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A Modified Technique for the Preparation of Specimens of Sternorryncha for Taxonomic Studies

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ABSTRACT. *The insects belonging to Order Hemiptera: Suborder Sternorrhyncha (aphids, scale insects and mealybugs, whiteflies and psyllids) are often pests on cultivated crops. Identification of these pests is necessary to ensure the application of appropriate control methods. Identification of these insects is based on microscopic morphological characters that can only be studied at high magnification. Different slide preparation techniques are being used for different insect groups, and many of the reagents used in these techniques are highly toxic. This paper provides methods for preservation and the preparation of temporary and permanent slide mounts, using less toxic reagents. The proposed slide-mounting procedure involves maceration, bleaching, acidification, staining, stain differentiation, de-waxing, and clearing of specimens before mounting them on slides. Specific conditions required at each step and modifications necessary to adapt the method for use with different insect families are discussed.*

Keywords: *Preservation, slide mounting, Sternorrhyncha, taxonomy*

INTRODUCTION

Members of the Hemiptera: Sternorrhyncha; aphids (Aphidoidea), whiteflies (Aleyrodoidea), mealybugs and scale insects (Coccoidea) and the immature stages of psyllids (Psylloidea) have soft membranous bodies. If these insects are preserved as dry specimens, they crumple and distort and the colour of the body, and sometimes of the waxy covering, changes so that they cannot be identified to genus or species level with confidence. The taxonomy of the Sternorrhyncha is based on microscopic characters on the cuticle that can only be studied and measured on slide-mounted specimens (Watson and Chandler, 2000).

When Sternorrhyncha are killed and preserved in alcohol at room temperature, the body contents sometimes turn black due to the action of enzymes that are not denatured by alcohol. This makes difficult or impossible to see taxonomic characters. The resultant black precipitate can be very difficult to remove from the body during slide preparation (e.g. in the papaya mealybug, *Paracoccus marginatus,* Williams and Granara de Willink), resulting in poor-quality slide mounts that are very difficult to identify.

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Several staining and slide-mounting techniques for Sternorrhynchous insects have been published (Table 1) and many of these techniques are adequate for identification purposes. However, the reagents and methodologies used are different between the insect families and for some of the most toxic reagents used, safer alternatives are now available.

Table 1. Published methods for slide-mounting of Sternorrhynchous insects

Target insect group	Authors
Aleyrodoidea	Martin, 1987; Watson and Chandler, 2000; Martin, 2004
Aphidoidea	Eastop, 1961; Martin, 1983; Blackman and Eastop, 2000
Coccoidea	Green, 1896; Newstead, 1903
Coccidae	Williams and Kosztarab, 1972; Ben-Dov and Hodgson, 1997
Diaspididae	McKenzie, 1957; Wilkey, 1990; Watson, 2002
Pseudococcidae	Williams and Granara de Willink, 1992; Watson and Chandler, 2000

This paper provides methods for preserving Sternorrhynchous insects and preparing them on microscope slides that require only a slight modification for use with different insect families. The methodology provided herein was developed using mealybugs collected in Sri Lanka for identification at the Plant Pest Diagnostic Center, California Department of Food and Agriculture, Sacramento, California, USA (CDFA-PPDC) in January-July, 2012.

MATERIALS AND METHODS

Collection and Preservation

In the field, Sternorrhynchous insects were collected on pieces of infested plant material and placed in labeled paper or plastic bags (15 x 20 cm Ziploc polythene bags). Live specimens were not picked off from the host-plant individually with forceps as this may damaged them. Bagged samples were taken to the laboratory for sorting and preservation using a dissection microscope. For sessile insects like mealybugs, small pieces of infested plant material were isolated and placed in labeled screw-topped Nalgene vials (3 & 5 ml) containing 80% alcohol, to kill and preserve them. In the case of mobile insects, a very fine paint brush wetted with alcohol was used to pick them up and transfer them to alcohol. To prevent body blackening, the labeled vials of freshly killed material were sealed and immediately stood in freshly boiled water (e.g. a water bath at 100 °C) for 15-20 minutes, to denature the enzymes and ensure optimal fixation of the body contents. Cooled samples were then stored in a refrigerator until they were required for slide preparation. The preserved samples were taken to the Plant Pest Diagnostic Center, California Department of Food and Agriculture, Sacramento, California, USA (CDFA-PPDC) for preparation and identification.

