A fast and effective method of preparing ants for scanning electron microscopy
Un método rápido y efectivo para preparar hormigas para microscopía electrónica de barrido

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Abstract

Regardless of the application of scanning electron microscopy (SEM) techniques, suitable preparation of the biological material to be analyzed is of paramount importance. In most ant studies that involve SEM, the ants have been desiccated by the critical-point technique (CPD), but most Dolichoderinae species have thin integuments and therefore tend to collapse easily. To evaluate a new method for potential advantages over the CPD technique, these thin-integument ants were treated with tetramethylsilane (TMS) and then air-dried. The results obtained in this study are presented in scanning microphotographs. Here, I detail a standardized protocol for the preparation of ants with TMS prior to SEM. The TMS technique enables the analysis of almost five to six times as many ants as CPD and is faster, easier, more efficient, and more economical than the CPD method.

Key words: Air-drying techniques; Formicidae; morphological analysis; Tetramethylsilane; SEM

Resumen

Independientemente de la aplicación de técnicas de microscopía electrónica de barrido (MEB), la preparación adecuada del material biológico a analizar es de suma importancia. En la mayoría de los estudios de hormigas que involucran SEM, las hormigas han sido desecadas por la técnica de punto crítico (CPD por sus siglas en inglés), pero la mayoría de las especies de Dolichoderinae tienen tegumentos delgados y, por lo tanto, tienden a colapsar fácilmente. Para evaluar las ventajas potenciales de un nuevo método sobre la técnica CPD, estas hormigas de tegumentos delgados se trataron con tetrametilsilano (TMS) y luego se secaron al aire. Los resultados obtenidos en este estudio se presentan en microfotografías de barrido. Aquí, detallo un protocolo estandarizado para la preparación de hormigas con TMS antes de SEM. La técnica TMS permite el análisis de casi cinco a seis veces más hormigas que CPD y es más rápido, más fácil, más eficiente y económico que el método CPD.

Palabras clave: técnicas de secado al aire; Formicidae, análisis morfológico; Tetrametilsilano; SEM

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Introducción

Scanning electron microscopy (SEM) is a specialized field of electron microscopy widely used in the analysis of biological problems (Bozzola and Russel, 1998). SEM has been employed in many studies of the anatomy and morphology of living organisms (Vukusic et al., 1999; Gorb et al., 2005; Ubero-Pascal and Puig, 2007; Fox et al. 2010; Camargo-Mathias *et al.,* 2011), taxonomy (Baroni Urbani and de Andrade, 2007), and phylogenetic relationships (Keller, 2011), among others. In every field within the biological sciences, SEM has enabled results that have improved the understanding of certain biological processes and mechanisms, in some cases laying the foundation for the creation of new technological tools (Lee and Szema, 2005).

Independent of the application of SEM techniques, adequate preparation of the biological material to be analyzed is very important; however, the methods used for this purpose may vary according to the type of biological material. The treatment most applied to biological tissues consists of a series of progressive phases (figure 1) whose final objective is to prepare a dried sample. The desiccation of the sample can also be achieved by alternative treatments, such as critical-point or air-drying techniques. Drying is a delicate task, as the stress due to the evaporation of internal liquids in the samples (e.g., water or ethanol) during the drying phase can result in partial or total damage to the shape and general appearance of the biological material (Bozzola and Russel, 1998, Ubero-Pascal et al., 2005) and, therefore, in an incorrect interpretation of the morphology or in the obscuration of taxonomically important characteristics.

The Critical Point Drying (CPD) technique is widely used to prepare ants that will be observed in the SEM. This technique is widely used because most ants have a relatively thick integument and are therefore resistant to drying (de Andrade and Baroni Urbani, 1999; Fernández, 2004; Keller, 2011). Some ants, however, respond very well to air-drying (Lucas e*t al.,* 2002) without prior chemical treatment or CPD. Despite the excellent performance of the CPD in the drying of most ants, some groups, such as the ants of the subfamily Dolichoderinae, exhibit very poor results (e.g., *Forelius* Emery 1888, fig 1 in Guerrero and Fernández, 2008:54) because of their very thin integument, which represents a challenge in the drying phase, especially by the CPD technique. Even after proper fixation, inadequate drying can cause shrinkage, collapse and even the loss of some useful taxonomic structures and thereby of potential understanding of the morphology of these ants.

