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ARTICLE

Dead bodies still tell tales: An alternative noninvasive and nondestructive protocol to extract cuticular hydrocarbons from museum specimens

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Abstract. Natural history museums represent valuable spaces for science outreach and as repositories of biodiversity data. The collections maintained in these institutions are often used as data sources for studies across various disciplines, such as ecology, phylogenetics, and population genetics. Another field that can also benefit from these resources is chemical ecology. Using insects stored in museums offers an alternative avenue for addressing new questions about the dynamics of their cuticular hydrocarbons (CHCs) with respect to different factors such as their interspecific variability, temporal stability and in chemotaxonomy. CHCs play a dual role in insects, serving as mechanical protection against desiccation and pathogens and mediators of communication processes. However, conventional extraction procedures can compromise the specimens given their invasive nature (*e.g.,* hexane immersion approach). Here (1) we propose an alternative, noninvasive and non-destructive method to study CHCs in pinned insects from museum collections and (2) we examine the efficacy of this alternative compared to a more conventional approach for determining CHC profiles. We used two model organisms to address our goals, the *Apis mellifera* Linnaeus, 1758 and the *Tetragonisca angustula* (Klug, 1807). We were able to detect CHCs in pinned specimens from both species though there was a qualitative reduction in pinned specimens when compared to fresh ones. The observed variability may reflect confounding differences associated with sampling locality. Our results suggest that this alternative protocol can be applied to assess the CHC composition of museum specimens without causing destruction and represent an effective method to be used in studies integrating taxonomy and chemical ecology.

Keywords. Museum collections; Cuticular Hydrocarbons; Alternative Protocol; SPME Fiber.

INTRODUCTION

Natural history museums (NHM) have an important role as alternative spaces for science communication (Falk *et al.,* 1986). Additionally, NHM serve as valuable repositories of biodiversity data (Ponder *et al.,* 2001). The museum specimens can be used by researchers belonging to different fields such as population genetics (Wandeler *et al.,* 2007), conservation (Lister & Climate Change Research Group, 2011), ecogeography (Miller & Sheehan, 2021) and phylogenetics (Payne & Sorenson, 2002). The DNA extraction from insect museum specimens is a common practice (Goldstein & Desalle, 2003; Gilbert *et al.,* 2007; Hernández-Triana *et al.,* 2014). For instance, all Lepidoptera specimens from AINC (Australian National Insects Collection) have been sequenced (Hebert *et al.,* 2013); in addition, Andersen & Mills (2012) extracted and amplified the genes COI and 28S from different parasitic Hymenoptera.

In the field of chemical ecology, some efforts have been made to understand the properties of cuticular hydrocarbons (CHCs) stability across the years in social wasps from NHM (Martin *et al.,* 2009). Due to their stability, CHCs stand out as promising components to complement more conventional approaches (*e.g.,* morphological

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or genetic data) for species identification. CHCs have dual functions in insects: they provide a protective barrier against desiccation and microbial infection (Gibbs & Pomonis, 1995) and act as cues or signals in communication among adults and between adults and brood, reflecting traits such as age, sex, task, nest origin and health status (Le Conte & Hefetz, 2008; Cappa *et al.,* 2016; da Silva *et al.,* 2021; de Souza *et al.,* 2023; da Silva *et al.,* 2025). Accordingly, NHM insect specimens offer valuable opportunities to investigate and clarify ecological and evolutionary questions related to CHC variation and dynamics.

Nevertheless, using conventional methods such as hexane immersion can be invasive and may compromise pinned specimens. To overcome this limitation, in this work we (1) propose an alternative, noninvasive and non-destructive methodology for sampling CHCs from NHM insect specimens and (2) assess its efficacy relative to a more conventional method for determining CHC profiles.