Preparation of Slide Mounts

The slide-preparation methods used were refined from the methods described by Watson and Chandler (2000), for preparation of both temporary and permanent slide mounts of Coccoidea (Asterolecaniidae, Coccidae, Diaspididae, Monophlebidae, Ortheziidae, Pseudococcidae); Aphidoidea (Aphididae) and Aleyrodoidea (Aleyrodidae). For quick identification of samples, a simplified preparation method was used to produce temporary

mounts in Hoyer's Fluid. However, when archival mounts were required for future reference, additional steps in the method allowed, preparation of permanent mounts in Canada balsam.

Handmade micro-spatulas and mounted needles were used for the preparation of specimens. Recipes for the reagents used for slide preparation are given in the Annexure 1. The chemical steps of slide preparation were carried out in glass cavity blocks (3 cm diameter) with 3.5 cm-diameter watch glasses for lids, labeled using a wax pencil. Under the dissection microscope (using x10 to x50 magnifications) the cuticle of each specimen was punctured in a part that lacked taxonomically important characters to allow reagents to penetrate the body - unless the body contents were black, in which case the puncture was only made later, just before cleaning out the body contents. Samples were heated to 60 °C on a thermostatically controlled dri-block.

The preparation of temporary mounts involved relatively a few steps: maceration; bleaching if required; acidification; dehydration; de-waxing and rehydration if required; and mounting (Fig. 1). Preparation of permanent, archival mounts involved: maceration; bleaching if required; acidification; staining and differentiation; dehydration; de-waxing; clearing; and mounting (Fig. 1). The time required in each step varied even between specimens from the same sample, from 5 minutes to several hours, so it was necessary to check progress regularly.

Steps in Slide Preparation

Maceration: Specimens were placed in 10% potassium hydroxide (KOH) at 60 °C, or at room temperature overnight followed by heating, until the body contents became transparent and liquified fat accumulated under the uppermost cuticle. Then the contents were all carefully expelled using a microspatula and a blunt mounted needle. If necessary, the hole in the cuticle was slightly enlarged to make the expulsion of solids easier.

Bleaching: Black whitefly pupae have to be partially bleached before they can be examined on microscope slides. After maceration and brief water rinse to remove KOH, specimens were soaked in freshly-mixed bleach made of equal parts of 30-volume hydrogen peroxide and 880 ammonium hydroxide. Bleaching sometimes progressed very rapidly, so the dish was watched under the dissection microscope using transmitted light. Bleaching was stopped as soon as the cuticle became light brown and completely transparent, by adding a few drops of glacial acetic acid. If specimens became over-bleached they required staining.

Acidification: Any traces of KOH left in the specimen prevented staining or caused it to de-colourize later. Specimens were therefore, rinsed in distilled water for at least 3 minutes to remove KOH from the insects and the spatula, before transfer to acidified 80% alcohol for at least 10 minutes to acidify the cuticle.

Staining: Temporary slides were mounted in a water-soluble mountant, so staining with water soluble Acid Fuchsin was not possible. For permanent mounts, all Sternorrhyncha with membranous, colourless cuticle (except aphids and psyllids, in which pigmentation patterns are taxonomically useful) were stained in a mixture of Acid Fuchsin stain and Essig's Aphid Fluid for 12-24 h. It is possible to stain in acidified 80% alcohol, but this evaporates quickly; Essig's Aphid Fluid was used instead, as it does not evaporate quickly and can be heated safely. If specimens refused to take colour (due to presence of traces of KOH), a few drops

of glacial acetic acid were added to the dish. Staining could be accelerated by several hours by warming at 60 °C.