As an alternative to desiccation by CPD, some specimens of several genera of Dolichoderinae ants were treated with tetramethylsilane ((CH3)4Si - TMS). The family of compounds derived from methylsilane (hexamethyldizilisane (HMDS) and TMS) are organic compounds characterized by their good miscibility with chemicals used in the fixation and preservation of biological material (e.g., ethanol) and their low surface tension (Yaws, 2014), the latter allowing rapid evaporation at room temperature (Ubero-Pascal *et al.,* 2005) and therefore decrease in pressure caused by drying and surface tension generated by that drying front. The physical characteristics of these compounds have led to their widespread use in the treatment of biological samples prior to air-drying for SEM analysis (Nation, 1983; Dey *et al.,* 1989; Bray *et al.,* 1993; Laforsc and Tollrian, 2000; Botes et al., 2002, Barré *et al.,* 2006; Ubero-Pascal *et al.,* 2005; Ubero-Pascal and Puig, 2007; Ubero-Pascal and Puig, 2009). However, TMS has thus far been used much less than HMDS (Dey *et al.,* 1989; Reville and Cotter, 1991; Ting-Beall *et al.,* 1995; Ubero-Pascal *et al.,* 2005). Accordingly, the implementation of alternative drying techniques to CPD (e.g., desiccation in air after previous chemical treatment) would allow better preservation and analysis of the external morphology of this type of ants.

To evaluate the suitability of this technique in ants with a thin integument, dolichoderine specimens were treated with Tetramethylsilane. The results are presented as qualitative comparison scanning micrographs obtained in this study. TMS, as an alternative technique, is shown to be faster, easier, more efficient, and less expensive than CPD.

Materials and methods

Biological material

The biological samples examined were ants of various genera of the subfamily Dolichoderinae, which are characterized by a relatively thin integument compared with that of other ants (Shattuck, 1992; Wild and Cuezzo, 2006). We used 22 specimens distributed as follows: two workers of *Linepithema angulatum* (Emery 1894) and two workers of *Linepithema piliferum* (Mayr 1870), two workers of *Forelius damiani* Guerrero and Fernández 2008 and two workers of *Forelius pruinosus* (Roger 1863), four workers of *Dorymyrmex biconis* Forel 1912, one worker and one male of *Azteca* Forel 1878, four workers of *Tapinoma litorale*

Wheeler 1913 and four workers of *Tapinoma melanocephalum* (Fabricius 1793).

All the ants were previously preserved in 80 % ethanol except the specimens of *L. angulatum* and *T. melanocephalum*, which were killed and preserved in 90 % ethanol. The preservation time before preparation was not the same for all samples; some samples were preserved for several months (e.g., *L. piliferum*) and others only for hours (e.g., *T. melanocephalu*m) prior to treatment with TMS. However, the results do not seem to be influenced by the preservation time.

Protocol

The protocol proposed here is a partial modification of the usual procedures used for the preparation of any biological sample (see figure 1). Likewise, it is an adaptation of the protocol used by Ubero-Pascal *et al.* (2005) for the preparation of Ephemeroptera and Plecoptera eggs with HMDS and TMS.

