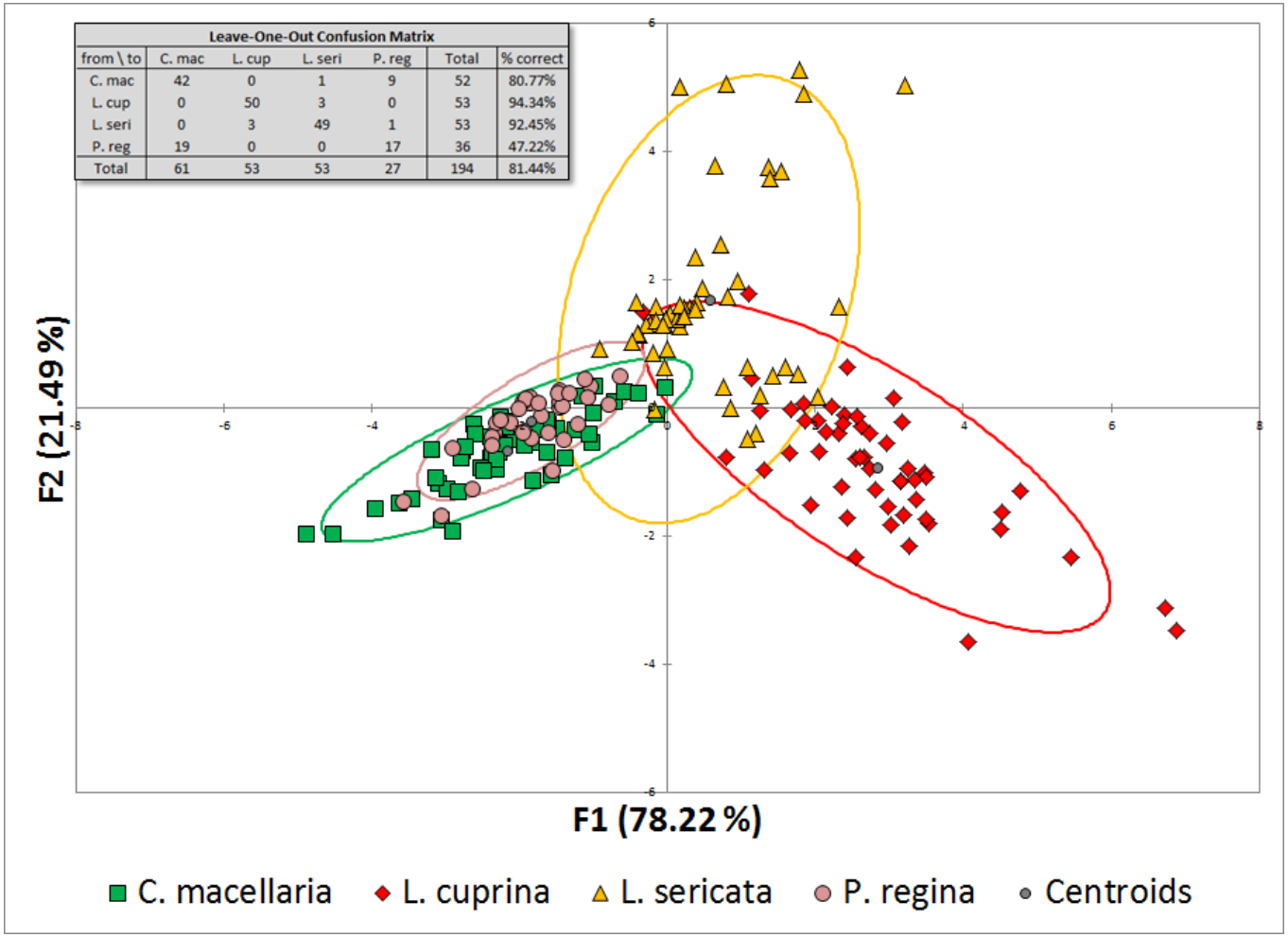

Figure 8. Discriminant analysis of the first three principal components for all four species, F1 generation, at all timepoints, with accompanying leave-one-out confusion matrix.

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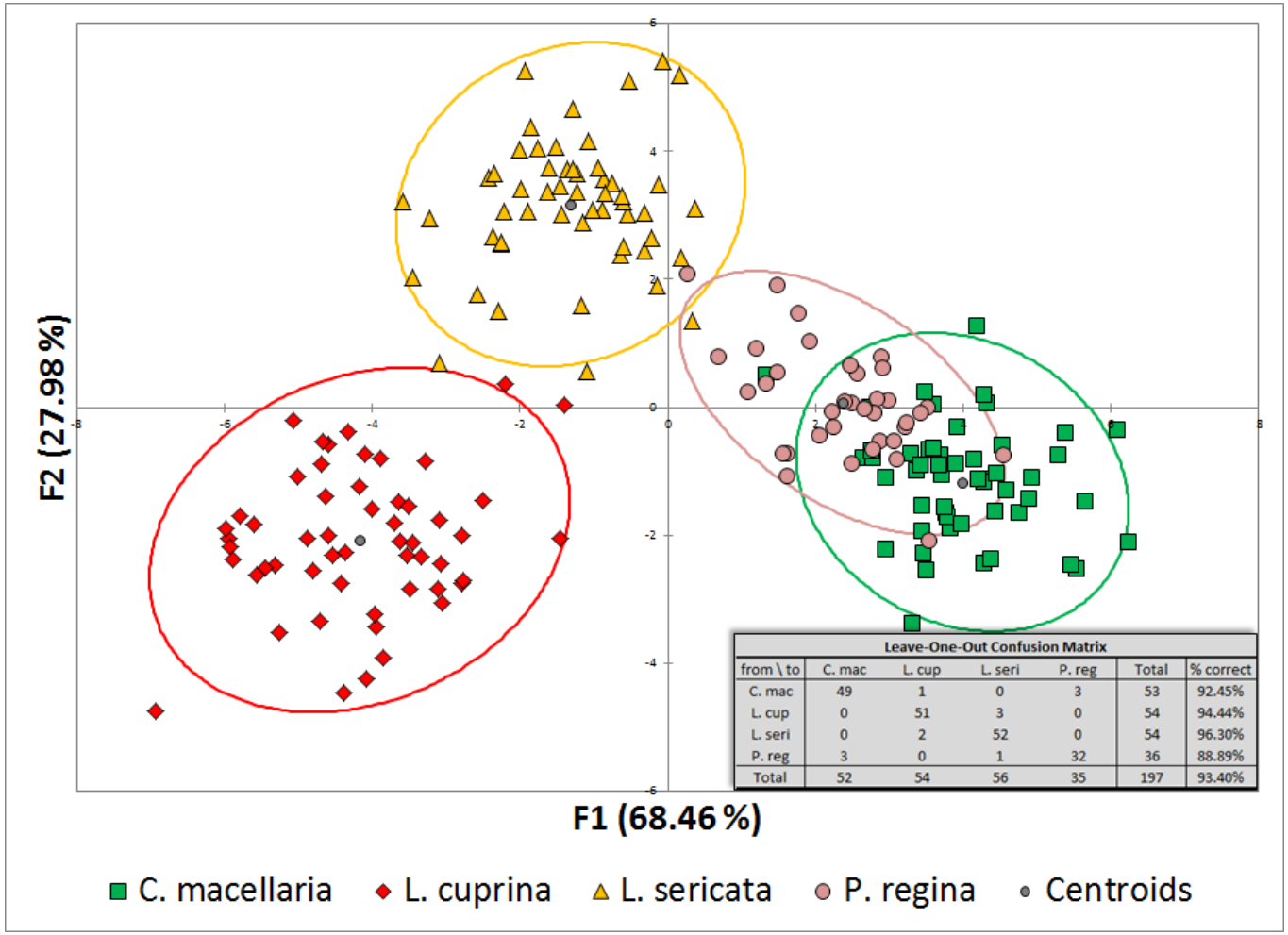