Differentiation: Specimens emerged from the stain entirely dark red. They were briefly rinsed in acidified 80% Isopropanol until the membranous cuticle became light pink while thick cuticle remained red. Then they were transferred quickly to 95% Isopropanol to fix the stain, and soaked for 15 minutes to dehydrate the cuticle.

De-waxing: Dehydrated specimens containing wax or oil droplets were transferred to histoclear phenol for at least 5 minutes to dissolve the lipids. Once the lipids had dissolved, specimens were rinsed in 95% isopropanol to remove dissolved waxes.

Clearing: For permanent mounts, de-waxed, dehydrated specimens were soaked in high quality, anhydrous clove oil for at least 10 minutes to clear the cuticle. Clove oil also removed any remaining water and lipids. At this stage, unsightly tracheae or egg shells could be carefully extracted from the body using a mounted micropin; however, the cuticle was very fragile at this stage and that greater care was necessary.

Mounting: Mounting was done under the dissection microscope. For temporary mounts, a drop of Hoyer's Fluid was placed on a labeled slide. Specimens were transferred from acidified alcohol to the mountant. After a short pause to allow any alcohol traces to evaporate, the specimens were pushed down into the mountant and positioned before a coverslip was lowered onto them.

For permanent mounts, a 2 mm-diameter drop of clove oil was placed in the centre of the slide. Specimens were transferred into the clove oil drop and roughly positioned before most of the oil was soaked into a folded tissue. After final positioning and arrangement of limbs, more clove oil was carefully removed until the specimens were almost dry. A small drop of well-liquefied Canada balsam was placed on the specimen and quickly spread in a circular pool around the specimens. This was dried for 15 minutes, to fix the specimens in position. Then a larger drop of Canada balsam was added, quickly spread around the specimens and a cover slip was lowered onto it using a needle. The cover slip was allowed to settle under its own weight. The slide was then placed in an incubator at 39 °C for a period of 12 weeks.

