OPTIMIZATION OF TOTAL VAPORIZATION SOLID PHASE MICROEXTRACTION (TV-SPME) FOR THE DETERMINATION OF LIPID PROFILES OF *PHORMIA REGINA*, A FORENSICALLY IMPORTANT BLOW FLY SPECIES.

William Kranz, Clinton Carroll, Darren Dixon, Christine Picard, John Goodpaster*

Indiana University Purdue University, Indianapolis

Department of Chemistry and Chemical Biology

402 N. Blackford Street LD326, Indianapolis, IN 46202

John V. Goodpaster, Corresponding Author

(317) 379-9699

jvgoodpa@iupui.edu

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Abstract

A new method has been developed for the determination of fatty acids, sterols, and other lipids which naturally occur within pupae of the blow fly *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 analysis 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 is approximately a factor of five more sensitive than traditional liquid injection, 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 lauric acid (12:0) to arachinoic acid (20:4), as well as cholesterol. The concentrations of myristic acid (14:0), palmitelaidic acid (16:2) and palmitoleic acid (16:1) were the most reliable indicators of the age of the pupae.

Keywords

Total Vaporization

Solid Phase Microextraction

Phormia regina

Lipids

Blow fly

Introduction

Solid phase microextraction (SPME) is an off-column pre-concentration technique that has successfully facilitated the trace analysis of an abundance of samples and matrices. The technique relies on a polymer fiber whose surface chemistry may be tailored to favor the adsorption of target compounds of interest. In headspace SPME, the fiber is inserted into the sample vial, the volatile organic compounds (VOC's) in the headspace bind to the coating, and then the fiber is transferred to the injection port of the GC for desorption and chromatography. In immersion SPME, the fiber is placed directly into a liquid (often aqueous) sample and the compounds of interest adsorb/absorb to the fiber coating. The fiber is then desorbed in the inlet of a GC or LC [1,2]. This paper will discuss the use of total vaporization solid phase microextraction (TV-SPME), which is a highly-sensitive technique whereby the totality of the liquid aliquot is vaporized prior to sampling, simplifying the equilibria inside the sample vial and maximizing the amount of analyte available in the headspace [3,4]. TV-SPME can analyze relatively large volumes (~50 – 100 μL) of organic extracts, and the choice of fiber coating chemistry can be used to advantage based upon the solvent and analyte(s) [5,3,6].

The samples of interest for this study were the pupae of *Phormia regina*, a species of blow fly that is commonly found by forensic entomologists during death investigations. Traditionally, the analysis of insect species in a forensic context has fallen within the purview of entomologists and biologists. However, there has been considerable effort in the chemistry sphere to evaluate these specimens by GC-MS, LC-MS, and similar analytical techniques.

Although other techniques exist for collecting the gaseous chemicals given off by the insects (for example, using a volatile trap [7]) 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 [8-13]. 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 , or it has relied on the headspace analysis of live samples in bulk [14]. Bulk analysis is not a practical method for forensic analysis – as individual pupa need to be identified in forensic casework samples.

It is much more common to use a work-up that relies upon liquid extraction. Typically, the insect is immersed in a nonpolar solvent for some length of time, allowing the cuticular and internal lipids to be extracted out. These extracts may be derivatized to improve sensitivity and performance during subsequent separation steps [15,16]. Inevitably, one or more rounds of chromatography follows: gas chromatography (GC) [17-22,16,23,24], liquid chromatography (LC) [18-24], and thin layer chromatography (TLC) [17] are among the various techniques that have been used. This general procedure has been applied to the analysis of pupae [19-22] and puparia [17,16,25] alike, and to the analysis of single specimens [19,20,16] as well as specimens in bulk [17,25].

A broader survey of the literature shows that the lipids, hydrocarbons, and other compounds that can be isolated from flies have been of concern to various authors for an 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 [7], while others have devoted themselves to elucidating the antimicrobial and bacteriostatic effects of

certain cuticular lipids [19]. Still others have made efforts to catalogue the insects' chemical profiles for chemotaxonomy purposes [17], 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 [16].

Our initial experiments, which sought to evaluate the VOC's emitted by pupae using headspace solid phase microextraction (HS-SPME) at elevated temperatures, were unsuccessful. Hence, attention turned to the development of a new method for the liquid extraction of pupae in order to isolate any lipids and hydrocarbons followed by TV-SPME analysis. A trimethlysilyl derivatization was also carried out inside the sample vial immediately prior to GC-MS analysis, offering a potential advantage to future analysts seeking to analyze blow fly pupae.

