



Article

The Impact of Entomological Sample Handling Techniques on a Single Larva Odor Profile

Ana Zoe Monogan, Joshua L. Smith and Paola A. Prada-Tiedemann * 

Department of Environmental Toxicology, Texas Tech University, Lubbock, TX 79416, USA;
joshua.l.smith@ttu.edu (J.L.S.)

* Correspondence: paola.tiedemann@ttu.edu

Abstract: Background: Chemical odor profiling within forensic entomology is an emerging tool given its potential for species identification and larval aging and its ability to identify decomposition stages. A volatile analysis of larval masses across species of distinctive developmental stages was carried out with extraction techniques to identify odor signatures. However, it is unknown how larval sample handling (i.e., live samples in research vs. hot-water-killed samples in casework) affects odor signatures or the possibility of obtaining relevant volatiles from a single larva. **Method:** This study utilized solid-phase microextraction (SPME) with gas chromatography–mass spectrometry (GC/MS) for the analysis of odor volatiles emanating from single larval samples of *Cochliomyia macellaria*. Fifty (50) larvae (25 live; 25 boiled) were analyzed. **Results:** The SPME-GC/MS method allowed for odor volatile detection from a single maggot regardless of the sample handling group. The main compounds identified across both groups included those previously reported as emanating from larvae and decomposition substrates. When comparing treatments, the boiled larval samples had a 6-fold decrease in compound abundance compared to the live samples. The identified odor volatiles observed in the hot-water-killed treatment group included indole, p-cresol, and phenol. **Conclusions:** These results suggest that the handling technique impacts odor detection. Additionally, the heterocyclic aromatics and alcohols identified in the boiled samples are potentially odor markers of a higher intrinsic nature to the maggot rather than a cross-transfer from the decomposition substrate given their survival post elevated temperature treatment. This work shows the plausibility of carrying out an odor analysis of a single maggot following both common research and casework handling practices.



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1. Introduction

Forensic entomology (specifically the area of medico-legal entomology) utilizes post-mortem insect colonizers of a body to gather information useful in estimating the time since death (ex: [1]), the identity of the body (ex: [2]), and/or the possible relocation of the body after death (exs: [3,4]). The time since death, commonly referred to as the post-mortem interval (PMI), can be estimated from entomological evidence in cases where advanced decomposition has rendered earlier body measurements (i.e., algor, livor, or rigor mortis) unreliable. A common approach to estimating time since death using entomological evidence is by estimating the age of a larva removed from a body [5]. Estimating the age of a larva is often determined through a comparison of a physical trait of a larva removed from a corpse to a reference growth curve for the same species under similar environmental

conditions [6]. The larval length, the most common physical trait used to estimate the age of a larva [5,7], is often measured after killing larvae with hot water [7]. Estimating the age of a larva provides an estimate of the minimum time since death, assuming insect colonization occurred after death and assuming that the larvae could not have crawled into or onto a body from a nearby source [8].

While measurements of size (ex: [9]) or development stage (ex: [10]) represent more common approaches to estimating time since death with entomological evidence, chemical composition analysis has become another tool in recent entomological practices. Previous work has estimated age [11–13] and allowed for species identification [14–17] of larvae and pupae based on hydrocarbon composition in the cuticle layer [12,15–19]. The cuticular hydrocarbon composition typically includes the identification of linear alkanes, branched alkanes, and alkenes to monitor changes in these volatile patterns as a function of age and/or species. Attention has been paid to these chemical compounds given they are a main component of the lipid layer of insects whose function is to prevent desiccation [20] and in chemical communication contexts [21]. Recent studies have further expanded volatile odor analysis to include a wider range of odor chemicals to monitor a more generalized volatolomic odor landscape using egg or larval masses as the target odor source for analysis. Using blowfly eggs as the odor substrate, studies have evaluated odor signature differences from two species of the Calliphoridae family [22]. Other studies have utilized volatile emissions to understand decomposition VOCs related to colonized or uncolonized carrion sources and highlighted high-concentration compounds as attractive for carrion-colonizing arthropods [23]. Moreover, volatile odor profiles from larval masses have also been evaluated for use as a potential substrate for understanding cadaveric VOC patterns associated with decomposition [11,24]. The use of larval specimens is advantageous as they are an abundant and easily portable source for cadaveric VOCs. Studies using larvae as odor sources for cadaveric VOCs, however, have relied upon multiple larvae for VOC generation [11,24]. A potential issue in using multiple larvae under casework conditions, however, is if more than one species is present, even a collection of many larvae from the scene may only include limited numbers of a particular species. Accurate species identification of larval forms can be challenging, especially at a crime scene [25]. Research has shown evidence of species-specific VOC patterns [22], so being able to obtain VOCs from a single larva improves the versatility of the analysis and removes variations caused by the presence of multiple species. As chemical odor profiling continues to be explored as an added tool for analytical entomological protocols, it is imperative to understand sample size variations for an optimal understanding of compound abundance and insect diversity to perform identification on the body. To date, however, no VOC studies have focused on analyzing a single larva for chemical odor analysis.