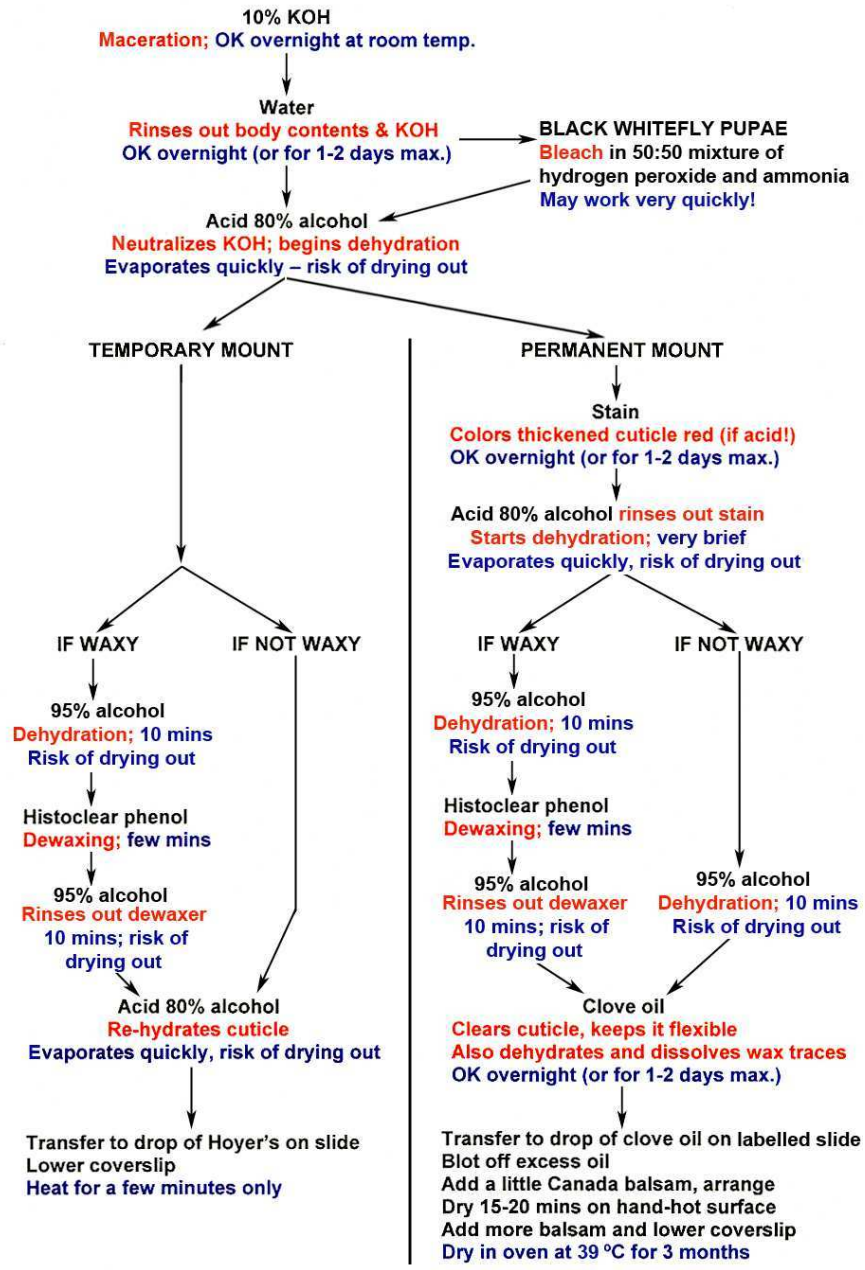
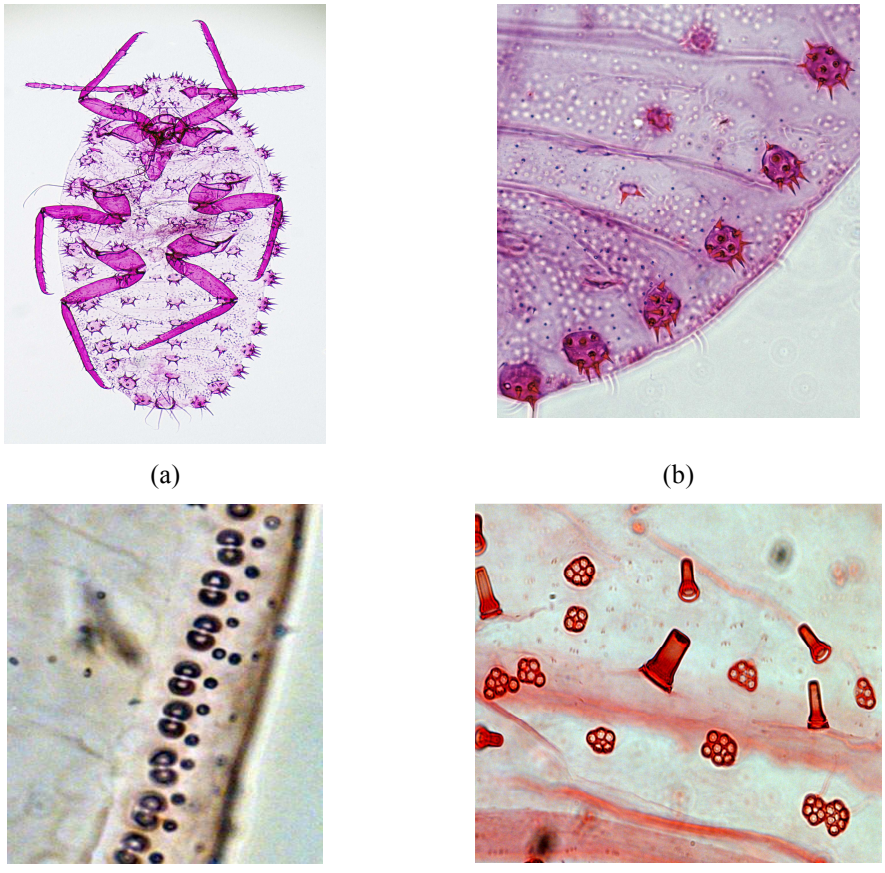


Fig. 1. A flow diagram of the technique for preparing temporary and permanent slide mounts of soft-bodied Sternorrhyncha (modified from the methods given in Watson and Chandler, 2000 and Martin, 2004).