Materials and 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. Compounds were identified via searches of the NIST/EPA Mass Spectral Database, as well as comparison to authentic standards.

Rearing of Fly Colonies

A colony of *Phormia regina* blow flies (progressing through at least 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 (25g) was provided to the colony for oviposition 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). The age of the pupae was tracked in accumulated degree hours (ADH). ADH is a common unit in entomology and it reflects the number of thermal units that are required for the pupae to grow and develop to a certain stage. After 1 – 4 days of 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.

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 [3,4].

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 were 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.

Results and Discussion

Initial HS-SPME Experiments

On the surface, HS-SPME would be the ideal method for analyzing pupal 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 did not originate with the pupa, but with the sawdust wherein pupation occurred. The major substrate contaminants were 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 vermiculite substrate instead of sawdust [26].

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 of interest to the project. Following these difficulties, attention turned to the development of a new method by TV-SPME.

TV-SPME Solvent Selectivity 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 [27-29,25,19]; pentane, whose chemical properties are similar, served as the non-polar solvent in this research. Dichloromethane is another solvent that has previously been cited for the extraction of biological compounds from pupae [18,19,21,30,22-24]. Ethanol is the solvent most commonly employed for the preservation and long-term storage of entomological specimens collected from crime scenes , and for this reason, it was included as a solvent of interest. To our knowledge, acetone has not previously been reported as an insect extraction solvent. It was included in this study as a solvent of intermediate polarity between dichloromethane and ethanol.

Table 1 conveys the results of this study, which relate directly to the selectivity of the method. Selectivity is defined by IUPAC as “The extent to which other substances interfere with the determination of a substance according to a given procedure” [31]. The selectivity of this method is primarily based upon *separation selectivity* (the gas chromatographic separation) rather than *detection selectivity* (the mass spectrometer). The latter is true because the total

ion current (TIC) from the mass spectrometer was used to determine peak areas, rather than extracted ion profiles (EIC). Of course, the use of full scan data directly contributes to the specificity of the method, which reflects the extent to which the mass spectrometer can uniquely identify each separated component.

As such, the number of chemical compounds that were successfully separated and identified from a given solvent extract became a proxy measure of selectivity and specificity. Ethanol and DCM proved particularly ill-suited to generating complex lipid profiles by TV-SPME, providing no more than 12 and 14 compounds at best. Performance for acetone was better, but still mediocre. Pentane proved the overall best solvent, providing a minimum of 40 compounds when paired with a PEG fiber, and a maximum of 63 compounds when paired with a PDMS fiber. The distribution of lipids was also comparable with what has been previously reported by Gołębiowski et al. [19]. Overall, our experiments showed pentane/PDMS was superior to all other alternatives, and this combination was selected as the foundation for further experiments.

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, 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. Figure 1 portrays the peak area ratios for palmitic acid normalized to the internal standard for simple extraction, sonication, and heating experiments. In all cases, there was an "induction period" of approximately 10 hours, where the concentration of palmitic acid increased modestly, followed by a more rapid increase of concentration that increased linearly without a clear plateau. The data obtained did not evince any significant difference in extraction efficiency between those samples which were heated or sonicated and those that 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.

Sensitivity toward other compounds of interest, including oleic acid, arachidonic acid, and cholesterol, was likewise unaffected by heating and sonication (data not shown). Furthermore, the peak area ratios for these compounds followed a similar trend of a ten-hour induction period followed by a steeper linear increase in concentration, as shown in Figure 2. Cholesterol was the exception in that it increased to a lesser extent, reached a maximum around 96 hours, and exhibited a modest decline. It was noted that even long periods of extraction did not cause the concentrations of all other analytes to reach a true plateau. Therefore, based upon the response of cholesterol the time of extraction was set at 96 hours for all future experiments. Figure 3 shows the effects of extraction time on the chromatograms obtained from *P. reg* pupae. Note that 96 hours was chosen for the maximum absolute response for cholesterol, but as a relative measure, the peak for cholesterol appears smaller in the chromatograms due to the larger increases in the fatty acids.

The inclusion of a derivatization step following extraction 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. To this end, derivatization using BSTFA w/ 1 % TMCS was carried out during 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. This is an improvement over many previously published methods, which have generally relied on the use of concentrated acids or rigorous work-ups (as in the case of Frere et al., wherein the derivatization is accomplished by a transesterification reaction in the presence of concentrated sulfuric acid [16]; or in the manner of Folch et al., where the derivatization is achieved by lengthy sonication in a methanol:chloroform solution maintained in a constant ice bath [32]).