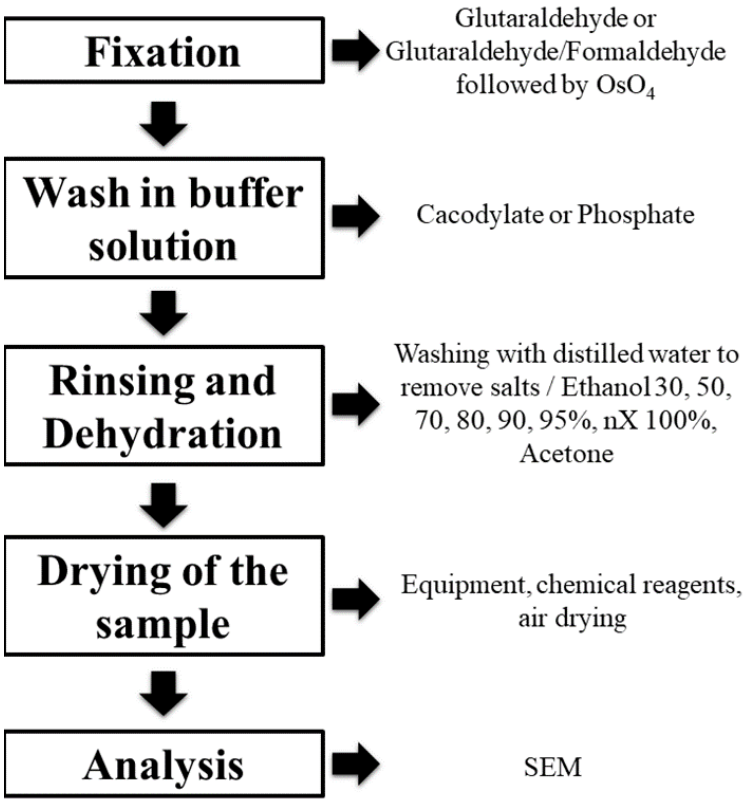

Figure 1. Summary of the steps followed to prepare a biological sample for SEM with procedural alternatives in each step. Here, nX refers to the treatment of the sample several times with 100 % ethanol.

The procedure is quick, simple, and useful for preparing many specimens at once depending on their size. For example, if the ants are ≤ 5 mm in size, at least 25 specimens can be prepared in each two-milliliter vial. The steps are detailed below:

1. Have a rack containing vials of a certain volume. Fill these vials with TMS to their maximum capacity and cover very well so that the TMS does not evaporate. The volume of the vials will be determined by the size of the ants or the number of ants to be prepared, that is, a) vials that allow complete coverage of the specimen with TMS, or b) several specimens that fit loosely in the vial can be used. Twenty-two ants less than 5 mm in size were analyzed in this study, so only one vial with a capacity of two milliliters was used.

2. Take ants preserved in 70-90 % ethanol and immerse them for 10 minutes in one of the vials filled with TMS. This first session is called the "first bath". The vial must remain hermetically sealed to avoid evaporation of the TMS.

3. Afterwards, take the samples from the first bath and immediately transfer them to a second vial containing TMS ("second bath"). The exposure time for this phase is 20 minutes.

4. After 20 minutes, remove the samples quickly and place them

on filter paper so that the TMS evaporates. This step must be performed in a well-ventilated environment or a gas extraction chamber. This procedure facilitates the subsequent manipulation of the ants for the indirect assembly of these insects.

5. Ants must be mounted on acid-free cardboard triangles according to the standard protocol for mounting ants in a museum (Lattke, 2000), which exposes a greater surface area to the metal when covering the specimen. Silver glue is recommended for attaching the ant to the cardboard because it provides electrical continuity and thus reduces charging, but any glue is useful as long as contact with the body of the ant is guaranteed; in this way, the metal coating will produce a conductive pathway for the electrons inside the scanning microscope.

Metal coating and SEM observation

The pinned ants were attached to a metal cylinder using electrically conductive double-sided adhesive tape, then covered with gold by an Ion Coater IB•2 device using an ionization current of 5 milliamps per 10 minutes. The ants were observed using a Hitachi S2400 scanning electron microscope with a working voltage of 10 KV. The images were obtained with various magnifications (30 to 60x) depending on the size of the sample, considering that the aim was not to observe specific structures but rather the relative preservation of the ant integument.

The preparation, metallization and observation of the samples was carried out at the Electron Microscopy Center of the Facultad de Ciencias of the Universidad Central de Venezuela.