RESULTS AND DISCUSSION

To ensure good slide preparation, only the most suitable specimens should be selected. For mealybugs and scale insects, this is the fairly newly moulted adult females, whereas for Aphidoidea and immature Aleyrodoidea and Psylloidea it is better to use specimens some time after moulting, to allow the pigmentation to develop. For all Sternorrhyncha except the Aphidoidea, all the body contents should be removed. Selection of specimens that do not contain eggs or embryos minimizes the work necessary to clean out the bodies (and the resultant damage). Small Coccoidea specimens are also easier to study microscopically than large ones, because the characters are closer together and therefore easier to locate.

Slide mounts prepared using the method given in Fig. 1 were optically superior to those prepared by methods in earlier publications. Even fine morphological structures could be seen easily (Plate 1), which is very important for taxonomic studies.



(a) (b)

(c) (d)

Plate 1: Fine morphological structures in slide mounts prepared using the method given in Fig. 1. (a) *Coccidohystrix insolita* (Green) (Pseudococcidae); (b) Cerarii of *Coccidohystrix insolita*; (c) 8-shaped pores of *Russellaspis pustulans* (Cockerell) (Asterolecaniidae); (d) Truncate dorsal setae and and pores of *Dactylopius confusus* (Cockerell).

The chemicals used for slide making in the references given in Table 1, are listed and annotated in Table 2. Scientists preparing insect slide mounts are likely to have received training in the safe handling of strong acids and alkalis, so that such chemicals are not regarded as being exceptionally dangerous. However, workers may not be familiar with the specific risks presented by those organic solvents, hence are regarded as potentially more hazardous to use.

Table 2. An annotated list of chemicals used in the slide-making methods given in the papers cited in Table 1.

Chemical	Main hazards presented
Potassium hydroxide	Strong alkali; corrosive
Ammonium hydroxide	Strong alkali; dangerous, toxic fumes
Concentrated lactic acid	Corrosive; irritating fumes
Glacial acetic acid	Corrosive; irritating fumes
Hydrogen peroxide	Powerful oxidant
Stains	Some stains may be carcinogenic
Alcohol	Flammable; toxic
Canada balsam	Flammable; toxic
Cellosolve	Flammable; may cause a low motile sperm count in men
Histoclear	Flammable; irritant; fumes moderately toxic
Clove oil	Flammable; moderately toxic
Phenol	Corrosive; irritating fumes; a strong neurotoxin
Chloral hydrate	Sedative and hypnotic, often requires a license to use
Xylene	Highly flammable; moderately toxic; hazardous fumes; can penetrate skin
Tetrahydrofuran	Highly flammable; moderately toxic; can penetrate skin; forms explosive peroxides

For most workers on Aphidoidea (Martin, 1983; Blackman & Eastop, 2000), clove oil was sufficient to dissolve the small amounts of fats and waxes in their specimens. The early workers on Coccoidea (Green, 1896; Newstead, 1903; Williams and Kosztarab, 1972) used clove oil to dissolve fats and waxes also. However, clove oil does not remove large amounts of wax very well, so later workers began to use stronger de-waxing reagents like tetrahydrofuran (Mckenzie, 1967; Wilkey, 1990); or mixtures of xylene and glacial acetic acid (Ben-Dov and Hodgson, 1997) or xylene and phenol (Williams and Granara de Willink, 1992; Watson and Chandler, 2000; Watson, 2002). With increasing awareness of the hazards presented by some of these organic solvents, the most recent publications have avoided using tetrahydrofuran, cellosolve and chloral hydrate, and have suggested using Histoclear as a safer alternative to xylene (Watson and Chandler, 2000; Watson, 2002; Martin, 2004). The modified method in Fig. 1 follows these recommendations as much as possible and it is recommended herein to use a fume hood when working with chemicals that give off potentially harmful fumes; e.g xylene, glacial acetic acid, ammonium hydroxide, Essig's Aphid Fluid and phenol.