Sensitivity and Detection Limit of TV-SPME vs. Liquid Injection

In a separate study by Bors et al., TV-SPME was found to improve sensitivity by an order of magnitude over liquid injection [4]. The practical result of this disparity is depicted in Figure 4, which indicates an improvement in sensitivity over the method employed by Frere et al. [16]. 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.

The compounds which can be extracted from the pupa using this method include: hydrocarbons (e.g., tetradecane, pentadecane, and higher branched and normal alkanes), free fatty acids (e.g., lauric, myristic, pentadecanoic, palmitelaidic, palmitoleic, palmitic, linolenic, linoleic, oleic, stearic, and arachidonic), sterols (e.g., cholesterol) and other insect-related compounds, including 2,2,4-trimethyl-3-carboxyisopropylpentanoic acid isobutyl ester. 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 calibration curve for the TV-SPME method clearly demonstrates the greater sensitivity of TV-SPME versus liquid injection, as evidenced by Figure 5. Overall sensitivity was approximately five times larger, with a calibration curve slope of 4.93×10^7 ppm^{-1} for TV-SPME and 1.03×10^7 ppm^{-1} for liquid injection. Linearity was essentially the same for both methods (i.e., R^2 of 0.999 for liquid injections and 0.998 for TV-SPME).

Limits of detection can be calculated in a number of ways, including analyses of blanks, calibration curve data, determining signal-to-noise, etc. [33,34]. In our case, the limit of detection (LOD) was calculated based upon the calibration curve, where LOD was defined as three times the standard deviation of the y residuals divided by the slope [35]. By this method, the calculated limit of detection was 196 ppb for TV-SPME. The signal-to-noise characteristics of the method were also assessed using the same calibration data (see Figure 6). By plotting signal-to-noise as a function of signal and determining the slope of this curve (0.48), it was

confirmed that the signal-to-noise is dependent upon the square root of the signal. In turn, this confirms that the system is generating the maximum signal-to-noise for any given signal (i.e, the signal-shot noise limit) [36].

Age Prediction

The goal of this methodology is to assess the age of a pupa, as well as help differentiate between the various species of blow fly based upon their pupae. Table 2 contains the results of a correlation analysis of various lipids with the age of the pupae in ADH. Five compounds (i.e., myristic, palmitelaidic, palmitoleic, linolenic and linoleic acid) exhibited statistically significant correlation coefficients with ADH. Of these, 3 compounds (i.e., myristic, palmitelaidic and palmitoleic acid) also exhibited a strong linear relationship with ADH, as assessed through linear regression. A detailed study of the relationship between pupae lipids and the species, age and diet of blow flies appears elsewhere [37]. That study required multi-variate statistical methods such as Principal Components Analysis (PCA) and Discriminant Analysis (DA). The DA models were then checked using leave-one-out cross validation.

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 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* [19] versus *Hydrotaea aenescens* [16]), or in sample type (e.g. pupae [19] versus discarded puparia [16]). A comparison to some of the previously-published methods is provided in Table 3.

In terms of 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 [21]. 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 derivatization 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.

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Table 1. TV-SPME solvent study. The number of detectable compounds (peaks present in each chromatogram) is listed for each combination of extraction solvent and SPME fiber coating.

	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

Table 2. Correlation coefficients for various extracted lipids versus accumulated degree hours (ADH) in *P reg.* Values in bold are different from 0 with a significance level $\alpha = 0.05$. For each significant variable, the results of a linear regression for samples between 3648 and 5016 ADH is also included.

Compound	Correlation Coefficient (r)	Slope (ADH^{-1})	R^2
Lauric Acid (12:0)	-0.232	-	-
Isopropyl myristate	-0.208	-	-
Myristic Acid (14:0)	**-0.417**	**-1.49×10^{-5}**	**0.991**
Pentadecanoic acid (15:0)	0.012	-	-
Palmitelaidic Acid (16:2)	**0.371**	**1.58×10^{-5}**	**0.995**
Palmitoleic Acid (16:1)	**-0.562**	**-8.23×10^{-5}**	**0.870**
Palmitic Acid (16:0)	0.061	-	-
Linolenic Acid (18:3)	**0.378**	**3.74×10^{-6}**	**0.704**
Linoleic Acid (18:2)	**0.374**	**6.17×10^{-5}**	**0.360**
Oleic Acid (18:1)	0.265	-	-
Stearic Acid (18:0)	0.156	-	-
Arachidonic Acid (20:4)	-0.120	-	-
Cholesterol (C)	0.287	-	-

Table 3. Comparison of assorted methods for the analysis of extracts from blow flies.