Leave-One-Out Confusion Matrix

from \ to	C. mac	L. cup	L. seri	P. reg	Total	% correct
C. mac	49	1	0	3	53	92.45%
L. cup	0	51	3	0	54	94.44%
L. seri	0	2	52	0	54	96.30%
P. reg	3	0	1	32	36	88.89%
Total	52	54	56	35	197	93.40%

Figure 9. Direct discriminant analysis of all four species, F1 generation, at all timepoints, with accompanying leave-one-out confusion matrix.

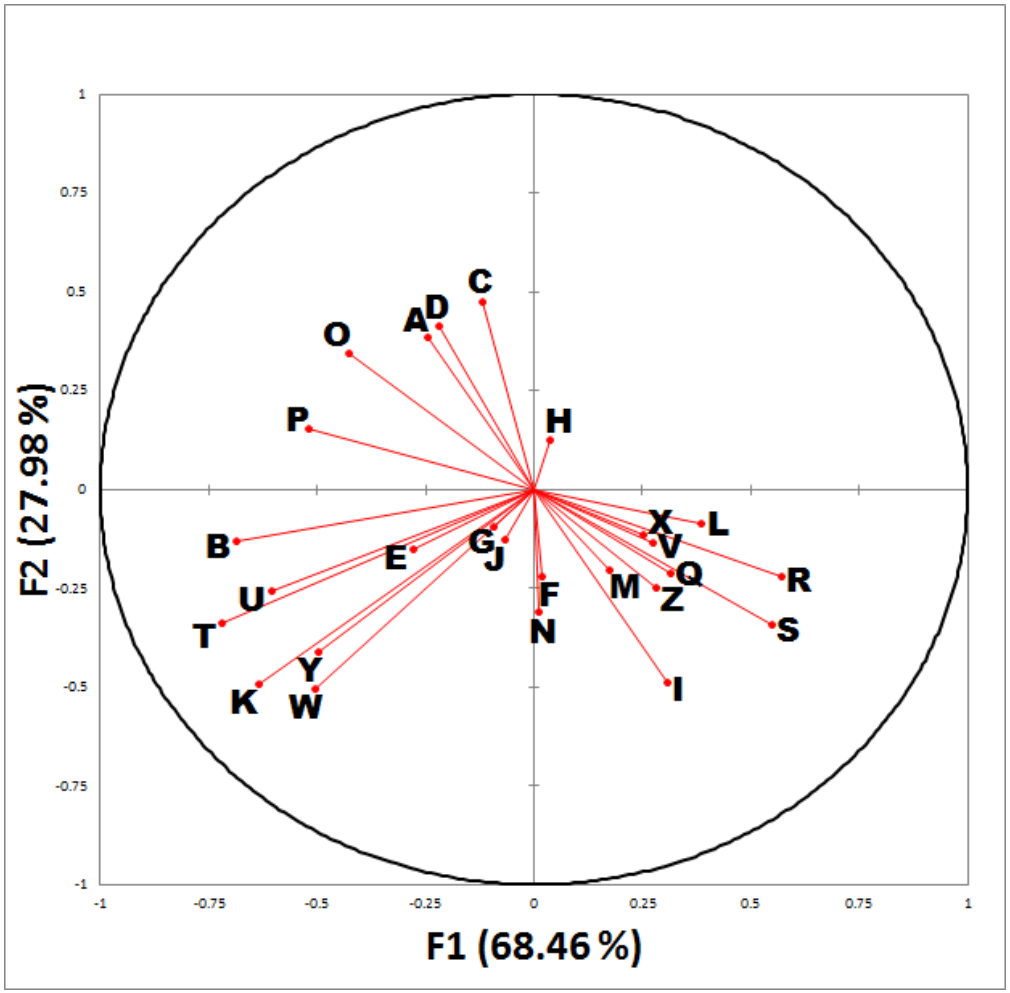

Figure 10. Variables plot for DA results in Figure 6.3. Identities for the rays projected from the origin correspond to the lettered compounds in Figure 6.1.

