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Author(s): Brown, P. A., De Boise, E.

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Procedures For The Preparation Of Whole Insects As Permanent Microscope Slides And For The Remounting Of Deteriorating Aphid Slides

- Paul A Brown* & Emma De Boise**
Department of Entomology, The Natural History Museum, London

*Slide Preparation Laboratory
**Soil Biodiversity Laboratory, Department of Entomology, The Natural History Museum, Cromwell Road, London, SW7 5BD. UK.

This paper has been prepared to increase the circulation of our slide mounting techniques beyond the usual expert entomologist readership to the natural history conservator and curator community. Slide making methods are published in taxonomic papers or in Entomological manuals such as Cranston & Gullan (199X). Carter and Walker (1999) do not cover slide-making techniques. If possible, it is worth referring to an expert taxonomist working on the group of insects or other small organisms concerned so as to learn the latest and best specific variations in preparation and dissection techniques.

Preparation for microscopic examination as used for Hemipteran bug whole mounts.

Many small insect specimens can only be accurately identified once they have been cleared of their body contents and mounted on microscope slides. Specimens preserved in Formaldehyde cannot be cleared and so are almost useless for taxonomic study. Standard 75 x 25 mm slides, preferably of 0.8-1.2 mm thickness are normally used. Cover-slips should be the thinnest possible, no.0 grade, but many workers have their own preferences for cover-slip dimensions. We use circular cover-slips of 13 mm, 16mm, 19mm and 22mm diameter, depending on the size of the specimens.

For rapid identification of a specimen, almost any mounting medium can be used with a good refractive index and protected by a cover-slip. For permanent museum preparations, it is important that an archival quality mountant is used. There are many slide mounting media available, as listed in Brown (1997) and after much experience of mountant deterioration (as mentioned below) we suggest that the best choices for permanent preparations are Canada balsam and Euparal, because of their proven long-term performance. Canada balsam is used for Hemiptera, Thysanoptera, Phthiraptera, Psocoptera and small Hymenoptera whole mounts and is manufactured from the resin of the conifer *Abies balsamea* which is usually thinned with xylene. Canada balsam is known to be stable for over 150 years. The refractive index of Canada balsam (1.48 when still wet and 1.52 after the Xylene has evaporated) is similar to insect cuticle so if you use Canada balsam, phase contrast microscopes can be used to successfully study the fine details of insect cuticular features. Euparal is used for Hemipteran and Dipteran whole mounts and Lepidopteran genitalia permanent collections and have not exhibited any deterioration in over 50 years. Euparal is reportedly a recipe of Eucalyptus oil, methyl salicylate, camsal, sandarac and paraldehyde but is now a trade secret. Euparal has a contrasting refractive index (1.48) to insect cuticle and so is more suitable for bright field microscopy.

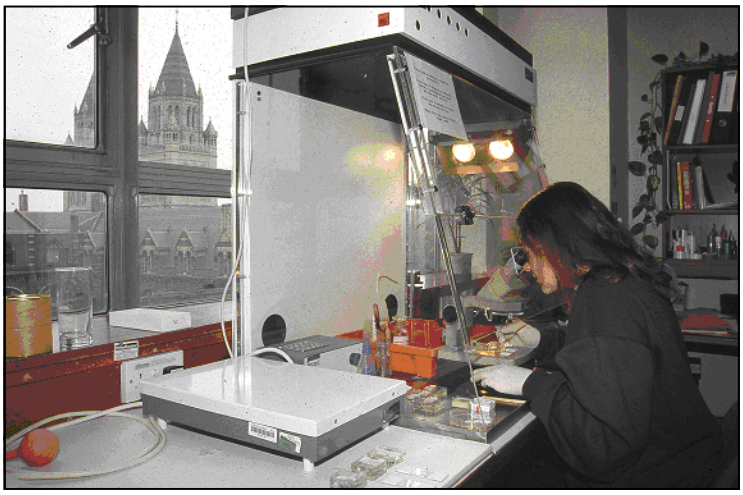

Fig.1 Hazardous chemicals such as xylene and phenol require the use of a portable fume hood.

The technique which we use at the NHM for whole mounting small Hemipteran bugs (aphids, whiteflies and coccids) is stated below and closely follows the method published by Martin (1999). For many small insects, all stages of this method can be carried out in a square-based watch glass or similar small receptacle with a wide top and a lid. Decanting fluids between stages of the procedure needs to be done whilst observing the pipette tip through the dissecting microscope (to

avoid the loss of specimens), the use of test tubes is not recommended. COSHH regulations for some of the reagents and chemicals used in this technique demand the use of suitably ventilated working areas, preferably with fume ducting or fume hoods.