Results

Independent of the ants treated here with TMS, the samples show little or no morphological distortion, especially in regions such as the head and the mesosoma. Slight deformations occurred in the propodeal dorsum of a sample of *Linepithema angulatum* (figure 2A) and the mesonotal dorsum of a worker of *Azteca* sp. (figure 4C). In both cases, the damage could have resulted from the handling of the specimens at collection or prior to treatment with TMS. Other specimens of *L. angultaum* and *L. piliferum* show no head or mesosomal distortion (figures 2B-D). Samples from both *Forelius* species (figure 3), *Dorymyrmex biconis* (figure 4A-B), and both *Tapinoma* species (figure 5) show no damage to any part of the mesosoma. The propodeal cones in *D. biconis* are fragile and prone to handling damage at the time of mounting (Cuezzo and Guerrero, 2012), but in this case they were completely preserved.

Some specimens of *Linepithem*a, *Forelius* and *Azteca* did show slight deformations in one or two tergites of the gaster (figures 2A, 2B, 3B, 3D, 4D), but these deformations were not important to the analysis of morphological trait associated with that region of the body. The gaster of the *Linepithema angulatum* samples was the one that showed depressions like a footprint in the first or second gastral tergite (figures 2A, 2B), but in *L. piliferum* there was no kind of damage in the gaster, maintaining the shape of this like an inflated balloon; although the tergite separated from each other (unlike *L. angulatum*), there was no damage to the cuticle or resilin among the tergal plates (figures 2C, 2D).

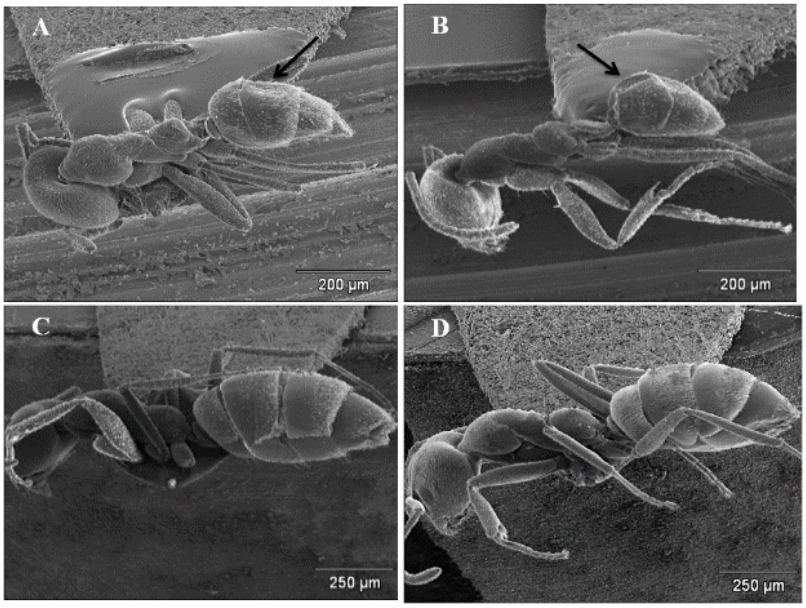

Figure 2. SEM images of *Linepithema* ants. A and B) Lateral-dorsal view of *L. angulatum*. C and D) Profile view of *L. piliferum*. The arrows indicate deformation in the integument of some gastral tergites.