	This work	**Golebiowski**	**Frere**
Specimen	Pupae	Pupae	Puparia
Target Compound Class	Lipids	Lipids and hydrocarbons	Lipids
Extraction	0–4 d Pentane	10 s petroleum ether 1 min DCM 10 d hexane	40 min ethanol + acid Extraction in pentane Evaporation and re-concentration in isooctane
Derivitization	Yes BSTFA w/ 1 % TMCS	Optional BSTFA w/ 1 % TMCS	Yes Acid-catalyzed ethyl esterification
Instrumental Analysis	SPME-GC-MS	HPLC for collection of fractions GC-MS of HPLC fractions	GC-MS

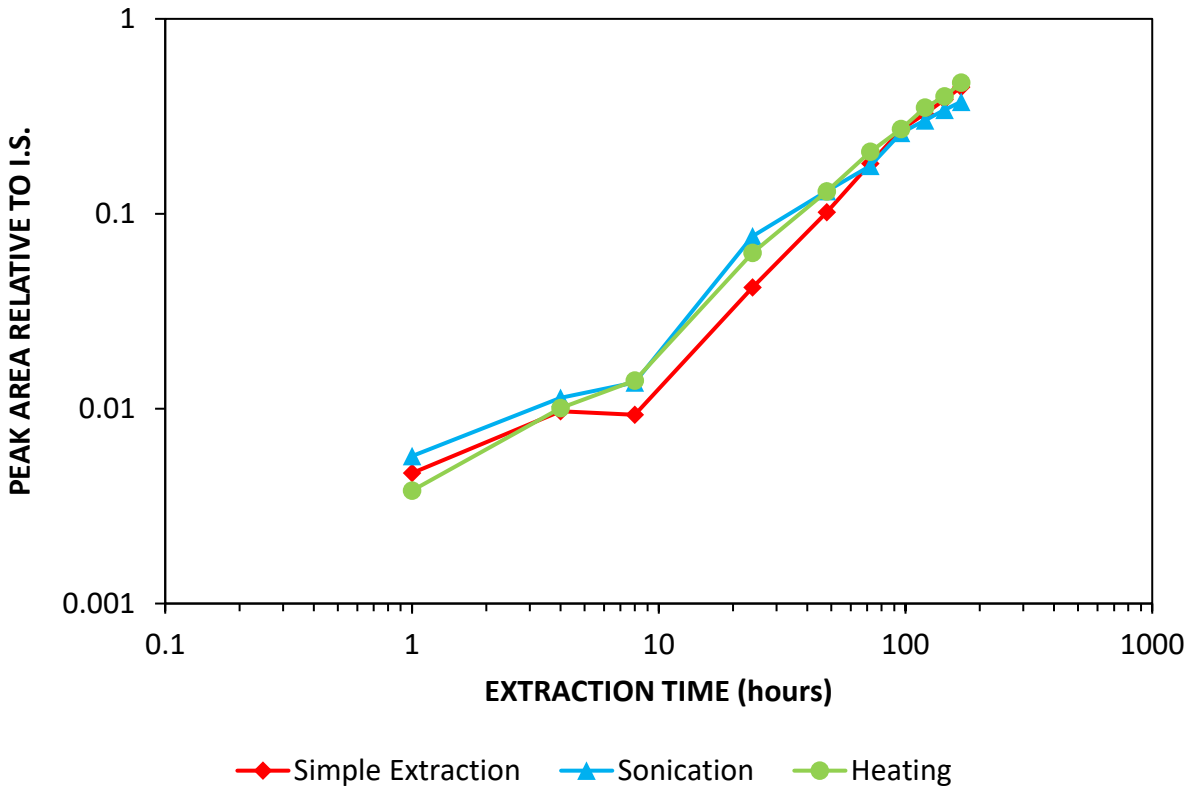


Figure 1. The peak area for palmitic acid (16:0) divided by the undecanoic acid (11:0) internal standard (I.S.). Results are shown for samples which were sonicated for the first four hours, heated for the first four hours, and extracted under normal conditions.