MATERIAL AND METHODS

Data collection

We conducted this study between July and December of 2016. We used both recently frozen (hereinafter = fresh samples) and pinned specimens (24 years old) for our analysis. The pinned individuals were stored at the *Laboratório de Ecologia e Comportamento de Insetos Sociais,* Universidade de São Paulo, in Ribeirão Preto. We worked with two bee species (Hymenoptera: Apidae): *Apis mellifera* Linnaeus, 1758, and *Tetragonisca angustula* (Klug, 1807). The pinned specimens were originally collected in 1992 at the Estação Ecológica Jataí (21°36′54″S, 47°48′02″W), Luís Antônio, São Paulo, Brazil (Mateus, 1998). Fresh specimens were collected on the campus of the Universidade de São Paulo in Ribeirão Preto (21°09′49.7″S, 47°51′30.9″W), São Paulo, Brazil. The mean distance between the two sampling sites was approximately 51 km.

Description of the conventional and alternative non-destructive protocols for the extraction of insect museum specimens

To prepare the fresh samples, we collected specimens and we kept them at a low temperature -20°C until their CHC extraction. We followed a standard protocol to extract the CHCs from the fresh samples (Martin *et al.,* 2009), which consisted of washing them individually in glass vials for 3 minutes with n-Hexane (Makron® 95%). Then, we removed the specimens from the glass vials, and we left the extracts to evaporate in a flow chamber under room temperature. Following the evaporation step, we suspended their content in 40 µL of n-Hexane and we left them in a sonic bath for 10 seconds. We later injected a total of 2 µL of their content in a system of gas-chromatography coupled with a mass spectrometer (GC-MS; Shimadzu, model QP2010 Plus). To extract the CHCs from the pinned individuals without compromising their integrity, we used a SPME fiber (Supelco 100 µm, red 24 ga) in combination with n-Hexane (Makron 95%) as a solvent. The following steps were followed (see Fig. 1):

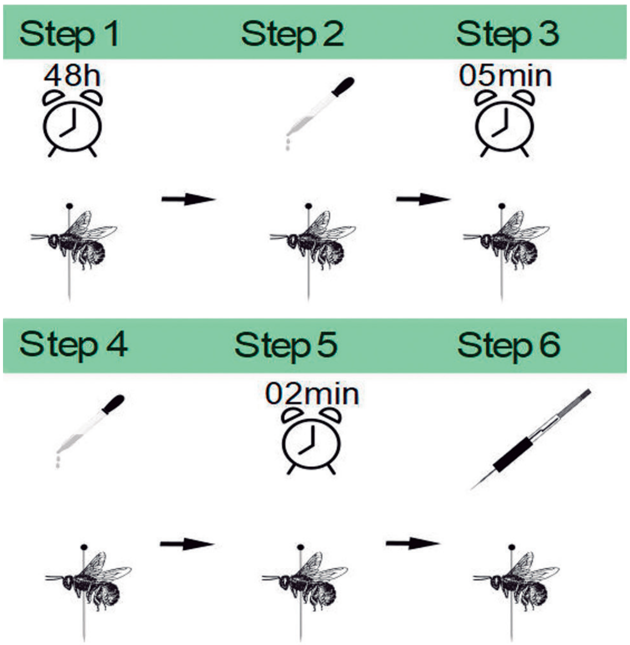

Figure 1. Steps to alternative protocol of chemical composition extraction from pinned insect specimens using SPME fiber. In the Steps 1, 3 and 5 the watch represents the time necessary to complete the procedure. In the Steps 2 and 4 the pipet represents the solvent drop procedure. In the Step 6 the SPME fiber represents the extraction using fiber.