Special Requirements for Different Insect Groups

Slight variations to the slide-making procedure are necessary for different groups of insects.

Aclerdidae, Cerococcidae, Eriococcidae, Lecanodiaspididae, Margarodidae, Monophlebidae and Pseudococcidae: Small adult females should be selected. Any lumps of external wax should be physically removed in 95% alcohol, before chemical processing is started. The hole is made in the dorsum of the metathorax or the most anterior abdominal segment. Thorough dehydration and de-waxing is essential and sometimes gentle heating in de-waxer and addition of a few drops of xylene, or even repeated changes of de-waxer, to dissolve all the wax. Also it is important to include the hind legs in the slide mount.

Ortheziidae: Adult females are prepared as for the Monophlebidae, but the hole is made in the ventral cuticle between the mesocoxae. Often the body contains gluey material that can only be removed by dissolving it in a warm mixture of histoclear phenol and xylene; sometimes overnight soaking and repeated changes of de-waxer are necessary.

Asterolecaniidae: Young adult females that have not yet started laying eggs should be used. The cuticle is extremely delicate, so that the tests should left on the insects, since they came off during maceration. The hole is made in the ventral cuticle of the mesothorax.

Coccidae and Keriidae: Small or medium-sized adult females are slide-mounted. If external resin or wax is visible, soaking in 95% alcohol will dissolve the resin cover of Kerriidae or harden the sticky covering of wax scales sufficiently to enable lumps of external wax to be physically removed, before chemical processing begins. In Kerriidae the hole is made in the dorsum of the abdomen, and in Coccidae it is made in the ventral cuticle on one side of the abdomen. Further de-waxing is often necessary later in the preparation process.

Conchaspididae, Diaspididae and Halimococcidae: Small to medium-sized adult females are slide-mounted. In alcohol, the scale cover should be removed and for pupillarial Diaspididae and Halimococcidae, the delicate adult female dissected out of the second instar exuviae. The hole is made in the dorsum of the anterior abdomen. In pupillarial diaspidids and Halimococcidae, both the second instar and adult cuticles are mounted on a slide together.

Aphidoidea and immature Psylloidea: For aphids, wingless adult females are required for identification, although winged adults should also be mounted if available. Any external lumps of wax are removed in alcohol before starting the chemical processing. The hole is made in the venter, between the hind coxae.

Aleyrodoidea: The final immature stage is slide-mounted. Any large external lumps of wax are removed in 95% alcohol before starting the chemical processing. A tiny prick is made in the venter, just posterior to the hind legs. After maceration, black specimens require bleaching until they are light brown and fully transparent.

CONCLUSIONS

The methods provided enable preservation of Sternorrhynchous insects and preparation of high-quality temporary and archival slide mounts suitable for identification and long-term reference purposes. These methods are less varied and use fewer toxic reagents than the methods currently available in the literature.

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Annexure 1. Recipes for slide-making reagents and mountants

10% KOH: Approximately 50g weighed pellets in 500 ml distilled water.

Acidified Alcohol: 6 drops of concentrated HCl in 100 ml of 80% alcohol

Acid Fuchsin Stain:

Distilled water	300 ml
10% HCl	25 ml
Acid Fuchsin stain powder	0.5 g

Histoclear Phenol:

Phenol	1 part
Histoclear	… 3 parts

Essig's Aphid Fluid:

Lactic acid (85%)	20 parts
Phenol liquid	2 parts
Glacial acetic acid	4 parts
Distilled water	1 part

Hoyers Fluid:

Distilled water	30 g
Chloral hydrate	200 g
Glycerin	20 g
Gum Arabic (crude, not refined)	30 g