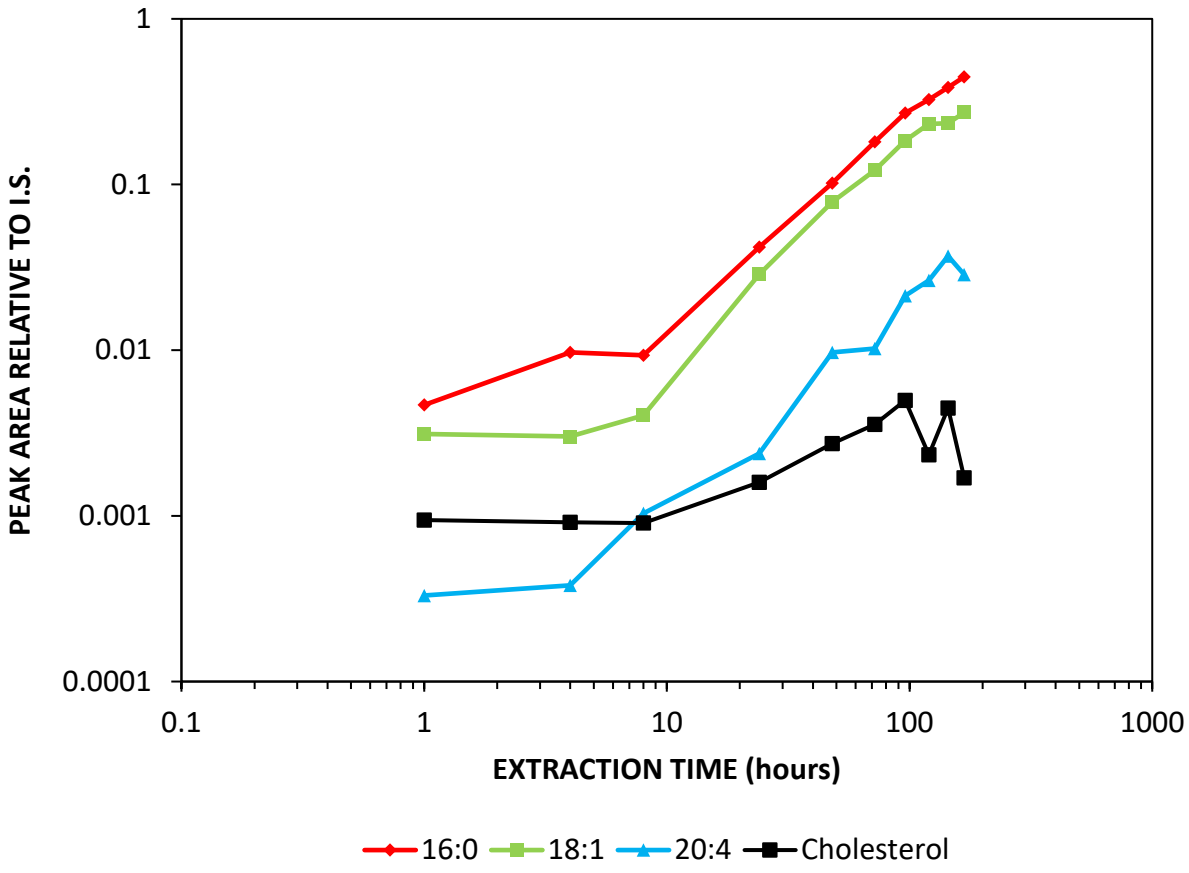
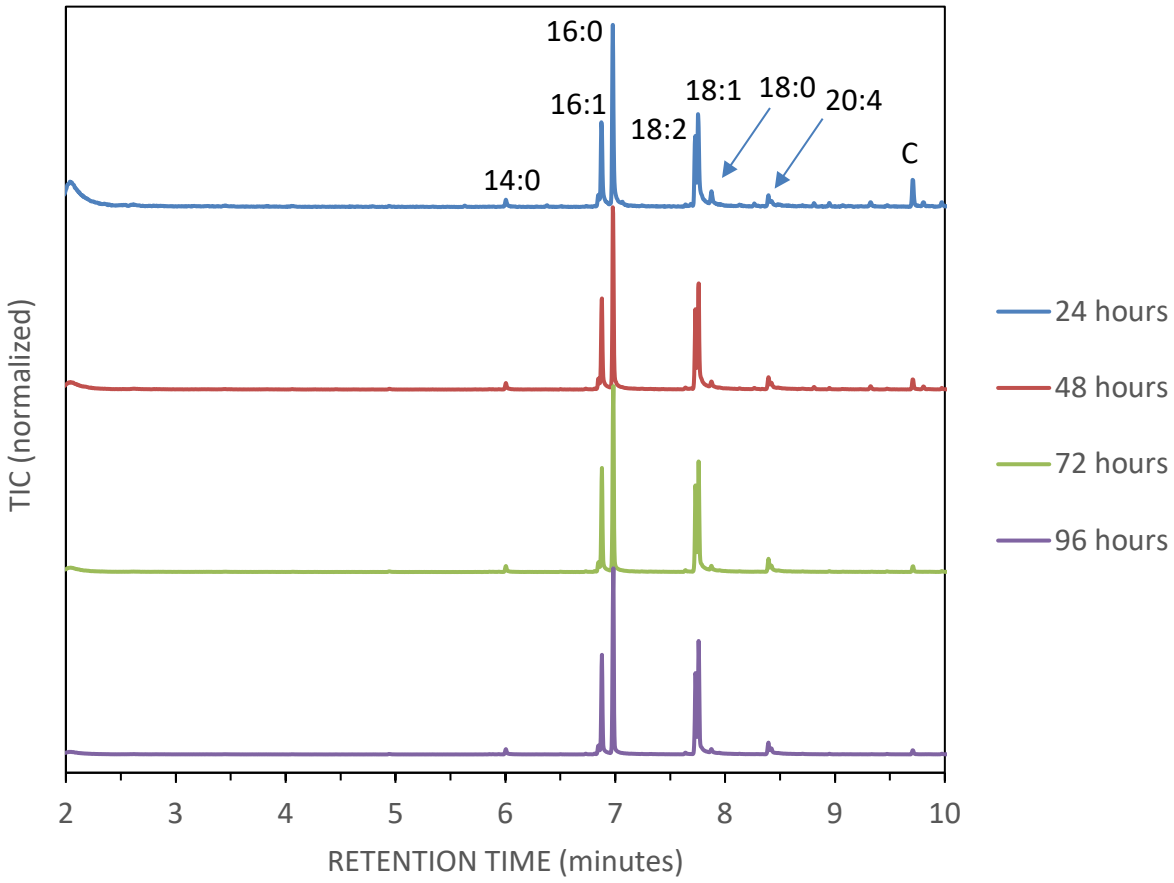