(1) Remove specimens from their collection boxes and leave them in a flow chamber for at least 48 hours. This step is essential to avoid any chemical compounds used for collection maintenance (such as naphthalene and camphor) from contaminating the extracts to be used in the GC-MS system.
(2) After 48 hours, apply one (or more, depending on the specimen size) n-Hexane drops on the specimen. This step is important because it will allow the lipids covering the body of the specimen to soften.
(3) Allow the n-hexane to evaporate (~ 5 minutes).
(4) Repeat step 2 and 3.
(5) With an SPME extraction fiber, gently rub the specimen for about 2 minutes. This step is important because the previously softened lipids will then adhere to the SPME fiber.
(6) Following step 5, we inserted the fiber into the GC-MS system and left it in place for 5 minutes to allow the adsorbed lipids to desorb and be analyzed by gas-chromatography.

Chemical Analysis

We analyzed all samples in a system of gas-chromatography coupled with a mass spectrometer (GC-MS;

Shimadzu, model QP2010 Plus) equipped with a column Rtx-5MS (thickness 0.25 µm; length 30 m; diameter 0.25 mm) and helium as carrier gas. We injected samples at 280°C in the splitless mode. We used a different temperature protocol for each study species to optimize the acquisition of their chemical profiles. We prepared a total of 39 samples (*A. mellifera* – *n fresh* = 10 and *n pinned* = 10; *T. angustula* – *n fresh* = 10 and *n pinned* = 9). For *A. mellifera* samples, the oven temperature started at 150°C and reached 250°C at a rate of 5°C/min, then it remained at 250°C for 5 min. Subsequently, the temperature increased at the rate of 1°C/min until 255°C, which was maintained for 2 minutes. Finally, the temperature increased at a rate of 2°C/min until 280°C. For *T. angustula* samples, the initial oven temperature started at of 150°C and it increased at a rate of 10°C/min until 280°C, and it remained at this temperature for 10 minutes. We identified peaks based on their expected mass spectrometric fragmentation patterns and retention indices.

Statistical Analysis

To compare the number of identified compounds between fresh and pinned samples, we performed an analysis of variance (ANOVA) using the *stats* package in R (R Core Team, 2024). We then evaluated whether the dispersion (variance) of CHC profiles differed between groups using a test of multivariate homogeneity of variances (PERMDISP; Anderson, 2006) implemented in the *vegan* package (Oksanen *et al.,* 2024). To visualize the clustering patterns of samples based on their CHC profiles, we conducted a non-metric multidimensional scaling (NMDS; Clarke, 1993) using Bray-Curtis distances, also with the *vegan* package. To statistically test for differences in CHC profiles between fresh and pinned samples, we performed a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) with *vegan.* Finally, to identify which compounds contributed most to the dissimilarities between groups, we used a similarity percentage analysis (SIMPER; Clarke, 1993) in *vegan.* All analyses were performed in R version 2024.12.1.

RESULTS

We were able to extract the CHCs from the studied species (Fig. 2), with a high compound abundance (10^7 to *A. mellifera* and 10^6 to *T. angustula*). We detected quantitative and qualitative differences in the CHC profiles of fresh and pinned samples from both species. We identified 64 compounds in *A. mellifera* and 47 in *T. angustula* samples. The carbon chain length ranged from 16 to 37 to pinned individuals and from 29 to 40 to fresh ones in *A. mellifera.* We detected a decrease of 25.225% in the number of compounds in pinned individuals of *A. mellifera.* The carbon chain length ranged from 12 to 23 in pinned individuals, and from 22 to 35 to fresh ones in *T. angustula* samples. We detected a decrease of 56.229% in the number of compounds found in *T. angustula* samples. Although we only focused on the CHCs to perform all our analysis, we also identified oleic acid in pinned individuals (Fig. 2). The number of compounds was significantly different in pinned and fresh individuals, in both species (*A. mellifera:* F = 12.24, df = 14.94, p = 0.003; *T. angustula:* F = 27.95, df = 14.63, p < 0.001). These differences were also evident in the relative proportions of different classes of compounds (Table 1).