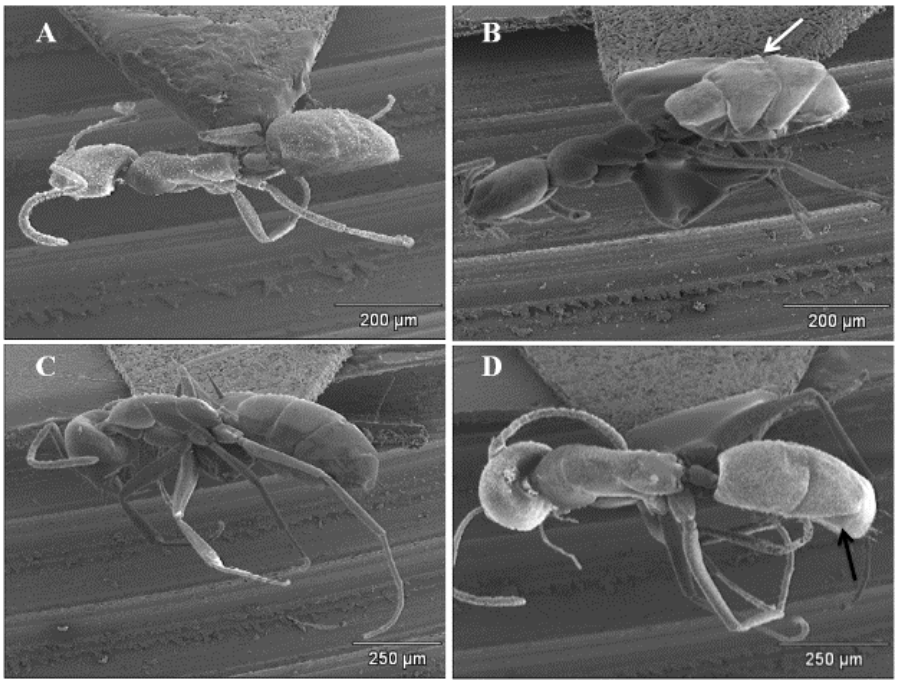

Figure 3. SEM images of *Forelius* ants. A) Dorsal view of *F. damiani*. B) Dorsal view of another specimen of *F. damiani*. C) Profile view of *F. pruinosus*. D) Dorsal view of another specimen of *F. pruinosus*. The arrows indicate deformation in the integument of some gastral tergites.

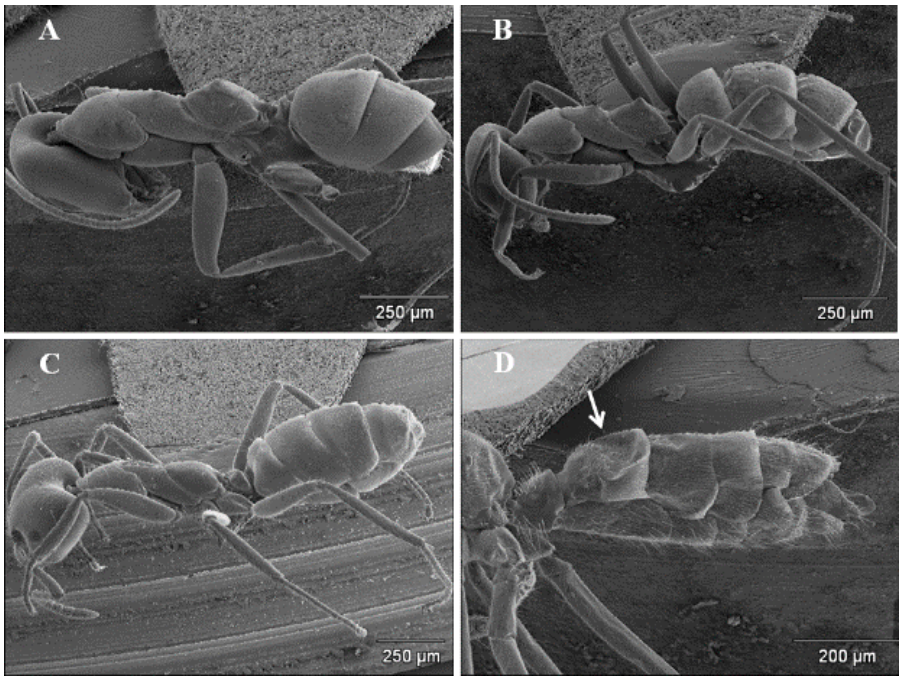

Figure 4. SEM images of ants of the genera *Dorymyrmex* and *Azteca*. A and B) Lateral view of *D. biconis*. C) Lateral view of *Azteca* sp. D) Propodeum, petiole and gaster of *Azteca* sp. male. The arrow indicates deformation in the integument of the first gastral tergite.