Figure 2. Extraction efficiency of five pupae in pentane as reflected in the relative peak areas for four selected compounds (palmitic acid (16:0), oleic acid (18:0), arachidonic acid (20:4) and cholesterol).



Extraction Time (hours)	Normalization Limit
24	357,189
48	1,575,088
72	2,484,099
96	4,032,476

Figure 3. The effect of solvent extraction time on the chromatograms obtained from *P. reg*. The major fatty acids as well as arachidonic acid (20:4) and cholesterol (C) have been labeled. As is reflected in the normalization limit, overall response increased with time. However, as is reflected in Figure 2, this increase was greater for the fatty acids relative to cholesterol.

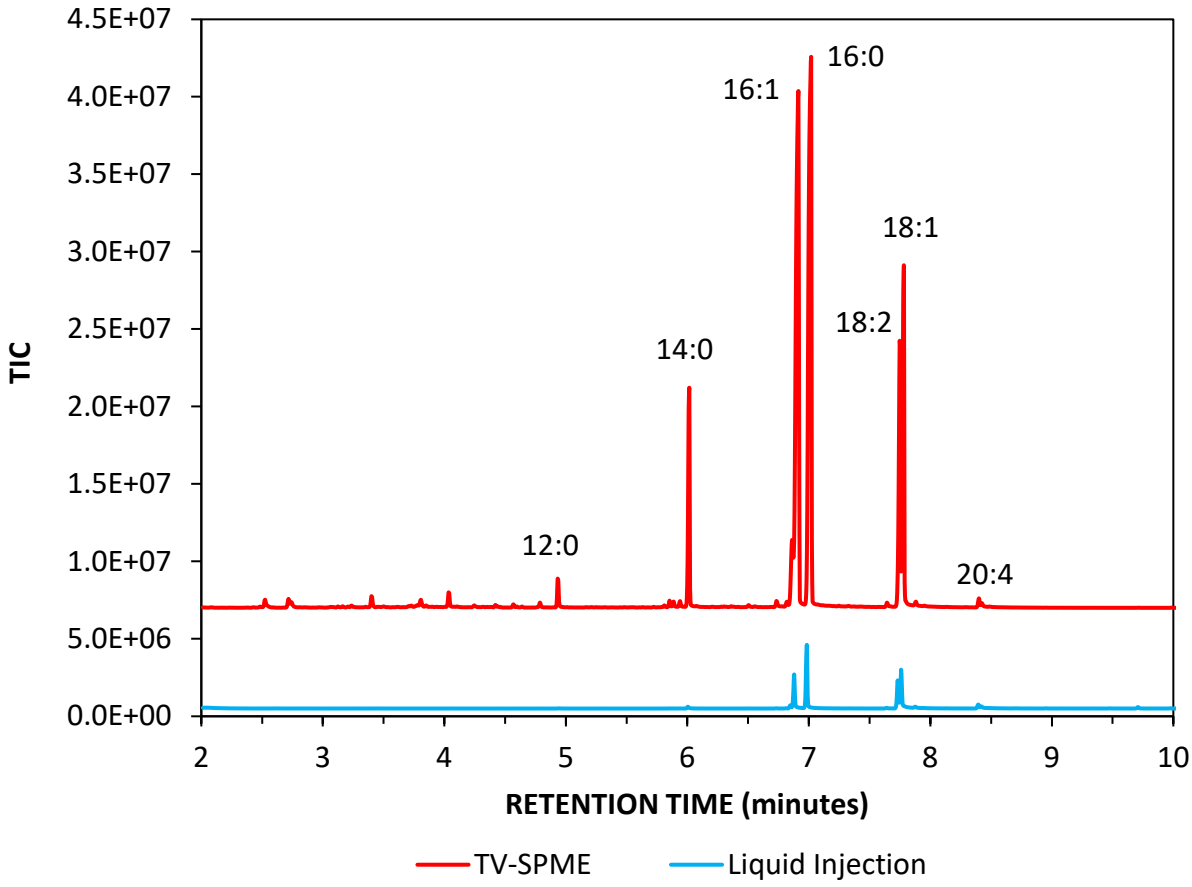


Figure 4. Sensitivity comparison between TV-SPME and liquid injection. The samples in this case were pupae that were extracted for four days. The major fatty acids have been labeled.