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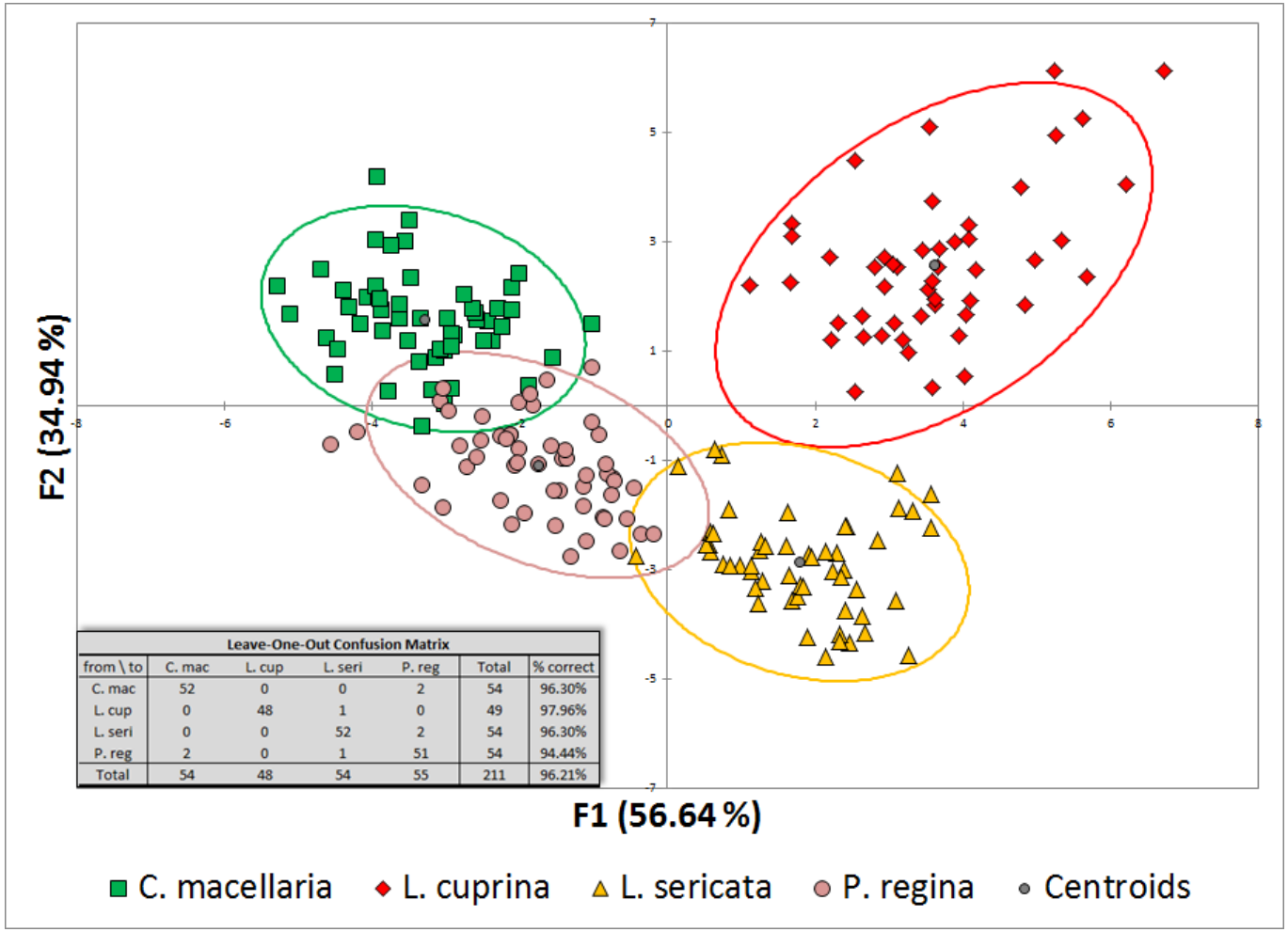

Leave-One-Out Confusion Matrix

from \ to	C. mac	L. cup	L. seri	P. reg	Total	% correct
C. mac	52	0	0	2	54	96.30%
L. cup	0	48	1	0	49	97.96%
L. seri	0	0	52	2	54	96.30%
P. reg	2	0	1	51	54	94.44%
Total	54	48	54	55	211	96.21%

Figure 11. Direct discriminant analysis of all four species, F10 generation, at all timepoints, with accompanying leave-one-out confusion matrix.

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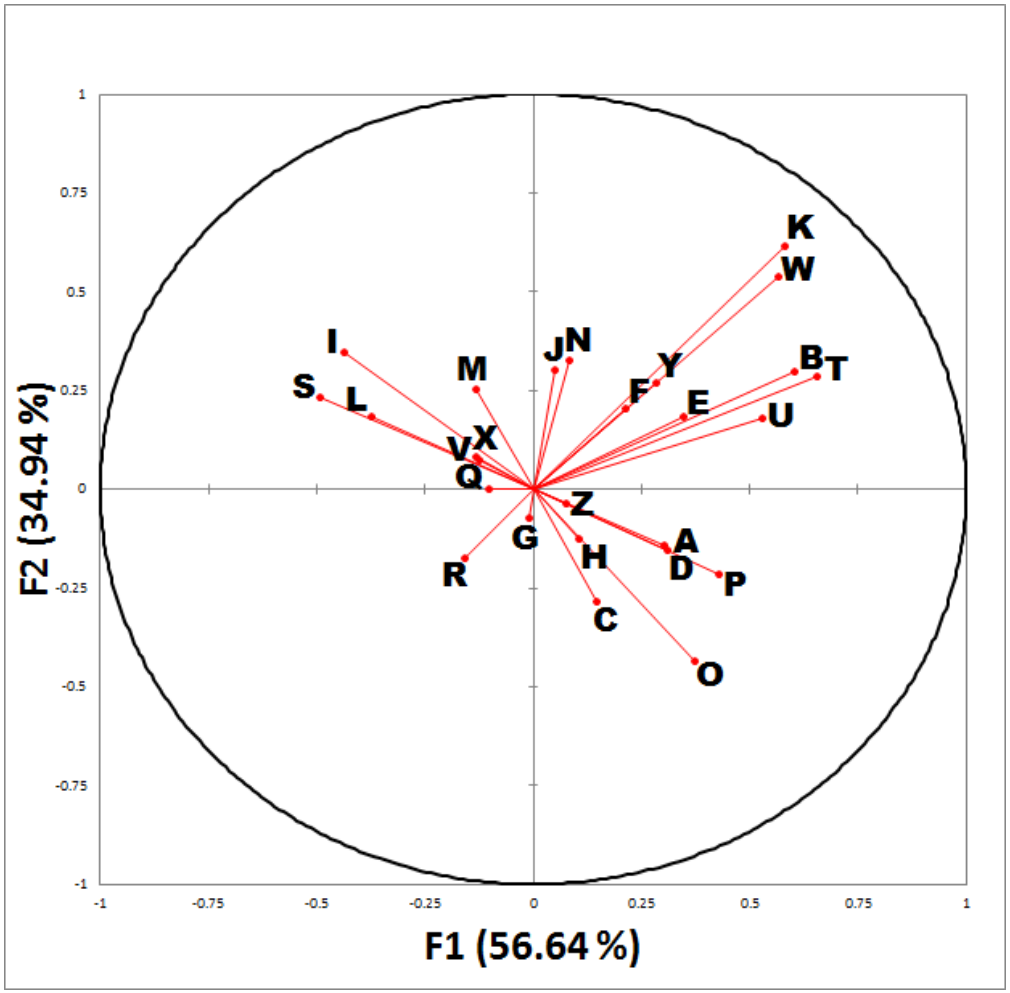

Figure 12. Variables plot for DA results in Figure 6.5. Identities for the rays projected from the origin correspond to the lettered compounds in Figure 6.1.

