Acetolysis Protocol

Materials:

- Herbarium samples
- Permanent marker
- 1.7 mL Microcentrifuge tubes
- Test tube racks
- Glacial acetic acid
- Concentrated sulfuric acid
- Acetic anhydride
- 10 mL Graduate cylinder
- Heat block with aluminum element for 1.7 mL tubes
- 100 - 1000 µl Pipette tips
- Centrifuge with inserts for 1.7 mL tubes
- Waste beaker
- 200 µm Nylon mesh filters
- 200 µm - 1000 µm Micropipette
- Glycerol
- Safranin
- 10 mL Pipetter
- 10 mL Pipette
- Vortex
- DI Water in wash bottle
- 95% ethanol

Filtering:

1. Place the filters inside microcentrifuge tubes, which then need to be placed into larger "buckets" (see Figure 1). Individual bucket assemblies are placed in the test tube rack next to each pollen sample, so each sample is paired with it's own bucket assembly.
2. Remove the parafilm.
3. Vortex the glass tube until the solid and liquid contents are well agitated.
4. Pour the contents of the tube into the bucket assembly. Removal of all liquid may require "tapping" of the glass tube.
5. Add 300 µl of glacial acetic acid (100%) to the glass tube.
6. Vortex the tube again and pour the liquid into the bucket assembly.
7. Fit the micropipette with the filter extension and main filter piece.
8. Draw the contents of the plastic tube up and down slowly several times to dislodge any remaining pollen from the filter. Drawing the liquid should be done slowly to avoid splashing liquid onto or into the end of the micropipette.
9. Remove the filter and place it in a waste beaker for washing.
10. Cap the microcentrifuge tube. A total volume of 700 µl should be present in the plastic microcentrifuge tube.
11. Repeat this process until all samples have been filtered.
12. Close the lid on each of the samples.
13. Place clean plastic micropipette tips into each bucket (Figure 2).

Figure 1: Bucket assembly

Figure 2: Test tube rack setup with clean pipette tips

Preparing the Samples for Acetolysis:

1. Centrifuge the samples at 2,000 rpm for 3 minutes to allow pollen to form a pellet at the bottom of the tube. If pollen sticks to the side of the tubes, rotate the tubes 180 degrees and re-centrifuge for 30 more seconds.
2. Fit the micropipette to the clean, plastic pipette tip that corresponds to the sample being drawn out.
3. Draw out the liquid from the tube and deposit it into a waste beaker. Draw out as much liquid as possible without disturbing the pellet. If the pollen is disturbed, return the liquid to the tube, re-centrifuge and draw out again.
4. Return the pipette tip to its bucket and attach a new tip.
5. Repeat this process until the liquid has been removed from all of the samples.

Preparing the Acetolysis Solution:

1. Add the necessary amount of acetic anhydride to a 10 mL graduated cylinder using a quick release pipette pump fitted with a DRY 10 mL glass pipette tip. The sulfuric acid and acetic anhydride must be used in a 9 (acetic anhydride) to 1 (sulfuric acid) ratio. It should be calculated based on the number of samples being processed.
2. Carefully remove the tip and rinse with glacial acetic acid over the waste beaker.
3. Fit a micropipette with a DRY glass pipette tip to add sulfuric acid to the graduated cylinder containing acetic anhydride.

Caution: Tips MUST be dry. Water reacts violently with the acids and may cause bodily harm.

4. Add the necessary amount of sulfuric acid. Be sure to follow the 9:1 ratio mentioned above.
5. Gently release the pipette tip into the graduated cylinder for use in the next step.

Acetolysis:

Caution: The Acetolysis process must be performed in a water-free environment. Be sure to remove any water containers from the fume hood prior to starting this process.

1. Uncap all samples being processed for a rapid addition of Acetolysis solution. The samples can be prepared 6 or 12 at a time depending on the user's preference.
2. Fit a micropipette to the glass tip from the graduated cylinder containing the Acetolysis solution. *Acetolysis is time sensitive. Reaction occurs immediately upon addition of the acids, so be prepared to work quickly.*
3. Add 300 µl of Acetolysis solution to each tube being processed and immediately transfer to the heat block. As mentioned above, it is best to work in batches of 6 or 12 samples.
4. Return the tip to the graduated cylinder and loosely cover the graduated cylinder with a small piece of parafilm.
5. Place the tubes into the heater in the order by which the acid solution was added (see Figure 3). During this step, leave the caps OPEN. Add and remove samples in the same order that they were placed in the heat block. The first sample into the heater must be the first sample removed from the heater.