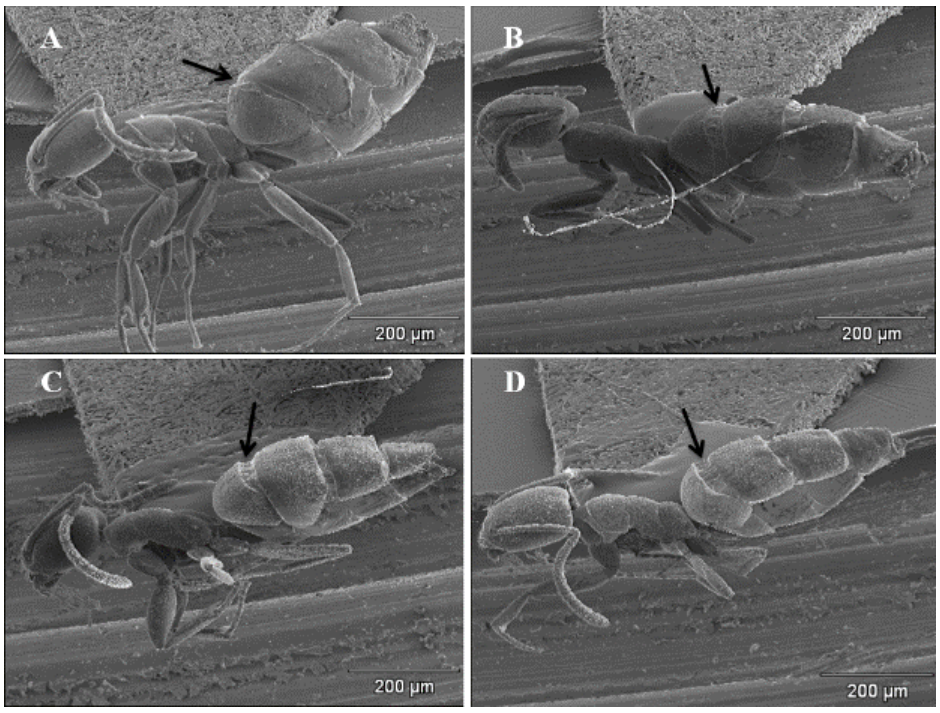

Figure 5. SEM images of ants of the genus *Tapinoma*. A) Lateral view of *T. melanocephalum*. B) Oblique-dorsal view of *T. litorale*. C and D) Lateral view of *T. litorale*. The arrows indicate strips of the protein resilin, which provides flexibility to the gastral sclerites. In all cases, the gaster remains very inflated and turgid.

Likewise, one sample of *F. damiani* (figure 3B) and another of F. pruinosus (figure 3D) showed bending in the second gastral tergite but in general in any of the *Forelius* samples the turgidity of the gastral tergites and sternites was maintained (figures 3A, 3C). Comparatively, the gaster of the *Azteca* male was the one that showed greater damage in the tergites of that tagma; the greatest deformation is seen in the first gastral tergite, but slight deformations occur in gastral tergites 2-5 (figure 4D).

Tapinoma ants examined here did not exhibit deformations in any of their tagma. As shown by the other dolichoderine genera examined, the region with the greatest possibility of deformation is the gaster but in all *Tapinoma* samples the gaster remains very inflated and turgid.

Discussion

Most studies using SEM to explore morphological characters in ants have used traditional drying techniques such as CPD (de Andrade and Baroni Urbani, 1999; Fernández, 2004; Keller, 2011) or air drying (Lucas *et al.,* 2002). This is the first study that attempts to assess the suitability of a chemical compound as a desiccant agent for ants prior to SEM imaging. The qualitative results (i.e., preservation of specimen morphology) suggest that TMS is an efficient chemical agent for use in preparing ants with thin integument prior to imaging in SEM. Most of the TMS-treated ants did not collapse or show substantial morphological damage, allowing the preservation of the cuticle of the specimen, and consequently the observation of important morphological characteristics in the taxonomy of these groups.