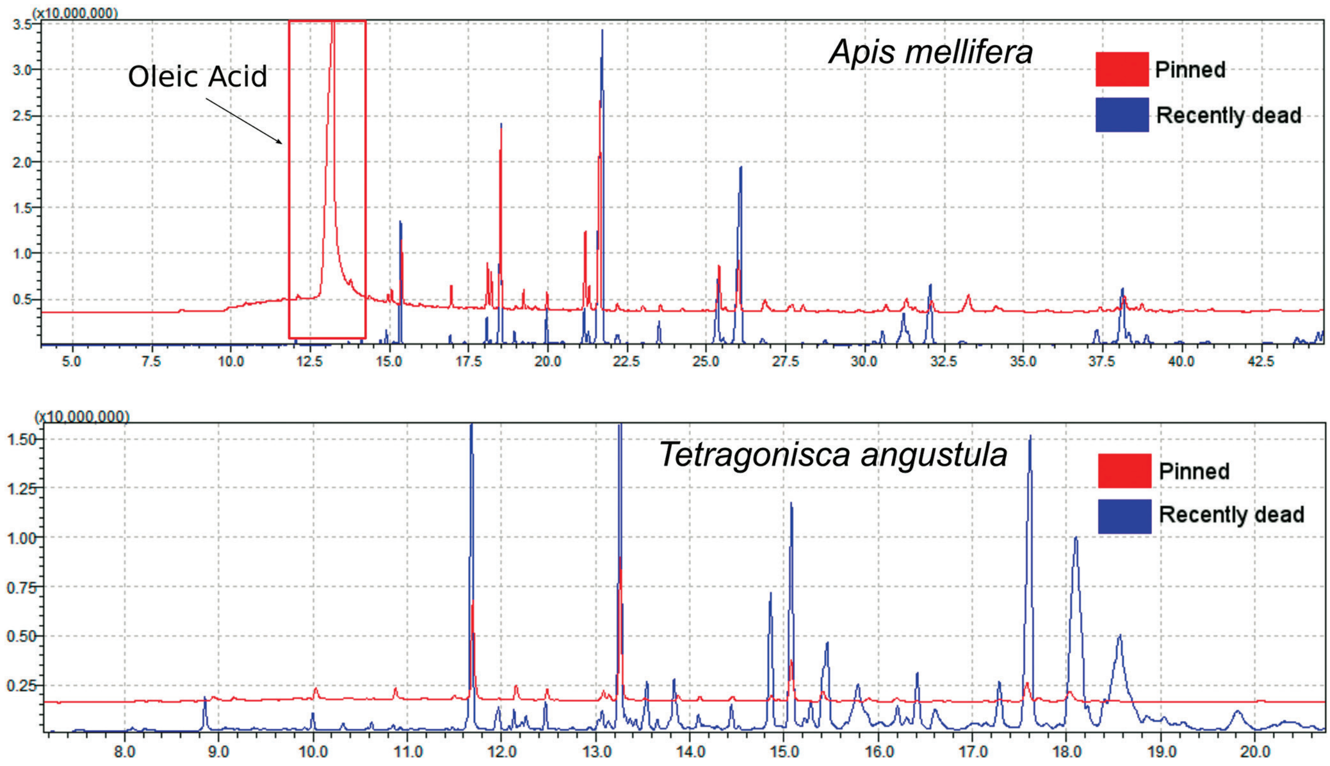

Figure 2. Chemical cuticular profile both study species, *Apis mellifera* and *Tetragonisca angustula* (pinned and fresh individuals).

Table 1. Class and quantity (mean ± SD) per class of compound in two bee species *Apis mellifera* and *Tetragonisca angustula*.