Outside of the absence of single larva odor analysis research, there is also a difference in sample handling techniques typically found in research versus casework [26,27]. Forensic entomology research has expanded with diverse analytical procedures investigated as complementary or standalone tools to enhance estimations of PMI made from larval evidence. Approaches including gene expression assays (ex: [28]), FTIR (ex: [29]), genome size estimation (ex: [30]), and the above-mentioned cuticular hydrocarbon (ex: [12]) and VOC analyses (ex: [24]) have been evaluated. A commonality among these approaches, however, is the absence of testing under the common casework condition of hot water killing as most approaches utilize either live (exs: [12,24,29]) or frozen larval specimens (exs: [28,30]). The omission of hot water killing means it is unknown whether these procedures can be complementary tools to traditional larval length analyses. It would be advantageous if new approaches to enhance information gained from entomological evidence were conducted with casework considerations in mind as it would make these

approaches more applicable in the field and maximize the amount of information gained from the same larval evidence specimen. Currently, a knowledge gap exists as to how common casework handling practices affect a chemical odor profile analysis.

The goals of this research are to (1) evaluate the plausibility of obtaining odor profiles from single larval samples for *Cochliomyia macellaria* (Fabricius) and (2) evaluate the effects of sample handling treatment (i.e., live versus killed with hot water) on the generated odor profile.

2. Materials and Methods

2.1. Insect Rearing

Colonies of *C. macellaria* were established as described in Smith [27]. Eggs were collected on Tyson® brand chicken gizzards. Chicken gizzards were initially frozen at $-20\text{ }^{\circ}\text{C}$ (K2 Scientific, Charlotte, NC, USA) and allowed to thaw overnight at $21\text{ }^{\circ}\text{C}$. Once thawed, chicken gizzards were placed in cages in Hefty® brand red solo cups for a period of 3–24 h to allow for oviposition. Collected eggs were placed into a DigiTherm® Incubator set to $25\text{ }^{\circ}\text{C}$ (TriTech Research, Inc., Los Angeles, CA, USA). The eggs were incubated for 3–4 days based on previous rearing times used within the laboratory. The developmental stage of all analyzed larvae was determined by counting the number of posterior spiracular slits.

2.2. Solid-Phase Microextraction (SPME) Headspace Sampling

Odor volatile identification from the sampled larvae was completed via SPME GC–MS. All samples were analyzed in 7 mL glass vials with a screw cap and PTFE/silicone septa (Supelco, Sigma Aldrich, Bellefonte, PA, USA) throughout the study phase. It was previously established that biologically sterile does not equate to an analytically clean vessel [31]; therefore, prior to any headspace sampling, a cleaning procedure was employed to remove any volatile contamination. Glass vials were sterilized using a methanol solvent (Fisher Scientific, Waltham, MA, USA) rinse followed by a heating period in a $105\text{ }^{\circ}\text{C}$ oven for 2 h. The septa and caps were sterilized via the same method for 15 min. All extractions were conducted in standard laboratory conditions with temperatures ranging from $14.8\text{ }^{\circ}\text{C}$ to $23.4\text{ }^{\circ}\text{C}$ with an average of $17.5\text{ }^{\circ}\text{C}$ and humidity ranging from 25% to 61% with an average of 40%.

After the incubation period mentioned above, some larvae were removed for sampling. For each sampling event, half of the removed larvae were boiled, and half were analyzed live. Boiled samples were individually killed in 150 mL of boiling deionized water on a Fisherbrand Isotemp hotplate (Fisherbrand, Thermo Orion, Chelmsford, MA, USA) and then dried on a paper towel, and single samples were placed into sterilized 7 mL clear screw cap vials with silicone septa for immediate use (Supelco, Sigma Aldrich, Bellefonte, PA, USA). Live samples were individually rinsed in 150 mL of deionized water and then dried and placed in vials as described for the boiled samples.