1. Maceration of body contents is carried out by warming to around 80°C in a 10 % Potassium Hydroxide solution (an alkali) for 5-10 minutes or longer, until visible contents have become translucent. A small puncture may be made in the ventral surface of each specimen in order to speed up this and subsequent processes, and to help prevent osmotic collapse.
2. Decant excess macerant.
3. If the insect is naturally waxy, De-waxing of cuticle is carried out by gently warming specimens in a medium such as Carbol-Xylol (Xylene with 10% dissolved Phenol), Carbol-Histoclear (Histoclear with dissolved Phenol) or Chloral-Phenol (equal weights of Phenol and Chloral Hydrate warmed to liquefy and remaining liquid when cooled).

4. Decant de-waxing fluid, making sure that as much chloral phenol, if used, is removed as possible as t his might cause blackening problems in the future.
5. a) for specimens with opaque black cuticle, rinse in strong alcohol and then partially bleach cuticle by immersing in a freshly prepared mixture of cold strong Ammonia and 30-volume Hydrogen Peroxide solutions. Bleaching should be monitored as it can be very rapid, and may be stopped quickly by adding a few drops of a water-soluble acid. Domestic bleach is unsatisfactory for controlled cuticular bleaching.
   OR
   b) for very pale unsclerotised specimens, staining may be carried out by adding an excess of Glacial Acetic Acid* or Acid Alcohol and a few drops of Acid Fuchsin stain solution. Staining is carried out cold and usually only takes a few minutes. Failure of staining may result if de-waxing has been inadequate (see stage 3).
6. Decant bleach or stain and twice rinse specimens in Glacial Acetic Acid* or 95% Ethanol.
7. *Final dehydration* of specimens may be carried out by soaking in Glacial Acetic Acid* or Absolute Ethanol for a few minutes. Decant dehydration fluid.
8. *Clear* specimens by adding a few drops of Clove Oil or Histoclear (for Canada balsam), Euparal Essence (for Euparal) or an appropriate product for any alternative mountant.
9. Place specimen(s) on a pre-cleaned and polished slide, in a drop of Canada balsam or chosen alternative mountant and arrange specimens as required. Depending on the group, it is a good idea to place some specimens dorsum-upwards and some venter-upwards, (as prescribed by taxonomists). This aids observation of discriminate characters, particularly, in the resolution of ventral characters of species with very ornate dorsa. When mountant has partially dried, gently lower a cover-slip with a small amount of fresh mountant: the drier mountant will hold the specimens in place, while the fresh mountant will spread to cover the entire lower surface of the cover-slip. A little practice will be needed to perfect the amount of mountant needed to provide a preparation which does not cause distortion through over-flattening, but is also not so thick that its optical quality is impaired.
10. Slides must be adequately dried, especially if vertical storage is to be employed. With Canada balsam, slides may require up to two months at 35-45°C prior to permanent vertical storage. If slides are provided with a pair of thick card labels, they can then be stacked immediately, and the cover-slip will also be protected throughout the life of the slide: for this reason, paper labels are not recom mended for permanent collections.

* The choice between Glacial Acetic Acid and alcohols, is influenced by economic and safety factors. Glacial Acetic Acid has the advantage of being a cheap means of neutralising alkalis and vigorously dehydrating material, and provides the acid medium necessary for staining; it has the disadvantages of its unpleasant, breath-catching smell and ability to cause skin burns. Whilst 95% industrial Ethanol (IMS) is cheap when it can be readily obtained, Absolute (100%) Ethanol is extremely expensive, and both are more pleasant to work with than Acetic Acid. Ethanol is extremely hygroscopic, however, and many workers prefer 100% iso-propanol, which is not. Iso-propyl alcohol is, thus, a better choice for dehydration in humid environments, as well being cheaper than Absolute Ethanol.

Labels, glues and inks should all be selected with archival quality in mind. Suitable materials and methods are suggested by Carter & Walker (1999), for slide-mounted material.

Conservation of Deteriorating Microscope Slide Preparations.

Upton (1994) and Brown (1997) have drawn attention to the problems of Gum Chloral and plastic mounted slides deteriorating over time. We here discuss the procedure used to rescue such material from the slide collections held in the Department of Entomology of The Natural History Museum, London.