Species	Compound Classes	Sample Type	Mean	SD
Apis mellifera	Alkane	Live	72.19	8.66
		Pinned	72.95	9.27
	Alkene	Live	21.67	1.64
		Pinned	19.96	1.76
	Branched-Alkanes	Live	1.21	0.67
		Pinned	5.05	0.18
	Alkadienes	Live	4.29	0.69
		Pinned	0.19	0.12
Tetragonisca angustula	Alkane	Live	43.90	6.18
		Pinned	77.41	10.13
	Alkene	Live	8.86	3.17
		Pinned	13.64	2.30
	Branched-Alkanes	Live	47.24	4.09
		Pinned	8.86	2.22
	Alkadienes	Live	0	0
		Pinned	0.09	0

There was no significant difference in the variance of profiles for *A. mellifera* (*pseudo*-F = 3.046, p = 0.103). However, for *T. angustula,* fresh individuals exhibited greater variance than pinned ones (*pseudo*-F = 10.754, p = 0.005). The NMDS analysis showed a clear separation between fresh vs pinned samples for both species (*A. mellifera:* stress = 0.096, Non-Metric fit R2 = 0.991, Linear fit R2 = 0.957; *T. angustula:* stress = 0.026, Non-Metric fit R2 = 0.999, Linear fit R2 = 0.998) (Fig. 3). According to the PERMANOVA, the CHC profiles of fresh and pinned samples were statistically different (*A. mellifera: pseudo*-F = 12.63, *p* = 0.001; *T. angustula: pseudo*-F = 19.495, *p* = 0.001). According to the SIMPER, the compounds that explained 70% of the CHC variance among different types of samples were: *A. mellifera:* linear alkanes (n-C23; n-C24; n-C25; n-C27; n-C29; C31), alkenes (C25:1-1; C25:1-2; C27:1; C29:1; C33:1; C33:1-2); *T. angustula:* linear alkanes (n-C25; n-C27; n-C29; n-C31), branched alkanes (15-; 13-; 11-; 9-MeC31; 15-; 13-; 11-; 9-; 7-MeC33; 11,19-; 9,15-diMeC31; 13,x-; 11,x-diMeC33), and alkenes (C27:1; C31:1; C33:1).

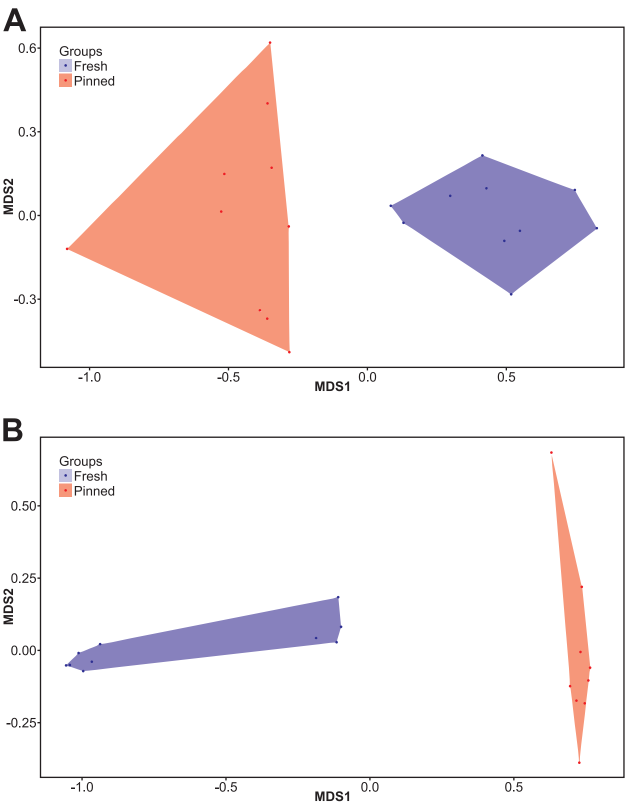

Figure 3. Results of Non-metric Multidimensional Scaling (NMDS) ordination to each species. (A) *Apis mellifera;* (B) *Tetragonisca angustula.*