2.3. Fiber Chemistry Optimization

A variety of five different commercially available fibers were tested for the extraction of odor volatiles from the distinctive larval treatment groups with an extraction time of 24 h. The tested fiber chemistries included 85 μm polyacrylate coating (white), 100 μm polydimethylsiloxane (red), 75 μm carboxen/polydimethylsiloxane (black), 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (gray), and 65 μm polydimethylsiloxane/divinylbenzene (blue). All fibers were from Supelco with a needle size of 24 ga (Supelco, Sigma Aldrich, Bellefonte, PA, USA). Each fiber chemistry type was used to sample the headspace of a live and boiled larval pair for a total of 3 extraction replicates per sample treatment per fiber chemistry type, with a grand total of 30 samples. To ensure

each fiber was free from any odor volatiles or potential contaminants prior to sampling procedures, a blank fiber instrument run was performed. The fiber chemistry that yielded a combination of the highest number of compounds and the highest peak area of identified analytes was deemed optimal for this extraction fiber selection and used in the rest of the study.

2.4. Extraction Time Optimization

Live and boiled larval pairs were sampled using the optimal fiber from the previous step, divinylbenzene/carboxen/polydimethylsiloxane, at five different time points of 30 min, 3 h, 15 h, 21 h, and 24 h. Three extraction replicates of each treatment group (i.e., live vs. boiled) were analyzed per extraction time for a grand total of 30 samples. The extraction time that yielded the highest number of compounds and highest peak area of analytes was deemed the optimal extraction time parameter. Weights for larvae in this group ranged from 207 mg to 853 mg with an average of 546 mg.

2.5. Population Sample Set

SPME fiber chemistry and extraction time parameter optimization for each treatment group yielded an optimal extraction time of 15 h for boiled samples and 21 h for live samples using the divinylbenzene/carboxen/polydimethylsiloxane fiber chemistry as the optimal selection for the larval groups. Using these optimized method variables, a total of 25 live and 25 boiled larval samples were evaluated for a larger population analysis dataset. For each sampling occurrence, a total of five live and five boiled larvae were extracted. This would allow for the monitoring of odor profile reproducibility. High frequency odor volatiles were identified if the volatile appeared in 75% or more of the 25 samples in each sample handling treatment. Larval weights ranged from 291 mg to 645 mg with an average of 452 mg for this group.

2.6. Gas Chromatography–Mass Spectrometry

All fibers were analyzed on an Agilent Technologies 7890A gas chromatograph system with an Agilent Technologies 5975C inert XL mass spectrometry detector with a triple-axis detector (Agilent Technologies, Santa Clara, CA, USA). Fibers were manually injected into the inlet of the GC system for 10 min at 250 °C. An Rtx®-5 capillary 30 m × 250 µm × 0.25 µm column (Restek Corporation, Bellefonte, PA, USA) was used with helium as the carrier gas at a flow rate of 1.0 mL/min. An oven temperature of 40 °C was held for 5 min with a 3 min solvent delay, and then the temperature ramped at a rate of 20 °C/min from 40 °C to 300 °C and was held for 2 min. The total run time was 20 min. Mass spectra were repeatedly scanned from 45 to 550 amu.

2.7. Data Analysis

Compounds were identified with the use of the Chemstation software, v. 10.1.49 (Agilent Technologies, Santa Clara, CA, USA) and the National Institute of Standards and Technology mass spectral library (NIST 2017) with a match spectral quality greater than or equal to 80%. The criteria for compounds identified as frequently occurring in the dataset were those odor volatiles detected in 75% of the overall sample number for each treatment group (n = 25). High-frequency compounds identified across both treatment groups were also used to create liquid solutions of various concentrations (5–100 ppm) to create external calibration curves for quantitation purposes. This was carried out by injecting the liquid solutions into the GC/MS system using the same method used for headspace sampling. An average response factor was then obtained and used to approximate the amount of VOCs being extracted by the SPME fiber. As the study yielded multivariate data, paired *t*-tests were used for comparing treatment groups using R [32].