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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 12, 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 12, 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 12, are therefore revealed to be highly-present in specimens of *Cochliomyia macellaria*, because that species likewise falls within the top-left quadrant in Figure 11. *Lucilia sericata*, which falls within the bottom-right quadrant in Figure 11, 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 12. 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.

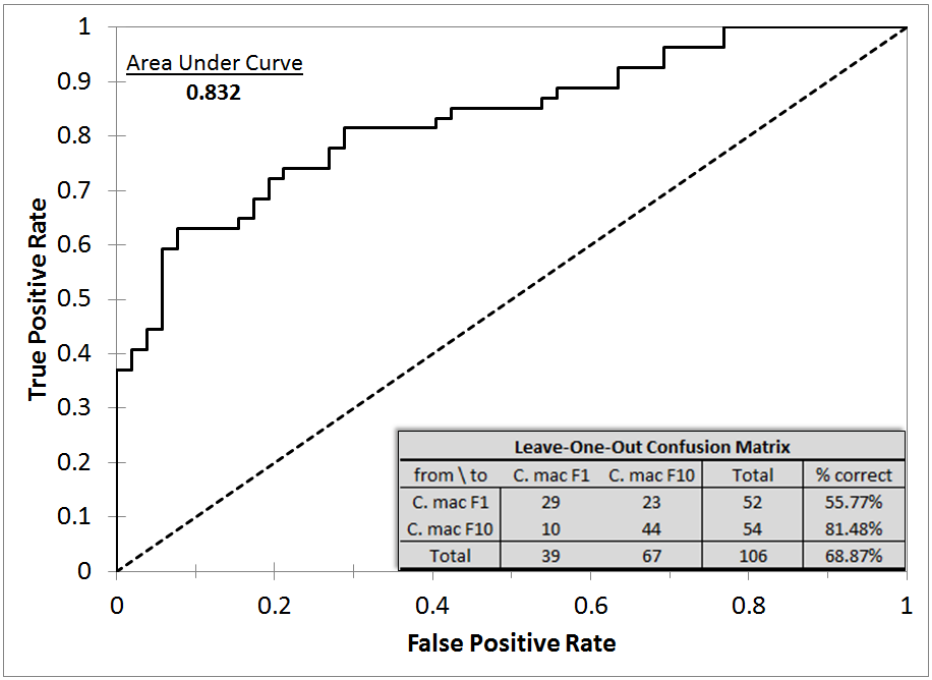
Determination of Generational Differences

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 13–16 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 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.

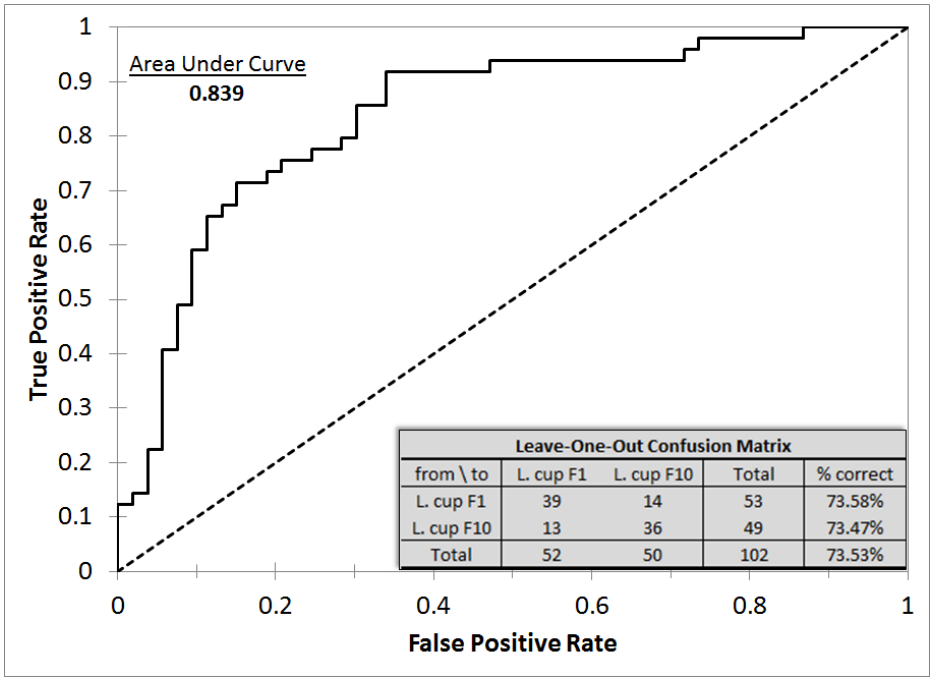
The fact that the ROC curves are well above the diagonal in each of the figures indicates there are intrinsic differences in the dataset between the F1 generation and the F10 generation. This is articulated by the area under curve values, which fall as high as 0.883 for *Phormia regina*. Thus, *Phormia 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 %).