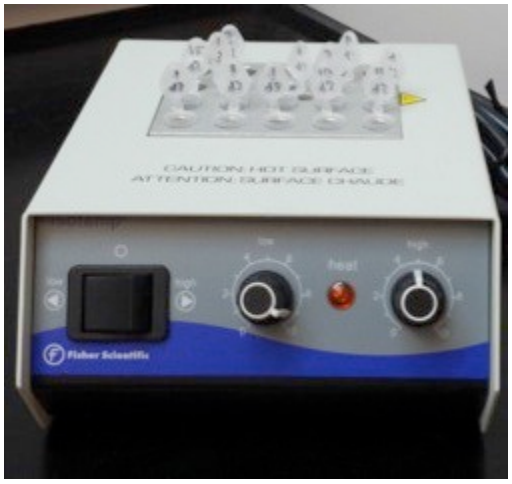

Figure 3: Heating of samples during Acetolysis

6. Keep the samples in the heating element for 3 minutes. The time may need to be adjusted, depending on the type of heater. Check the samples throughout this step by gently lifting them out of the heating element. Finished samples should appear light brown in color (see Figure 4).
7. After approximately 3 minutes, remove the samples from the heater and return to the test tube rack.
8. Using a clean glass pipette, immediately add 300 µl of glacial acetic acid to all heated samples to stop the reaction.
9. Cap all finished samples and vortex thoroughly.
10. Repeat the Acetolysis procedure with the remaining samples.
11. Once all samples are completed, turn off the heater and put away acetic anhydride and sulfuric acid containers.
12. Rinse all tips and glassware with glacial acetic acid, and dispose of the waste in an approved and labeled waste disposal container.
13. Glassware can now be safely washed with soap and water.

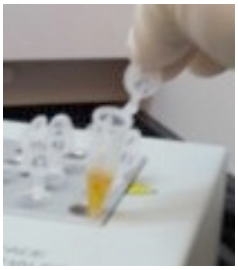

Figure 4: Color change after 3 minutes

Water Wash:

1. Centrifuge the samples for 2 to 4 minutes at 2,000 rpm.
2. Using the tips from each bucket, draw out the liquid from each tube and deposit into a waste beaker.
3. Rinse off the tips and accompanying buckets with water over the waste beaker. Return to the test tube rack.
4. Using a clean, glass pipette tip, add 500 µl of water to each tube.
5. Cap the tubes, then vortex and centrifuge them.
6. Once again, wash all tips and buckets and return them to the test tube rack.
7. Using the rinsed plastic tips, add 500 µl of water to the tubes. Plastic tips no longer need to be washed between each step.
8. Repeat this water wash process two more times with 500 µl of water. A total of four complete washes are required. Washes can be recorded on the preparation protocol sheet.
9. After the final wash, vortex and centrifuge the samples.
10. Draw off the water down to the pollen pellet.
11. Properly dispose of the waste by placing it into an approved waste container.

Addition of Glycerol:

1. Fill each sample to the 100 µl (0.1 mL) line on the plastic tube with water.
2. Vortex the tubes, then uncap them.
3. Add one drop of glycerol with safranin and one drop of plain glycerol to each tube. Be careful not to drop the glycerol down the side of the tube. The viscosity may inhibit the stain from reaching the pollen at the bottom. If this occurs, add another drop of the required liquid into the water at the bottom of the tube.
4. Re-cap all tubes. Vortex for 10 seconds.
5. Place tubes back into the test tube racks and leave them to rest over night (see Figure 5).

Figure 5: Sample with glycerol and safranin

Ethanol Washes:

1. After allowing the samples to rest overnight, add 500 µl of ethanol to each sample tube.
2. Vortex each sample thoroughly.
3. Centrifuge for 2 to 4 minutes at 2,000 rpm.
4. Use plastic pipette tips to draw each sample down to the pellet.
5. Repeat this washing procedure twice more to ensure the removal of all glycerol.
6. Using a clean glass pipette, add ethanol to the samples to fill to the 100 µl (0.1 mL) mark on the tube.
7. Re-cap the samples and place in the proper storage box for mounting and/or SEM photography.