3. Results

All larvae utilized throughout the study were third instar. The live larval weights in the fiber chemistry optimization group ranged from 166 mg to 616 mg with an average of 365 mg, and the boiled larval weights ranged from 124 mg to 665 mg with an average of 381 mg. The weights ranged from 207 mg to 831 mg with an average of 545 mg for live larvae and ranged from 258 mg to 853 mg with an average of 549 mg for boiled larvae in the extraction time optimization group. For the population sample set, the live weights ranged from 294 mg to 585 mg with an average of 453 mg, and the boiled weights ranged from 291 mg to 645 mg with an average of 452 mg. Using paired t-tests, no statistical differences between the live and boiled treatment weights were observed for fiber ($p = 0.2411$, $\alpha = 0.05$), time ($p = 0.4002$, $\alpha = 0.05$), or the population sample set ($p = 0.5177$, $\alpha = 0.05$) groups.

3.1. SPME Fiber Chemistry Optimization

Three replicates from each larval handling treatment group were extracted for 24 h with each of the five fiber chemistries. The resulting odor profiles were then evaluated based on the abundance and number of detected compounds. Figure 1 depicts the mean peak area response of compounds extracted across the two larval treatment groups to determine the suitability of the five (5) fiber chemistries tested. It was determined that for both larval handling treatment groups, the 50/30 μm DVB/CAR/PDMS fiber chemistry was deemed optimal for extraction purposes based on both the ability of the fiber to extract the highest number of odor volatiles as well as the highest abundances of the selected compounds. While the PDMS/DVB fiber was able to extract a higher average abundance in the live treatment group, there was a lower average number of compounds, five, compared to the DVB/CAR/PDMS fiber chemistry, which was able to extract seven compounds on average. Similarly, DVB/CAR/PDMS extracted three compounds on average compared to the one to two compounds extracted using the other fiber chemistries.

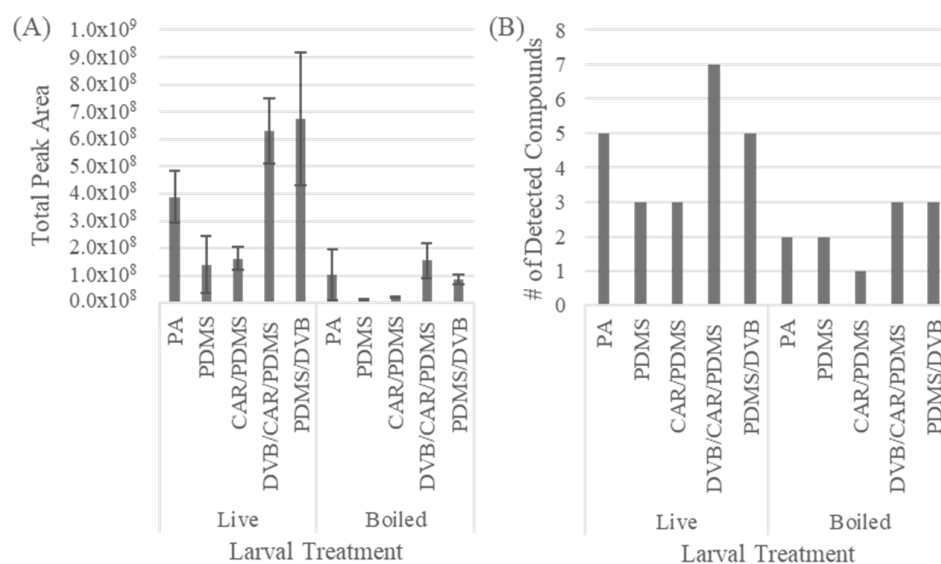


Figure 1. SPME fiber optimization results showing (A) total average peak area response as function of fiber chemistry type for larval treatment groups and (B) average number of detected compounds as function of fiber chemistry type for larval treatment groups ($n = 3 \pm \text{SE}$).

3.2. Extraction Time Optimization

The extraction time optimization results are summarized in Figure 2, showing the ratio of compounds per extraction time in the live and boiled samples. For the live samples, an extraction time of 21 h was selected due to the greatest number of seven compounds

collected in the headspace compared to the five compounds collected in the 15 h and 24 h extraction times. For the boiled samples, an extraction time of 15 h was selected, also due to the greatest number of four compounds being observed in the headspace for this extraction time with the highest average peak area.