Leave-One-Out Confusion Matrix

from \ to	C. mac F1	C. mac F10	Total	% correct
C. mac F1	29	23	52	55.77%
C. mac F10	10	44	54	81.48%
Total	39	67	106	68.87%

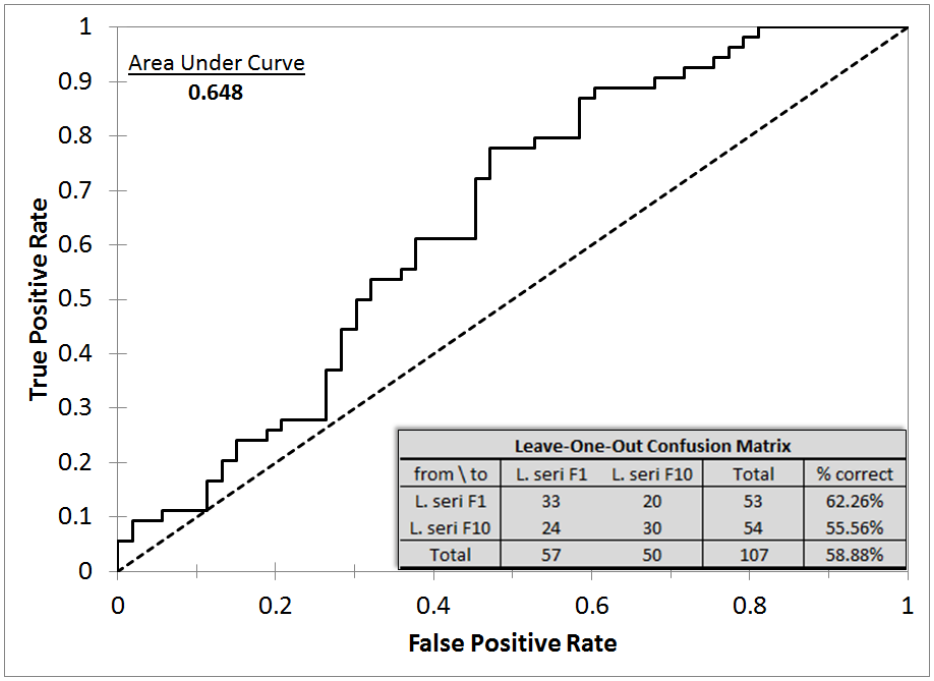
Figure 13. ROC curve for *Cochliomyia macellaria* with leave-one-out confusion matrix.



Leave-One-Out Confusion Matrix

from \ to	L. cup F1	L. cup F10	Total	% correct
L. cup F1	39	14	53	73.58%
L. cup F10	13	36	49	73.47%
Total	52	50	102	73.53%

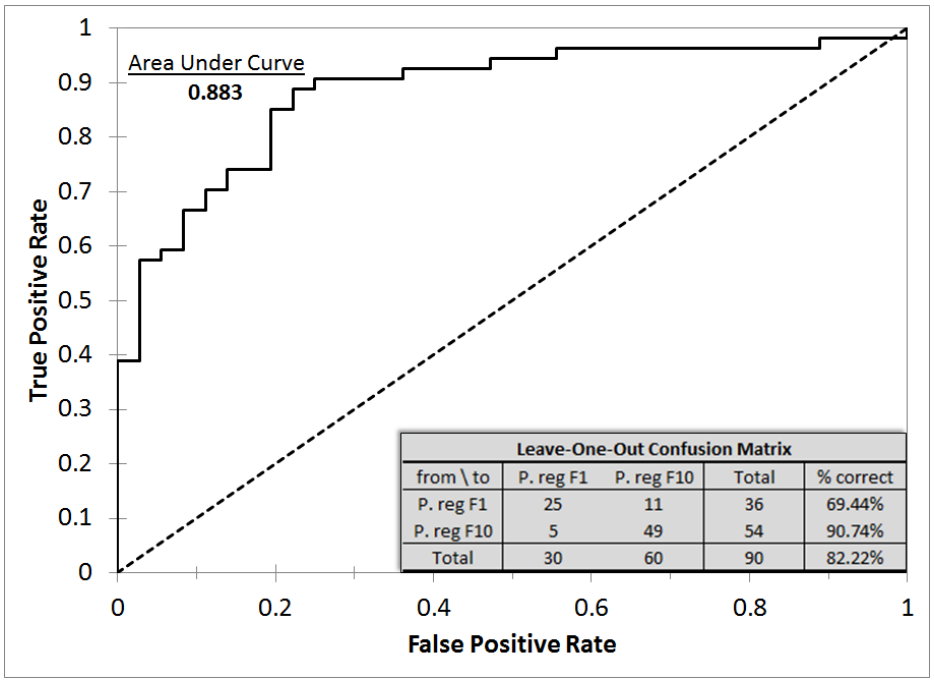
Figure 14. ROC curve for *Lucilia cuprina* with leave-one-out confusion matrix.



Leave-One-Out Confusion Matrix

from \ to	L. seri F1	L. seri F10	Total	% correct
L. seri F1	33	20	53	62.26%
L. seri F10	24	30	54	55.56%
Total	57	50	107	58.88%

Figure 15. ROC curve for *Lucilia sericata* with leave-one-out confusion matrix.



Leave-One-Out Confusion Matrix

from \ to	P. reg F1	P. reg F10	Total	% correct
P. reg F1	25	11	36	69.44%
P. reg F10	5	49	54	90.74%
Total	30	60	90	82.22%

Figure 16. 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 per the DA model constructed from F10 pupae. If the genetic differences were irrelevant, then the F1 pupae would be expected to be grouped per 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 agree 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 2. Leave-one-out confusion matrix for F1 pupae grouped per 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.

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 17. 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 18 and Figure 19 showcase the change over time for palmitoleic acid and linoleic acid in *Phormia regina* F1. Palmitoleic acid steadily decreases, while