In most cases, the effect of TMS on the cuticle enhanced the turgidity of gastral tergites and sternites, thus preventing the collapse of this tagma, a result that has never previously been recorded in Dolichoderinae ant specimens treated with CPD or other drying methods. For example, *Dorymyrmex, Forelius*, and *Tapinoma* specimens frequently suffer gastral collapse when treated with CPD (Guerrero and Fernández, 2008) or air-dried but this type of damage does not occur in thick-cuticled ants such as *Dolichoderus* (personal observations). In this experiment some specimens of *Linepithema, Forelius* and *Azteca* did show slight deformations in at least one gastral tergite, while each of the *Tapinoma* ants examined here did not exhibit deformations and the gaster remains very inflated and turgid. The shrinkage or deformation in the gaster of these dolichoderine ants could be derived from an incomplete dehydration process prior to treatment with TMS; all the samples, however, were preserved with Ethanol (ranging from 80-90 %) which is a chemical agent

with the ability to remove water from the sample and preserve the original structure of the biological sample (Mehdizadeh *et al.,* 2014). Likewise, the preservation time of the samples could have influenced the results; although the preservation time was different between samples (see Material and Methods), the results seem not to be influenced by this factor, since samples with a few hours (*T. melanocephalum*) or months (*L. piliferum*) of preservation did not show any damage to the gaster.

Differences in cuticle thickness between species may influence the results between CPD and TMS, due to differential deposition of chitin layers, as recorded in species of the genus *Zasphinctus* (Hita-García *et al.,* 2017). Surface deformations may also be due to non-biological factors, i.e., physicochemical properties of the different reagents used to dehydrate and desiccate the sample. Of all these properties, the most important are surface tension forces and evaporation rates of the liquids used during the process (Dahl, 1972; Yaws, 2014). In this case, TMS is chemically inert, and it has very low solubility in water; its boiling point is 23.6 °C, much lower than that of several dehydrating chemicals such as ethanol (78.1 °C) and acetone (53.6 °C). Moreover, the surface tension of the TMS is 10.2 dynes/cm at 20 °C, while ethanol and acetone have surface tension approximately two orders of magnitude higher (22.75 dynes/cm and 23.70 dynes/cm, at 20 °C, respectively). The properties of TMS make it the most effective desiccant liquid for use in preventing deformation or collapse of ant exoskeletons. These properties have also been recorded as being suitable for the preparation of more delicate biological samples, such as mammalian tissues (Dey, 1993), aquatic insect eggs (Ubero-Pascal *et al.,* 2005) and other types of thin-integument aquatic arthropods (Laforsch and Tollrian, 2000).

Addition, the treatment of ants with TMS is fast and very economical. Air-drying ants after treatment with TMS is simple, and much faster than for other techniques such as CPD, the latter ranging from a few hours to several days depending on the biological material (Jung *et al.,* 2010). Although CPD was not used here, these results with TMS suggest that it could significantly increase the number of samples processed, a benefit that would translate into substantial time savings (e.g., 25 samples/~45 minutes). Time efficiency of TMS as a preparation agent has also been recorded in other groups of animals, plants, protozoa, and multiple types of cells and living tissues (Reville and Cotter, 1991; Ting-Beall *et al.,* 1995; Ubero-Pascal *et al.,* 2005), but this work is the first to evaluate it in ants. In economic terms, the TMS technique would cost approximately 45 times less than the CPD technique; TMS drying technique requires only the chemical agent (100 ml of TMS ranging from USD $ 103 to USD $ 230) while the CPD technique uses expensive specialized equipment (USD $ ~10.000).

In conclusion, the use of TMS as a drying agent permits optimal results in Dolichoderinae ants prior to SEM imaging. This approach is an efficient and effective method in terms of morphological preservation, sample preparation time, the number of samples to analyze, and cost due to the use of a single reagent and no equipment for drying.
This new method should be evaluated for use with ant larvae, since their body is much softer, and susceptible to collapse due to tissue desiccation.

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