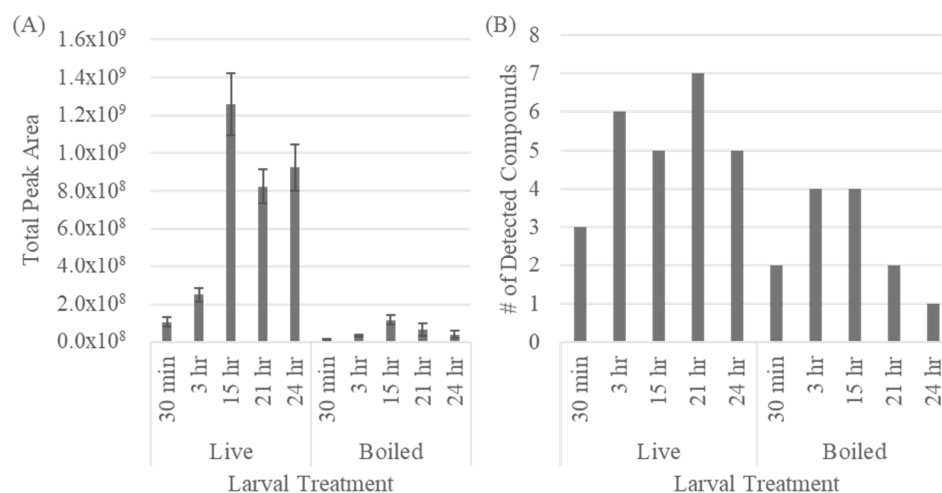


Figure 2. SPME extraction time optimization (A) peak area response as function of extraction time and (B) average number of compounds extracted as function of extraction time ($n = 3 \pm \text{SE}$).

3.3. Population Sample Set

Chemical odor characterization was performed for a total of 25 live larvae and 25 boiled larvae using the optimized SPME-GC/MS method developed. There were six overall volatile organic compounds that were identified as having a high frequency in the total population, defined by an occurrence of over 75% within the sample handling treatment. The compounds included dimethyl disulfide, dimethyl trisulfide, indole, p-cresol, phenol, and phenylethyl alcohol. Table 1 summarizes the data in terms of the volatile frequency of occurrence for each larval treatment sample group. These highly occurring compounds are categorized into the following functional groups: sulfur compounds, aromatic heterocyclic, and aromatic alcohols. All of these compounds have previously been reported in the literature either from entomological origin or as decomposition odor traces (Table 1). Figure 3 depicts an example of the total ion chromatograms for both larval treatment groups, highlighting these frequently occurring compounds.

Table 1. High-frequency odor volatiles in single larvae samples across treatments (occurrence > 75%).

Compound	Frequency of Occurrence					Previously Reported in the Literature
	Live	Percent	Boiled	Percent	Total	
Dimethyl disulfide	20	80%	18	72%	38	[22–24,33–55]
Dimethyl trisulfide	24	96%	13	52%	37	[22–24,33,34,37,39,44,45,47–49,51–61]
Indole	25	100%	19	76%	44	[11,23,24,34,35,37,38,45,48,49,57,59,62–64]
p-Cresol	23	92%	21	84%	44	[24,57]
Phenol	25	100%	25	100%	50	[11,22,24,34,37,38,45,48,49,51,52,57–59,62,63,65]
Phenylethyl alcohol	22	88%	10	40%	32	[22–24,59]