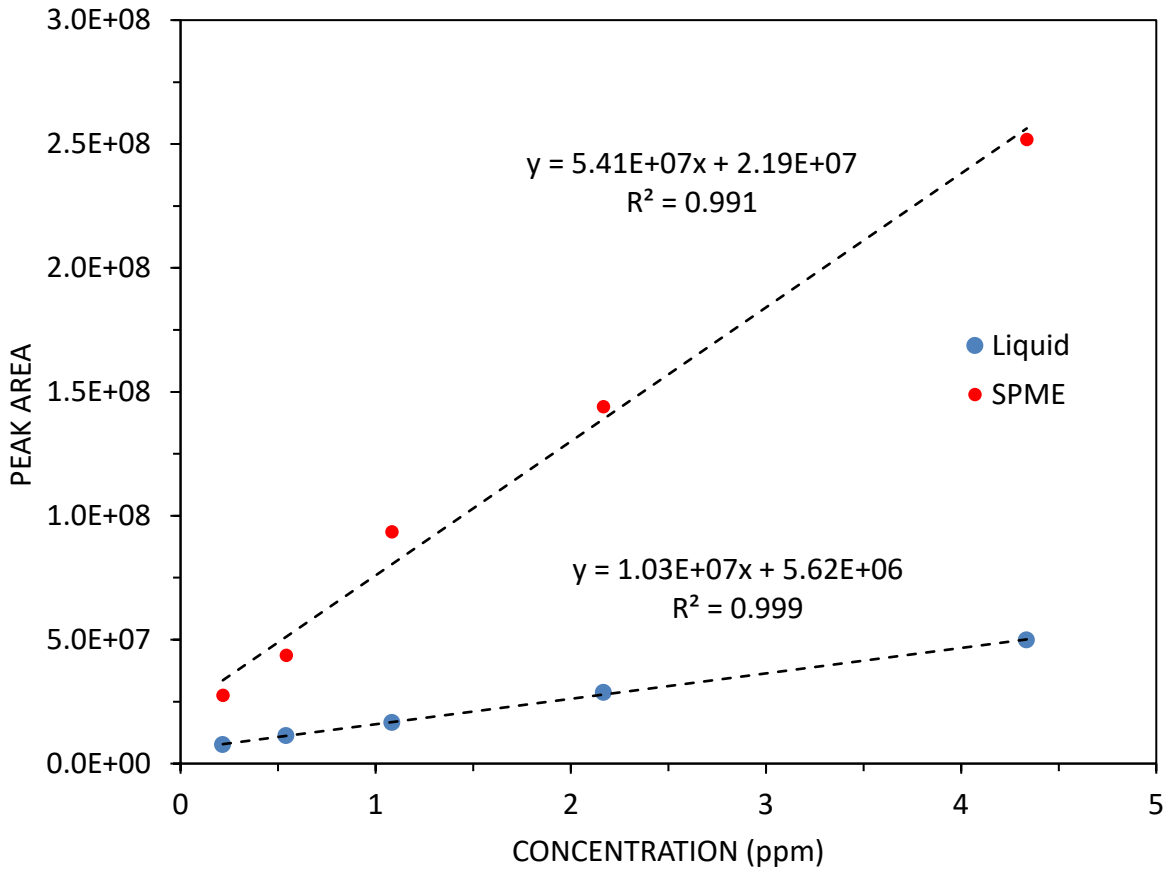


Figure 5. Comparison of TV-SPME and liquid injection calibration curves.

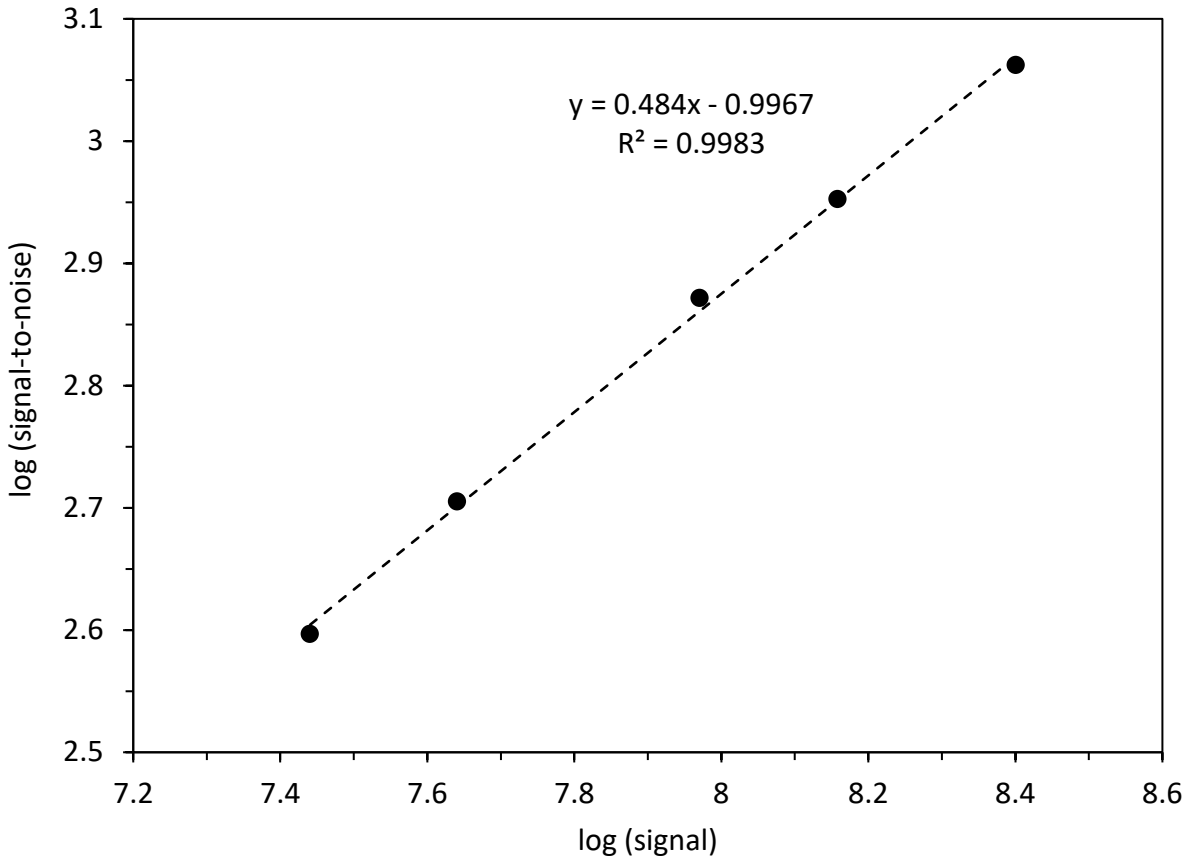


Figure 6. Log-log plot of signal-to-noise as a function of signal.

Conflict of Interest

The authors declare that they have no conflict of interest.

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