

## Phytolith Extraction Guidelines for Modern Plant comparative material

### Extraction Methods

There are several methods for extracting phytoliths from modern plants; these are discussed extensively in the "bibles" of Pearsall (2000) and Piperno (1988). Essentially they can be divided regardless of specific protocol into a few major groups:

1. Dry-Ashing (Using dry, high temperature heat to ignite and remove non-siliceous material through incineration)
2. Epidermal Peels (Chemically breaking down outer layers of plant tissue to "peel" away outer epidermis and view phytoliths in the physical location in which they occur in the plant)
3. Spodograms (Using incineration techniques to prepare a similar "in-situ" view of plant material)
4. Chemical Oxidation (Using strong chemicals, usually acids, to destroy non-siliceous tissues and other organic components of plant material).

As with all methods, each of the above has advantages and disadvantages and may be used depending on your particular question relating to phytolith production in your sample(s).

Dry-Ashing has the advantage of quite efficient, and cheap, if one has access to a muffle furnace, in that one can quickly process a number of samples at once. The disadvantage is that the ashy residue often contains a great deal of other particulate matter and makes for "dirtier" slides for viewing. Some tissue fragments in dry ashing often survive as "silica skeletons" or de facto "spodograms" so sometimes you may see phytoliths as they occur in tissues.

Epidermal peels and spodograms (I have no real expertise in these) are of great value if you want to really understand the particular locus in the plant your phytoliths are deriving from; e.g., if it is important to know they are occurring in vascular tissue, in mesophyll, etc. It can help mnemonically in understanding the 3-D morphology of phytoliths by seeing how phytoliths articulate with their surrounding tissue.

Chemical Oxidation is slightly more involved than dry-ashing but produces much cleaner end results. A number of reagents can be used, again see Pearsall (2000) or Piperno (1988) for sample protocols.

## Preparing Your Plant Sample

Since we will be doing Chemical oxidation today, I will discuss the specific choices in regards to those, but the guidelines generally hold for whatever method you choose. In no specific order here are some “tips and tricks”

1. Mature plants are better than young plants. Plants accumulate silica throughout their life, so an older grass or fruit is better than a young sprout.
2. Subsample the plant based on its anatomy. Leaves, wood/stem, fruit, and inflorescences should all be run as separate samples. Phytoliths should occur in aerial parts of the plant, roots rarely if ever produce phytoliths (I have never seen a reference to a root derived phytolith). Depending on your question, you may even want to subsample within anatomical parts (e.g. for leaves separate petiole from blade, or various parts of a flower or inflorescence as separate samples). I would recommend doing this detail work only after you have verified that the plant produces phytoliths.
3. In relation to #2, if you are running a generalized “leaf” sample, make sure you include a good cross-section of the anatomy, don’t just sample one tiny corner of a broad leaf, but get edges, central tissue, etc.
4. If the specimen is noticeably dirty, clean it to remove adhering soil particles, dust, etc. Distilled water and/or dilute HCl are good choices. Unless the plant material is really caked in crud, however, contamination is not a really significant issue with comparative material. Others are more compulsive about pretreatment with detergents, ultrasonic baths, etc.

**My Chemical Oxidation protocol, adapted from Lisa Kealhofer, who adapted it in turn from Dolores Piperno, to whom it was handed down on Stone Tablets from the Lord directly:**

**NB: Samples should be run in multiples of TWO to balance the centrifuge; i.e., you can’t do a run of three samples!**

1. Use 16X100 size borosilicate glass disposable test tubes. Use Masking Tape (standard 19 x 54mm [thin] size) and **PENCIL** (ink dissolves in the steam, acid and acetone used in this procedure), to label one test tube with your sample information. I would recommend using some kind of log to record complete information and a shorthand sample number to place on the tubes. Make sure the masking tape completely encircles the top of the test tube and overlaps a bit to keep it from slipping off (some masking tape brands work better than others – experiment).