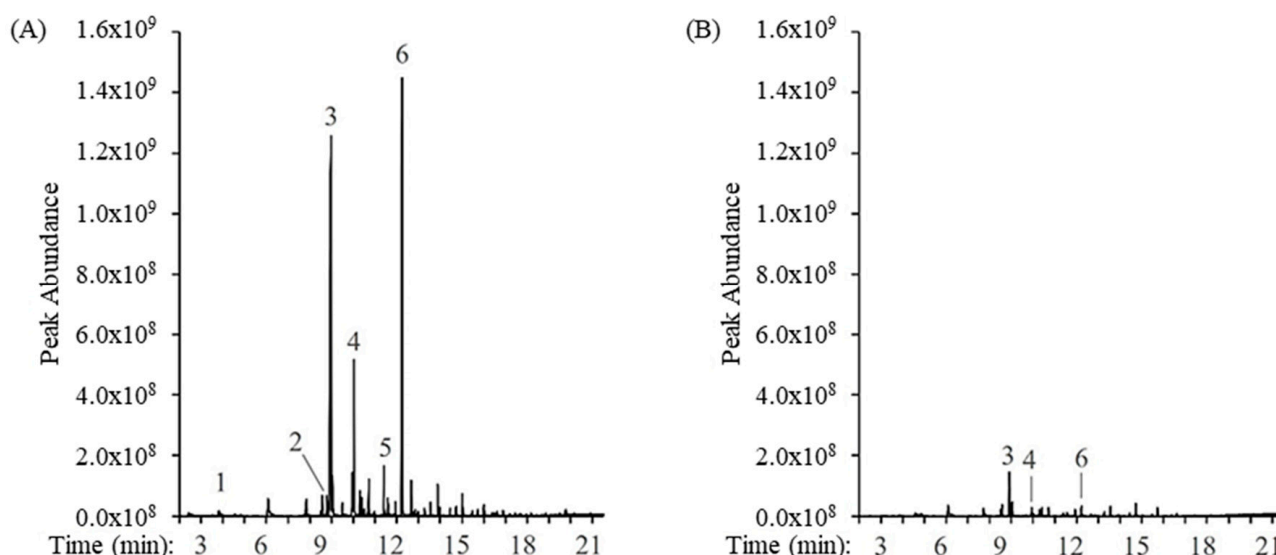


Figure 3. Total ion chromatograms for a single larva sample for both handling treatment groups: (A) live and (B) killed with hot water. The labeled compounds are as follows: dimethyl disulfide (1), dimethyl trisulfide (2), phenol (3), p-cresol (4), phenylethyl alcohol (5), and indole (6).

To further evaluate the distribution of highly occurring compounds across both sample treatment groups, Figure 4 depicts a color odor chart comparing the average peak area of individual volatiles to the overall chemical profile. For both treatment groups, phenol and indole are large contributors to the chemical odor profile. However, an important trend to observe is the decrease in the peak area response (6x fold decrease) in the boiled sample treatment group compared to the live samples.

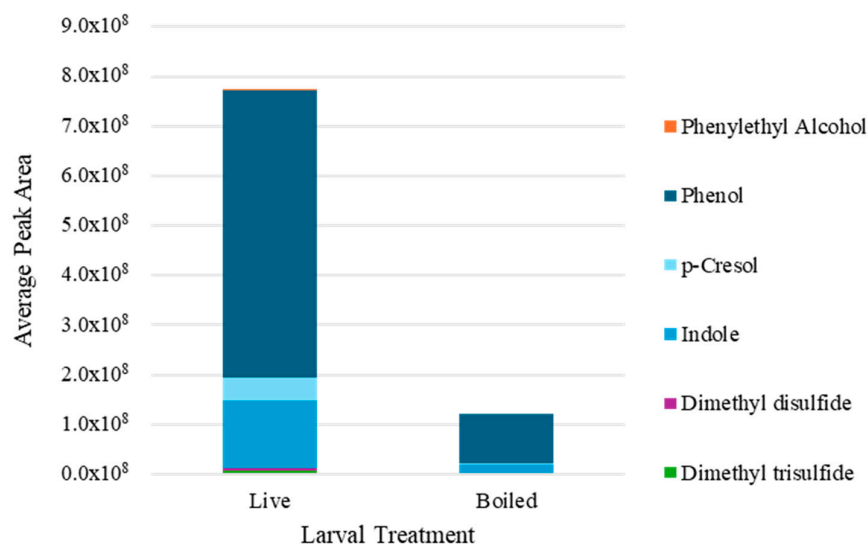


Figure 4. Color chart of odor distribution across sample treatment groups.

Using compound vapor concentration for each frequently occurring compound across both treatment groups, it can be noted from Figure 5 that phenol yields the highest average concentration (10.57 ppm live; 2.92 ppm boiled), followed by indole (2.81 ppm; 1.45 ppm) and p-cresol (1.74 ppm; 1.29 ppm). Dimethyl disulfide (DMDs), dimethyl trisulfide (DMTS), and phenylethyl alcohol (PEA) were only present in the live odor profile at relatively low average concentrations of 1.36 ppm, 1.30 ppm, and 1.25 ppm, respectively.