DISCUSSION

In this work, we present an alternative approach for determining CHC profiles from pinned insect specimens that avoids specimen destruction – an issue often associated with more conventional approaches. The profiles of fresh and pinned individuals differed in both CHC composition and the number of compounds detected. This difference may stem from variation in collection sites, a factor known to influence insect chemical profiles (Jennings *et al.,* 2014; Rajpurohit *et al.,* 2017; Otte *et al.,* 2018). The pinned individuals were sampled in a natural reserve, where environmental conditions such as temperature, humidity, and the availability and diversity of food resources differ markedly from those in a more anthropic environment, which are often warmer, drier, and subject to greater habitat fragmentation and human disturbance. These ecological factors can influence insect physiology and behavior, thereby shaping their CHC profiles – for example, through differences in desiccation stress, metabolic rates, and access to diverse floral or nesting resources (*e.g.,* warmer vs. cooler sites, dense vs. sparse vegetation, variable resource availability). For instance, orchid bees occurring in different biomes – Caatinga and Atlantic Forest – do not present qualitative differentiation in their CHC profiles, only subtle quantitative variation of some alkanes (Santos & Nascimento 2017). Similarly, foragers of the stingless bee species *Nannotrigona perilampoides* consistently show differences across eight sampling regions in Mexico based on the quantity of their CHCs (Méndez *et al.,* 2024). Martin and colleagues (2009) did not report significant differences among the four wasp species they studied, which had different preservation periods (0, 1, and 20 years), likely because all specimens were sampled from the same population and location. Altogether, these reports strengthen the idea that CHC profiles reflect the environmental and geographical conditions of the sites where specimens are sampled.

Nevertheless, the number of compounds detected was lower in pinned insects compared to fresh ones. Since the chromatogram signals from pinned specimens were weaker, it is possible that low signal intensity masked the presence of some cuticular compounds – particularly in smaller species like *T. angustula* (4-5 mm). This likely represents the main limitation of our technique, the need to enhance signal strength and compound abundance in the chromatograms whenever dealing with small specimens. Yet, we detected slightly more compounds in *T. angustula* than previously reported (n = 19; Balbuena *et al.,* 2018), a difference that may simply arise from variation in extraction and processing procedures (*e.g.,* use of dichloromethane vs. hexane, differences in gas-chromatography ramping programs). Therefore, when CHCs are employed as markers, such as in chemotaxonomy, these methodological constraints must be considered. A practical way to reduce such biases is to combine multiple extraction techniques whenever feasible to increase the likelihood of capturing the full spectrum of cuticular compounds.

In any case, the alternative technique we present here is effective for extracting chemical compounds from pinned insect specimens. It has proven particularly valuable for obtaining CHCs without compromising the specimens. Conventional methods such as the one used by Martin and colleagues (2009) involves removing both fore and hind wings. Thus, the methodology we present here has the potential to support researchers in incorporating additional types of data into their analyses (*e.g.,* chemotaxonomy approaches), while still preserving their specimens, as our method is noninvasive and non-destructive. Additionally, by applying this technique to museum specimens, we can gain insights into the evolutionary dynamics of CHCs over time – and, by extension, the evolution of communication in insects. This alternative approach may be particularly valuable for researchers working with rare or even extinct species.

We detected peaks of oleic acid in some pinned individuals. This compound was identified by Wilson *et al.* (1958) as the principal compound released by insects upon death. Therefore, its presence in our pinned samples is not surprising, as the specimens come from collections made 24 years ago. In summary, our technique provides an alternative approach for accessing the chemical diversity of insects stored in museum collections without compromising their integrity. This method serves as a valuable tool to help chemical ecologists and evolutionary biologists investigate the evolutionary dynamics of chemical communication in insects.

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CONFLICT OF INTEREST: Authors declare there are no conflicts of interest.

ETHICS STATEMENT: Not applicable given the type of study conducted.

AI USE: Not applicable; no artificial intelligence tools were used in this study.

DATA AVAILABILITY: The data will be made available upon request sent directly to the senior author (fsnascim@usp.br)

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