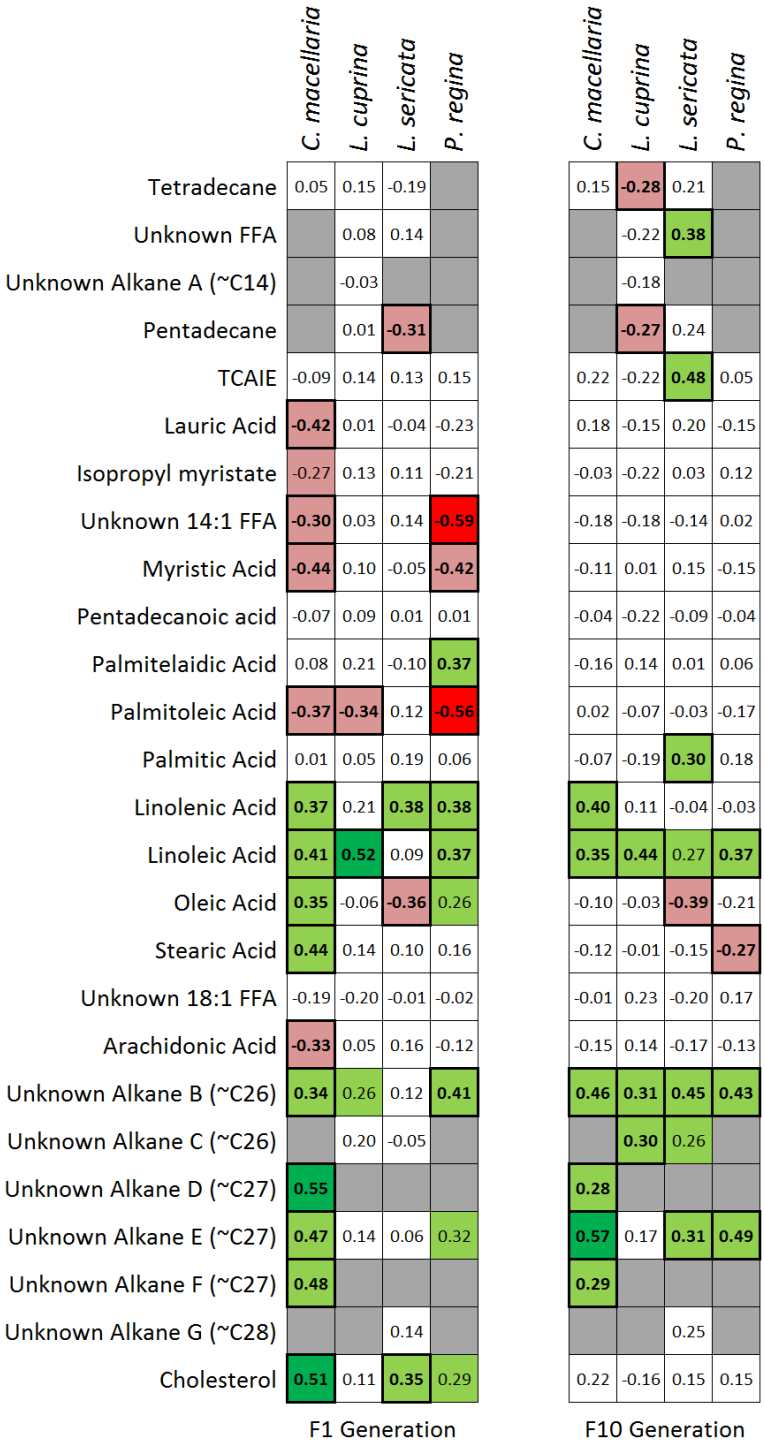


Figure 17. Correlations between selected compounds and ADH. Compounds in green increase markedly with time; compounds in red decrease markedly with time. Compounds highlighted in bold are ascribed a statistically-significant p-value < 0.05.

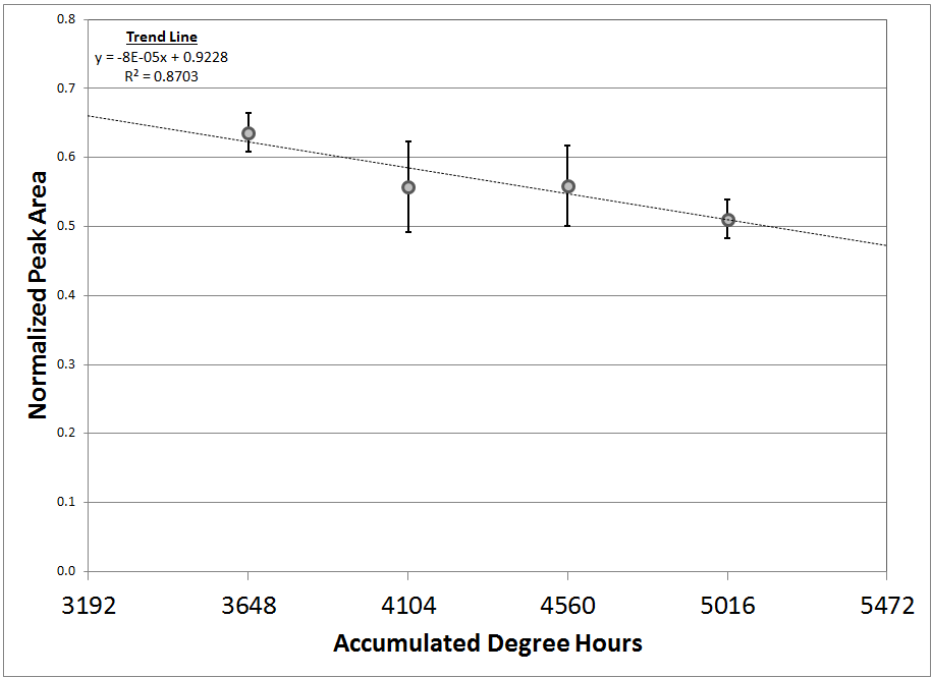


Figure 18. Normalized peak areas for palmitoleic acid in *Phormia regina* F1 as a function of ADH.

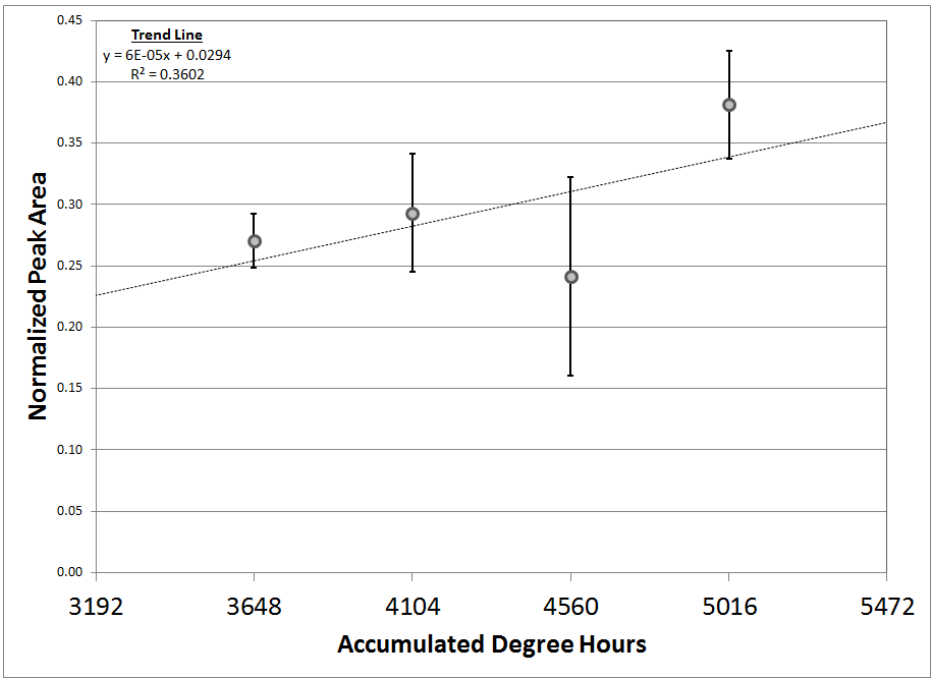


Figure 19. Normalized peak areas for linoleic acid in *Phormia regina* F1 as a function of ADH.