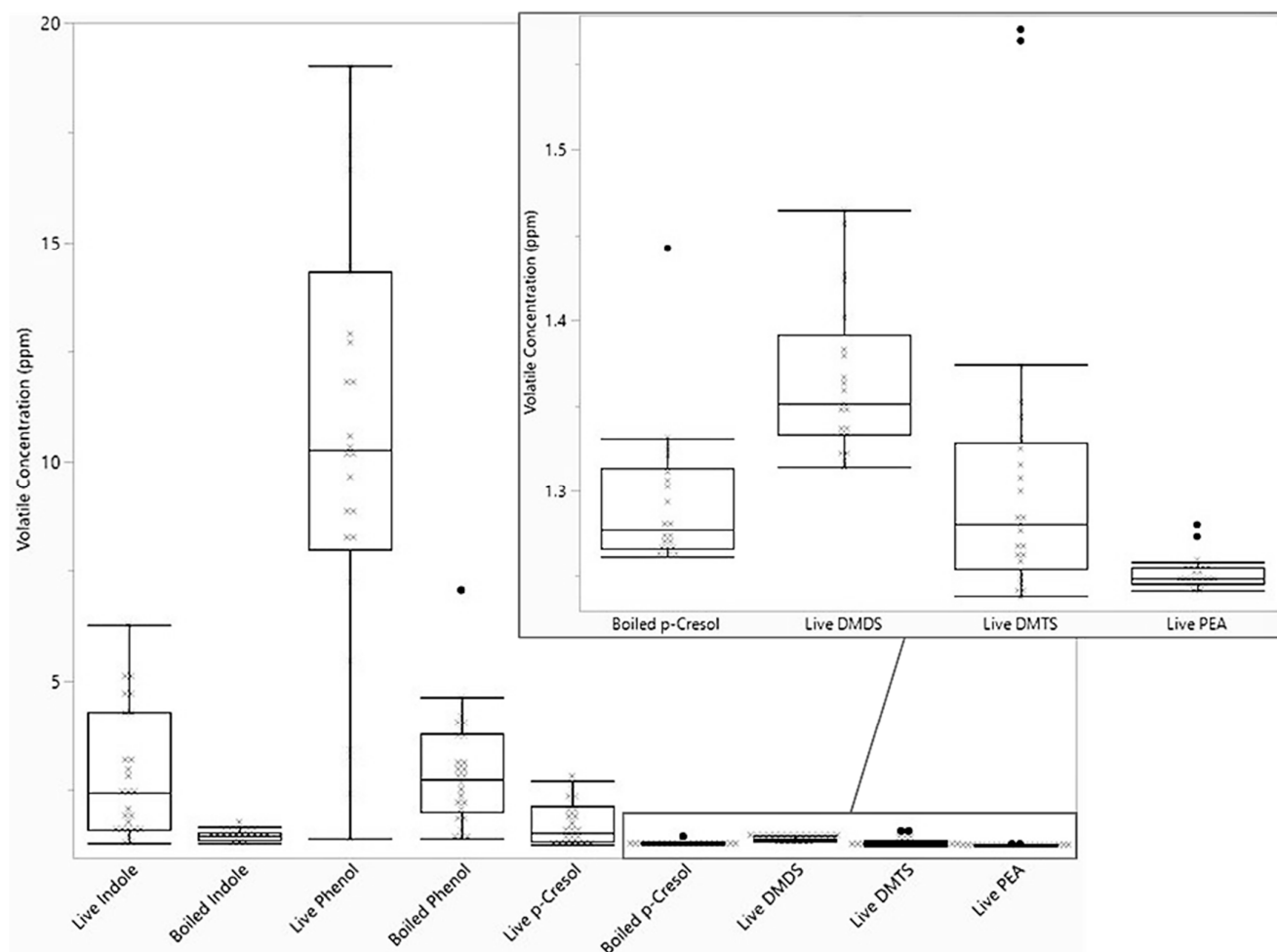


Figure 5. Box plot concentration distribution of frequently occurring compounds across both treatment groups. Solid black dots are potential outliers in the data sets.

4. Discussion

This study aimed to characterize the volatile odor profile of a single *C. macellaria* larva and highlight the difference between sample handling treatments in the field compared to those in traditional research settings. The developed SPME-GC/MS methodology proved to be viable for the detection of a chemical odor profile within a single larval sample. Prominent compounds in this study across the two sample treatment groups include dimethyl disulfide, dimethyl trisulfide, indole, p-cresol, phenol, and phenylethyl alcohol. These frequently occurring VOCs have also been observed throughout decomposition processes and reported within the literature on cadaveric odor [24]. Foundational work in entomological odor analysis encompass the age and taxonomic identification of forensically relevant species [11,17,19,66]; however, these studies focused on larval masses rather than single larval samples. Furthermore, most studies have targeted hydrocarbon or fatty acid composition using solvent extraction procedures [15,67,68] for the chemical analysis rather than direct headspace techniques. The characterization of odor volatiles from our study confirm the results obtained by Frederickx et al. (2012) [11], who also utilized SPME extraction procedures and reported compounds such as phenol and indole. In contrast to their study, which utilized a shorter heated extraction time (1 h) as well as live larvae masses, our study encompassed longer extraction times on the single maggot sample without solvent or heat enhancement during extraction procedures and still yielded compound detection.