Only a well-trained slide-preparator or conservator should undertake remounting of slide material. When in doubt please employ an expert to assess the problem first and to train staff if necessary or subcontract the work to a conservator. Carter and Walker (1999) briefly mention restoration of insect specimens on slides but the procedure mentions "easing the cover-slip off the slide" which we would not recommend as damage to the specimen(s) may occur.

Within the NHM Aphidoidea collection, a continuing survey of the collection is carried out to reveal deteriorating slides. Due to lack of time and man-power only deteriorating 'Type' slides and slides of species not well represented in the collection (i.e. if there are less than 50 slides of a given species) are chosen for remounting. Slides selected for remounting are those Gum Chloral mounts showing signs of Phenol blackening with pink, bluish or black areas emanating from the specimens. Other Gum Chloral mounts showing signs of crystallisation are also selected where Chloral Hydrate crystallises from the edge of the cover-slip as water evaporates from the Berlese due to a failed sealant ring.

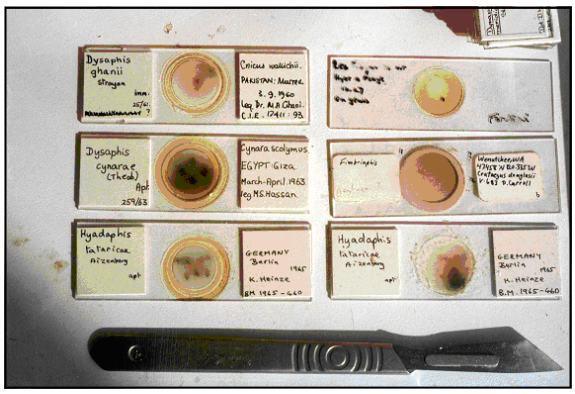

Fig. 2 deteriorating aphid slides showing blackening and crystallisation of Berlese gum chloral mountant.

Blackening in the Diptera collection slides has been blamed by some to be a reaction of the ringing medium Euparal with Gum Chloral. In the aphid slides, insufficient washing of the clearing mixture of chloral phenol might cause blackening and bleaching from the specimens before placing in the Berlese mountant, as the blackening emanates from the specimens and not from the edge of the cover-slip. Phenol is used in photography as a blackening agent!

Both aphids and Diptera were mounted in the same Berlese recipe that as quoted in Eastop & Van Emden, 1972:-

Gum Arabic	48g.
Chloral hydrate	80g.
50% w/w Glucose syrup	20ml.
Glacial Acetic Acid	20 ml.
distilled water	120 ml.

Berlese and other new mounting media were chosen because of the contrast in refractive index with insect cuticle, Berlese has an RI of 1.48.

Phenol balsam slides in the Diptera collection are satisfactory at the moment but a small number of aphid slides in this medium have turned black with cuticular degradation and have been rescued by soaking out in Xylene. This may indicate a future problem for this mountant. After dehydration, the specimens are soaked in a phenol/100% ethanol mix before placing in Canada balsam, which is dissolved in Phenol/Ethanol instead of Xylene.

Plastic (Polyvinyl Lactophenol) and gelatine mounts, showing signs of shrinking where air is entering under the cover-slips in long fingers, are also rescued. Canada balsam mounts are also occasionally rescued when the slide is broken or when the body contents need to be cleared for further taxonomic study using warm 10% potassium hydroxide. The Canada balsam slides in the Entomology Department are mostly not

showing signs of deterioration apart from yellowing and many are of a great age.

Methodology

Forty slides make up one batch and these are prepared by the scraping off of the ringing medium (Euparal and Murrayite) with a sharp scalpel, being careful not to damage the cover-slip in the process. These slides are then marked into three using a diamond stylus and the thirds are carefully snapped along the score lines, which usually do not splinter. The central third with the specimens under the cover-slip are placed into a watch glass with 30% alcohol and the two ends are put into water in a tub on top of the watch glass so as to keep the labels associated with the specimens. The labels float off the glass and are affixed to a new slide, which is again placed with the associated specimens and watch-glass. If the labels are paper, these are glued to an already carded slide using neutral pH Lineco PVA adhesive. If the labels are card, these are fixed with the same glue to an uncarded slide and if the card laminates in the water, the top label surface can be glued to a new card square. Future removal of such labels is done by carefully removing a layer of the card below the label, which strengthens it for further affixing. Occasionally non-permanent ink runs in this treatment so the soaking is watched carefully so those labels deteriorating can be removed quickly and then removed with careful use of a sharp scalpel. The new labelled but as yet blank slides are then left to dry on top of the watch glass with the associated specimens. Especially when dealing with 40 slides, one must be careful not to disassociate the specimens from the labels. A dedicated slide mounting-conservation area is prerequisite to avoid other people disturbing this system.