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 20. 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.

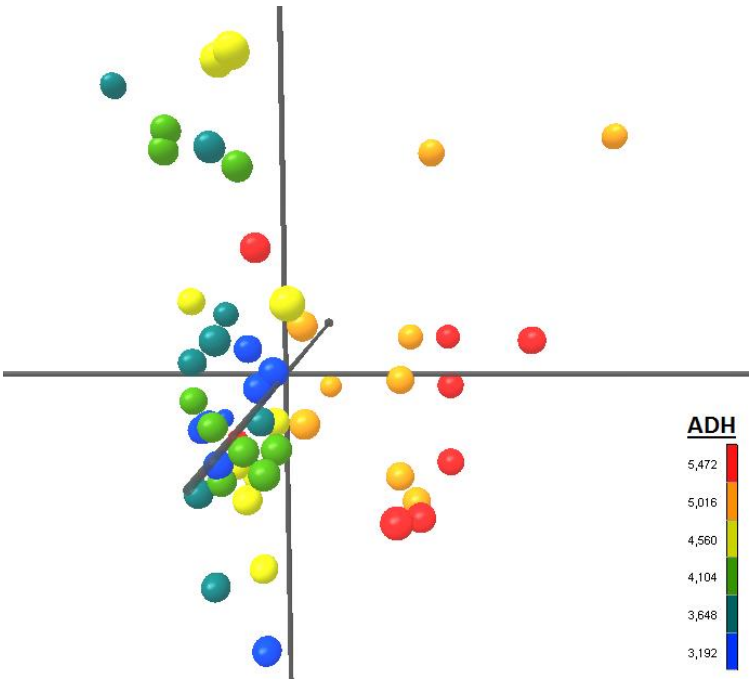


Figure 20. 3D PCA plot of *Cochliomyia macellaria* samples, categorized per ADH.

EFFECT OF ABIOTIC VARIABLES (*P. REGINA*)

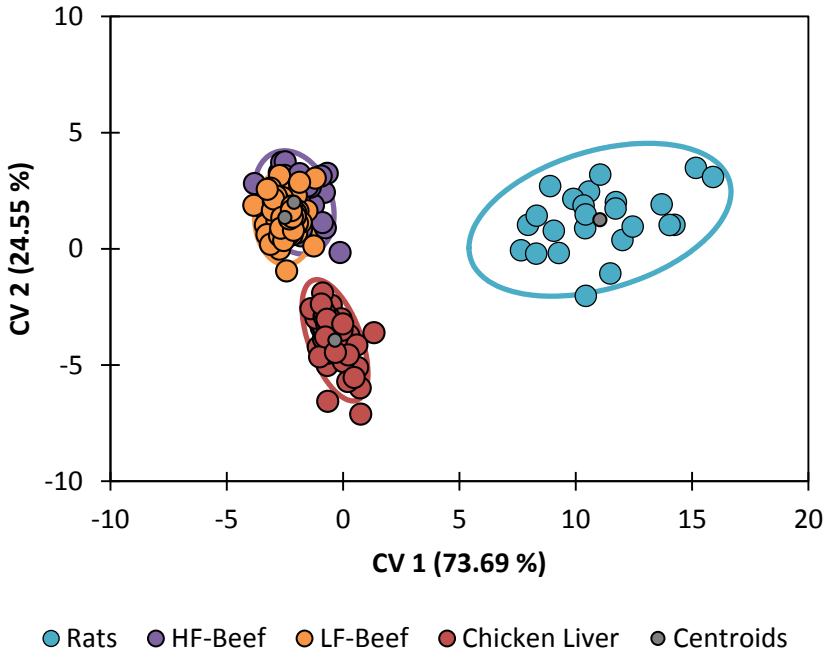
DA was carried out and confusion matrices were generated for the data from the studies of temperature, humidity, substrate, and diet. Although overall classification accuracies of 60 % or more were achieved for all four experiments, in most cases the actual differences observed among the classes were marginal. For example, almost no difference in the lipid profile was manifest among specimens allowed to pupate in sawdust, in sand, or in vermiculite (data not shown). Classification accuracies were therefore mediocre-to-poor, with a third of samples misclassified in the cases of pupae reared in sawdust and sand, and half of all samples in the case of vermiculite.

Results for pupae reared under different humidity conditions were almost as poor (data not shown), with discrimination only achieved for the sample set reared under normal 55 % humidity conditions. This is perhaps a surprising result, as the cuticular hydrocarbons of insects are known to play a part in resistance to desiccation, and might be expected to vary in response to adverse environmental conditions such as low humidity. It should be noted that although discrimination could not be achieved in these cases, this is, in fact, beneficial. In the context of insect specimens recovered from a crime scene, it is to the advantage of an investigator if the chemical profiles do not vary significantly with temperature, humidity, and other uncontrollable factors. A reliable method for age and species determination that is not undermined or biased by biotic and abiotic variables is the gold standard for any analyst. Therefore, the lack of discrimination is, in this case, the desired result.

Temperature appeared to have more of an impact on the lipid profiles, although its influence was still relatively small, as attested by the poor classification accuracies of 41.7 % and 62.3 % for pupae reared at 15 °C and 20 °C, respectively (data not shown). More substantial chemical differences were observed at

25 °C and 30 °C. In general, lauric acid, myristic acid, pentadecanoic acid, palmitic acid, and alkane G increased with temperature.

Figure 7: Discriminant analysis of *P. regina* pupae reared on various diets.