2. Tare your test tube and weigh between 0.1 and 1 gram of plant material and record the weight. These are “ballpark” figures; as we know plants vary in density, moisture content, etc. The goal is to get enough plant material to recover phytoliths but not overload the test tube and create too much chemical reaction, or, conversely, take overly long to digest. Record the weight.
3. Start a hot water bath, using small beakers half-full of tap water on a hot plate; water should be just below simmering, not boiling.
4. Wearing **GLOVES AND SAFETY GLASSES AND WORKING IN THE FUME HOOD** Fill the sample test tubes with concentrated Nitric Acid (70% strength) to the base of the masking tape line. Add a glass stir rod to each test tube, and place them in the hot water baths. If your plants want to float out, tamp them down with the stir rod. Watch the reaction as they begin to warm. If the reaction is already rapid, or bubbling, wait some time before proceeding to step 5.
5. Still wearing your **GLOVES AND SAFETY GLASSES**, add a very small pinch of Potassium Chlorate (crystalline or granular  $KClO_3$ ), and agitate the sample with the stir rod. If you get an angry reaction, again wait longer before repeating.
6. Repeat Step #5 above every 15-30 minutes, depending on the reaction until all the plant material is dissolved. If the samples are taking longer than your life or schedule permits, you can turn off the hot water bath at any point and just return to the samples when you have time. Watch the water level in the beakers so it doesn't evaporate; refill as necessary.
7. Once the material is dissolved, remove the tubes from the heat (still keeping them in the fume hood). Remove the stir rods and place them in a beaker with a little Sodium Bicarbonate (baking soda) in the bottom to neutralize the acid drips. Also neutralize any acid spills with baking soda.
8. Place the test tubes in the centrifuge, making sure they are properly balanced. Our ARF centrifuge is old and cranky and tends to make horrible noises around 1000 RPM. Try 2000 RPM. Centrifuge the samples for 10 minutes at 2000 RPM to collect the phytoliths (if any) at the bottom of each test tube.
9. While your samples are centrifuging away, fill the largest beaker you have about 1/3 full of plain tap water to dispose of the acid.
10. After 10 minutes, quickly decant the acid from each test tube into the large beaker with water. Obviously you are doing this wearing your **GLOVES AND SAFETY GLASSES** and working as much as possible within the **FUME HOOD**,

11. After you have poured off the acid from each sample, fill each test tube back up to the tape line with distilled water. Use clean stir rods and re-agitate/resuspend the material in the tube in the water.
12. Repeat Step #8, to collect the phytoliths in the bottom of the test tube again (2000 RPM for 10 minutes). Again decant the test tubes into the acid waste beaker. If there is still a yellowish tinge to the liquid, you should repeat steps 8-10 once again. Any nitric acid remaining in the sample can react with your mounting media and make ugly slides.
13. Once all of the acid is removed, you need to neutralize the waste acid before pouring it down the drain. Place a "catch pan" of some sort in the sink with a bit of baking soda in the bottom for overflow. Place the waste acid beaker in your "catch pan" and slowly add baking soda until the solution stops reacting and is neutralized. As this splashes and fizzes quite a bit, you should **WEAR SAFETY GLASSES** for this and if you are smart probably **GLOVES** too.
14. Now back to your exciting phytolith test tubes. Once they are clear, and the water has been poured off, add acetone to each tube. You do not need to fill the tubes completely, half to 2/3 is fine (to conserve the acetone), although all tubes should be filled to an equal height to keep the centrifuge balanced.
15. Again using a clean stir rod, agitate and resuspend your phytoliths in the acetone.
16. Centrifuge them again for 10 minutes at 2000 RPM.
17. Decant the acetone from each test tube into a beaker for waste acetone. Let the waste acetone evaporate in the fume hood or otherwise dispose of it properly. Acetone does NOT go down the sink.
18. Let your samples dry for several hours or overnight. The acetone will evaporate leaving, if you are lucky, pristine white powdery phytolith extract.

### **Mounting the Phytoliths**

1. Using a clean stir rod, gently grind up the phytolith extract (if any) at the bottom of your test tube, breaking up any caked together lumps, mortar and pestle style
2. You may have more phytolith extract than you need. If there is more than about this much: ●, you should save the remainder somewhere else. Dump all the rest more than ● size into some storage vessel for later fun and games. I like the little Nalgene cryo-tubes (1.8ml size) for storing my phyto extract. Forget the books, etc. that tell you to store them in water, I see no point to that at all. Dry is good.
3. Add your mounting media into the tube containing your phytolith extract. Use about 2/3 of a pipette worth of mountant, or enough to fill your test tube about 1-2 centimeters.

4. Using a glass stir rod (better, slower) or the tip of your pipette (lazier, more prone to produce bubbles if you aren't careful, but faster and less cleanup), suspend the phytolith extract in the mountant and suck it back up the pipette. **NEVER place an used pipette back in the mounting media bottle!**
5. Pipette a thin layer of extract onto your glass slide in the shape of your cover slip, leaving no air spaces (this takes practice).
6. Place the coverslip edge on one side of your extract square, making sure the liquid is in contact with the entire edge of the coverslip. Gently drop the coverslip onto the rest of your extract, and hopefully you have no bubbles. If you do, you can try tapping gently on the coverslip to move them to the edge of your slide.
7. You **can** look at the slide at this point, although the phytoliths will be at several different focal planes in the microscope. In a couple of hours they will settle down. After an hour or so you should seal the edges of your slide with nail polish. This will keep the mountant fluid for longer (important when "rolling" your phytoliths around to see their morphology).
8. **NEVER** store your phytoliths on their edge; if you are keeping the slides in slide boxes, the boxes need to be stood on their edge. All the phytoliths will sink to the bottom and make a crusty line of uselessness at the bottom of your slide. Store them flat (parallel to the ground!).