The specimens are left for a few days to soak in watch glasses with in 30% Ethyl Alcohol (the alcohol stopping fungal growth). These are placed in trays with suitable warning signs to avoid disturbance and covered to reduce evaporation and exposure to light. Some water-soluble mounts soak out quickly and the slide-square and cover-slip can easily be removed and disposed of in the sharps bin. Other mounts often need a further soak in cold 10% Potassium Hydroxide that digests the remaining mountant without damaging the specimens. This soak can be for 5 to 30 minutes. If the mountant is still intransigent, a further soak in warm KOH, or warm acetone will usually work. Acetone has a low boiling point so care must be taken to avoid over-evaporation or fire. The much thicker Lewis Diptera slides mounts in the NHM have successfully been soaked out of the possibly, slightly different Gum Chloral mountant by using warm acetone. The insect cuticle in this mountant has not deteriorated or bleached to the same extant as in the aphid 'Berlese' mountant.

Occasionally damaged Canada balsam slides can be soaked in Histo-clear 'orange' oil and or in xylene but the latter should be done in a fume hood. Cover-slips and specimens should not be helped out of the mountant as this often can damage the specimen with appendages breaking off. Often old Canada balsam slides have uncleared specimens with body contents still opaquely present. For taxonomic study, features of the cuticle need to be viewed so the body contents should be cleared. A careful heating in 10% KOH can clear these specimens before they are washed and dehydrated. Specimens that have previously been stored in formalin before mounting will not clear – this is another reason for not using formalin as a preservative.

The freed specimens can then be soaked in 30% alcohol in the watch glasses for a further period to wash any remaining KOH. The 30% alcohol is then decanted off and glacial acetic acid added for a short period of 2-5 minutes or changes of 50%, 80%, 95% and 100% alcohol, to dehydrate the specimens. If the cuticle of the specimens has been badly bleached by the deterioration process, Acid Fuchsin can be added in the glacial acetic stage to stain the specimens. The acid or alcohol is then decanted off and drops of clove oil are added to the specimens in the watch glass. From the clove oil the specimens can be removed to a drop of clove oil on the new slide and the appendages arranged suitably if the specimen is not already too rigid to allow this. Do not force the specimen if rigid as damage may occur. Different needle forceps, flattened pins and small spatulas can be used to remove the specimens from

Fig.3 Rescue of a microscope slide mount. Cutting of slide with diamond stylus and soaking off of labels and specimens from mounting medium (beware water soluble inks).

the watch glass to the slide, which is the most delicate part of the operation. Any appendages that become detached, should be placed close to the
specimen from which they came. The clove oil is then carefully soaked from the arranged specimens using the rolled corner of a tissue, taking care not to remove the specimens or their appendages in the process. Sometimes specimens will disintegrate through no fault of the conservator. If this occurs, place the fragments on the slide as even these can still be of taxonomic use. Add a drop of Canada balsam to the specimens and ensure that they are still arranged correctly and then place a cover-slip over the balsam and specimens ensuring that there are no or few air bubbles. Small air bubbles will often vanish when the slide is placed in the oven and attempting to remove bubbles by pressing on the cover-slip may cause damage to the specimens. This process is routinely carried out in a fume hood, especially if glacial acetic acid or xylene is used.

Place the slides in an oven at 30°C for three-four weeks to harden. If the balsam slides are not incubated then the balsam may well never harden sufficiently so that, if the slides are to be stored vertically, the mountant will run to the bottom of the slide under the influence of gravity. Very large numbers of 'thin-mount' slides are stored vertically in the NHM Entomology collections and no properly hardened slides have slumped. Thick mounts should always be stored horizontally as the centre of a mount often does not harden sufficiently even after baking.

Liquid mount conservation is not discussed here, as we do not have such mounts in our direct care. Simon Moore discusses the conservation of liquid slide mounts in his paper reporting on a project undertaken to save drying slides held within the Royal College of Surgeons in London (Moore, 1979).

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