Diet was the single-largest contributing factor to differences in the lipid profile (Figure 7). Classification accuracies (Table 8) were 100% for pupae reared on chicken liver and 87.5 % for pupae reared on feeder rats. The statistical model was unsuccessful at distinguishing between pupae raised on high-fat ground beef and pupae fed on low-fat ground beef. Neglecting that minor distinction, pupae raised on ground beef were still correctly categorized 100.00 % of the time. The following trends are clear:

- Pupae reared on chicken liver have lipid profiles with higher quantities of palimitic acid, palimitoleic acid, unknown alkane B, and cholesterol.
- Pupae reared on ground beef have lipid profiles with higher quantities of lauric acid, myristic acid, palmitelaidic acid, linolenic acid, and stearic acid.
- All other compounds appear in greatest abundance in feeder rats.

Table 8: Leave-one-out confusion matrix for *P. regina* pupae reared on larval diets.

From/to	Rats	HF-Beef	LF-Beef	Chicken Liver	Total	% correct
Rats	21	2	0	1	24	87.5
HF-Beef	0	36	17	0	53	67.9
LF-Beef	0	13	41	0	54	75.9
Chicken Liver	0	0	0	53	53	100
Total	21	51	58	54	184	**82.0**

The most meaningful takeaway from this series of experiments is that while humidity, substrate, and temperature may be negligible enough to be ignored in future studies, diet should always be carefully considered and controlled, as it has much larger capacity to bias the data and obfuscate accurate age and species determinations. Therefore, this experiment highlights the need for further research into the effect of larval diet on chemical profiles for the purposes of species identification.

CONCLUSIONS

METHODOLOGY

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 all cuticular lipids and hydrocarbons, even at elevated temperatures. It was our experience that HS-SPME was ill-suited to the analysis of pupae without workup. For useful chemical information to be gleaned, the analysis needed to be preceded by an extraction to separate the fatty acids, hydrocarbons, sterols, and other compounds of interest from the matrix. Hence, a new method was developed for the analysis of pupal liquid extracts by TV-SPME. The method offers a considerable improvement in sensitivity over traditional liquid injection techniques, which may potentially alleviate the need for rotary evaporation, reconstitution, and many of the other pre-concentration steps which are commonplace in the current literature.

Note that previous publications have typically focused on the entomological significance of the results rather than the method itself, and as such, discussions of analytical figures of merit have not often been included. In the absence of a direct comparison of sensitivities and limits of detection, it appears the method described in this paper offers advantages over alternatives—although here, too, comparisons can be difficult: frequently, the samples themselves are not identical, differing in species (e.g. *Caliphora vicina* (52) versus *Hydrotaea aenescens* (63)), or in sample type (e.g. pupae (52) versus discarded puparia (63)). A comparison to some of the previously-published methods is provided in Table 2.

In terms of sheer sensitivity, the method of Golbiowski is likely unmatched, as no sampling technique SPME or otherwise will be able to provide the resolution of a two-dimensional chromatographic separation involving the GC-MS analysis of HPLC fractions. It is possible, however, that such methods could be further refined by the substitution of TV-SPME for liquid injection in the secondary GC-MS analysis, enabling even more substantial improvements in sensitivity and greater clarity of the internal lipids. Compared to other one-dimensional separations, the TV-SPME method described in this paper appears to be more than adequate. Quantitative derivitization accomplished using BSTFA w/ 1 % TMCS is well-suited to rapid analysis, doesn't require incubation, demands no concentrated acids, and eliminates extraction efficiency sample losses. Moreover, the ability to fully-saturate a sample vial and pre-concentrate the analyte on the fiber surface prior to administering it to the GC offers a substantial improvement in sensitivity over traditional liquid injection techniques.

SPECIES AND AGE DETERMINATION

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 89 – 94% were obtained for the F1 generation, and classification accuracies of 93 – 98% were obtained for the F10 generation. The compounds most correlated and anti-correlated with species were identified. Data from the F10 generations were shown to be a suitable model for F1 pupae, although performance on *Phormia regina* suffered, with classification accuracy falling to 75%. 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 several unknown alkanes 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. Humidity and pupation substrate were found to have minimal impact on the lipid profile, whereas small chemical changes were observed with increases in temperature, and meaningful changes were observed with variations in diet.

Abiotic Factors

The most meaningful takeaway from this series of experiments is that while humidity, substrate, and temperature may be negligible enough to be ignored in future studies, diet should always be carefully considered and controlled, as it has much larger capacity to bias the data and obfuscate accurate age and species determinations. Therefore, this experiment highlights the need for further research into the effect of larval diet on chemical profiles for the purposes of species identification.

Implications for policy and practice

This study sought to provide a useful forensic tool for an under-utilized life stage of insect evidence – the pupa. Characterizing the organic compounds extracted from a puparium to determine both species and age provides increased precision to age estimations of pupae recovered from remains. The evaluation of biotic and abiotic factors and how these effect chemical profiles will greatly benefit the forensic science community since we will be looking at the most likely scenarios in which these types of forensic samples are most likely to be collected and useful. The development of standard operating procedures for this type of analysis will allow for individuals who are not necessarily trained as forensic entomologists to analyze samples collected from scenes to determine the species and approximate age using common equipment found in most forensic laboratories.

Implications for further research

A 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. 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 (65). 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 (65). 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 (65). 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 (65).

Past studies have seen DART utilized for the analysis of fatty acids (66) as well as sterols (67), 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.

Dissemination of Research Findings

Our results were presented at the NIJ grantees meeting in 2015:

Frontiers in Forensic Entomology – In Pursuit of Novel Methods for Age and Species Identification W. Kranz, C. Picard, and J. Goodpaster. 67th Meeting of the American Academy of Forensic Sciences. Talk. Orlando, FL. February 16-21, 2015.

The results of this project are the basis for two manuscripts that are in preparation:

1. William Kranz, Clinton Carroll, Hillary Veron, Darren Dixon, Christine Picard, John Goodpaster, "Optimization of Total Vaporization Solid Phase Microextraction (TV-SPME) for the Determination of Lipid Profiles of Phormia Regina, a Forensically Important Blowfly Species"

   We have written a manuscript describing our methodology and this will be submitted to a forensic journal such as the Journal of Forensic Science or Forensic Science International.

2. William Kranz, Clinton Carroll, Christine Picard, John Goodpaster "Differentiating the Age and Species of Blowfly Pupae by Chromatographic Profiles and Multivariate Statistics"

   We intend to send this manuscript to a journal that would have a significant readership in forensic entomology (e.g., Insects).

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