It is important to note that the live single larval treatment group depicted sulfur-containing compounds such as dimethyl disulfide and dimethyl trisulfide, both of which have been cited as a highly prominent volatile within decomposition substrates [33,34,44,45,47,48,51,54]. These sulfur-rich compounds originate from bacterial decomposition products and have been majorly identified in the bloat stage of the decomposition timeline [69]. Both dimethyl disulfide and dimethyl trisulfide have also been identified as carrion attractants during behavioral response testing across various insect species [70–72]. The identification of these cadaveric origin compounds in this population set corroborates the complex host–substrate interaction between the maggot and the decomposing odor source, suggesting that the identified sulfur odor volatiles may in fact undergo direct transfer during feeding and oviposition activities. Given that the hot-water-killed treatment group did not depict the same sulfur-containing compounds, the results suggest that compounds such as indole, p-cresol, and phenol are more resistant to elevated temperatures and could therefore be indicative of volatile odor markers for future studies. While this study did not report new odor volatiles from the single larva sample matrix, further studies should investigate whether these reported odor chemicals are intrinsic to *C. macellaria* or are common across multiple species for enhanced volatolomic identification purposes.

The second aim of our study was to investigate another parameter in insect odor analysis: sample handling effects on odor signatures. The preservation and storage of insect samples plays an integral part in entomological procedures, hence the common practice of boiling as a routine killing mechanism for length measurement and long-term storage [7,26,73]. However, to date, no research work has studied the effect of this practice on chemical odor signatures of the collected samples within or across species of forensic importance. The results demonstrate that the hot water killing process reduces the vapor concentrations of reported volatiles in a single larva sample and depicts a decrease in the number of compounds detected. As previously mentioned, it was shown that sulfur-containing compounds found in the parallel live sample treatment group were not detected in the hot-water-killed samples, thereby demonstrating that boiling temperatures affect the subsequent emission of these odor chemicals. This result could verify that while the interaction between the insect–decomposition matrix provides a platform for odor volatile transfer, laboratory procedures such as these boiling processes may remove sulfur-containing compounds from insect specimens. While there was a reduction in the number and concentration of compounds, the fact that some compounds were still detected in hot-water-killed samples means that larval specimens used for traditional length analysis in age estimation can also be used as a source of cadaveric VOC, increasing the information gained from a single specimen.

The gap between research practices and casework in forensic entomology is known [26]. The current work, by analyzing single larval specimens and directly comparing results between live and hot-water-killed specimens, aids in better connecting casework and research practices for forensic entomological volatile odor analysis. While this study demonstrates the feasibility of the chromatographic method for the volatile odor detection of single larval samples across two distinctive sample treatments, further studies using both animal analogs and human models are needed to extrapolate the obtained results to practical operational contexts in forensic taphonomy applications. Furthermore, this study needs to be replicated across multiple species, geographical locations, and environmental conditions to verify the robustness of the identified volatile odor chemicals across a range of pivotal variables encountered in the decomposition landscape. Additionally, a broader size and development stage range, specifically targeting smaller individuals in earlier instars, will allow for the determination of any possible size-based threshold for single larva odor

analysis. Finally, the impact of larvae preserved in ethanol on the odor profile, and the recommended storage solution in entomological casework [7], needs to be investigated as well.

5. Conclusions

This study evaluated headspace odor volatiles from single larval samples of *C. macellaria*. The chemical characterization targeted two distinct treatment groups, live and killed with hot water, which has provided valuable insights into the impact of sample handling techniques on volatile odor abundance and composition. Using SPME-GC/MS, we observed a six-fold decrease in the overall abundance of volatile compounds in the hot-water-killed (boiled) samples compared to the live ones. Notably, the live samples were characterized by the presence of sulfur-containing compounds, whereas the boiled samples primarily exhibited indole, p-cresol, and phenol. This shift in chemical profile highlights the significant effect of heat treatment on the volatilization process. The analytical method proved to be a robust tool for detecting and quantifying these differences, suggesting its applicability to volatolomic analysis within forensic entomology practices. Further research may expand upon this by exploring other insect species and translating the analytical method to both human analog models and human cadavers in order to validate the technique within field contexts and extend beyond laboratory insect rearing. This study provides a foundational framework depicting the capability of a single larval sample to yield a chemical odor profile and provides insights into the effect of sample handling on volatile pattern emissions.

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