SAMPLE COLLECTION AND PRESERVATION

A. For nutritional analysis, samples should be collected to closely mimic how the animal is selecting and consuming the food. Plant parts should be separated if the animal is selectively feeding on only certain parts. Both the consumed parts and the unconsumed parts may or may not be analyzed, depending on the questions being posed by the researcher. Dissections of plants into various parts (pulp, seeds, husk, etc.) are usually best done with fresh material and each part weighed, fresh. However, picking out fig seeds is better done on dry figs; too much pulp tends to be included with the seed fraction when scooping them out of fresh figs.

Slice or dice the already weighed fresh material before preserving, to facilitate drying and later grinding.

B. There are three preservation methods commonly in use:

1. analysis of fresh plant material – that is to say, no preservation at all, analyzes done immediately upon collection,
2. analysis of dried material, stored in plastic bags or paper (eg. manila) envelopes, and
3. analysis of fresh material that has been preserved in alcohol, eg. commercial produced methanol or ethanol – use sufficient alcohol to cover the sample in either dark glass bottles or protect from the light by wrapping bottle with aluminum foil.

C. All sample containers must be clearly labeled. It is best to label the container directly and label a boxboard ticket that is dropped inside with a dry sample. Alcohol preserved samples should be labeled twice on the bottle and once on the lid. All identifications should also be recorded on paper.

SAMPLE PROCESSING

A. Weight the whole sample either fresh, or “as eaten”, or in whatever condition you receive it (seeds and grains will be naturally dried, some human foods are eaten dry eg. bread).

1. Dry the samples in a 40-50°C circulating hot air oven.
   a. In the field, if this is not possible, dry the sample as quickly as possible in a warm, dry room with good ventilation and out of direct sunlight, on thin sheets of aluminum foil on some kind of drying rack and heat source (eg. kerosene lamps or stones). Stir the drying samples several times during the first 24 hours.
   b. Take at least 2 robust thermometers with you to the field.

If you receive samples at the lab in alcohol, first ask whether they were weighed fresh when collected. If not, filtering off the alcohol (but saving it) and weigh the solid sample, then pour the alcohol back in.

b. alcohol-preserved samples - evaporate the sample with its alcohol.
2. fresh samples that you analyze without drying, still need a calculation of dry matter and water content, so a small subsample should be dried.

This initial drying step is performed both as a preservation step, the sample can be stored after having been well dried at this temperature, and to allow for easier grinding, described in step C. The temperature must not be higher than 50°C because at higher temperatures the carbohydrate will either caramelize or bind with the protein in the sample and create what is called Maillard Product. Potato chips are a good example of Maillard product = the darker brown parts of a chip. Maillard will change the results of the analyses (specifically it increases % fiber and decreases % soluble nutrients, protein and carbs). If you are drying in a homemade oven, keep a couple thermometers placed at the same levels as the samples in the oven to monitor the temperature.

If you plan on analyzing for tannins, dry samples at 40°C, if you have molding problems (visible mold) or fermentation (blackened, charred looking sample), throw out the samples and start again - increase the temperature a little to dry quickly, cut into smaller pieces, and stir the diced or sliced sample more often to ensure even drying. Record what temperature you use.

B. Weigh the sample after drying it.

1. Field Dry Matter (RTDM) is calculated as:
   \[
   \%\text{FDM} = \frac{\text{total dry weight}}{\text{total wet (fresh) weight}} \times 100
   \]

2. Field % water = 100 - %FDM

A recommended method for storing samples in the field after the initial drying is to place them into manila envelopes and store them in a basket hanging close to the ceiling of the room where the initial drying occurs. The heat from the drying source (oven, kerosene lamps, kerosene oven, etc.) collects under the roof and will keep the samples from re-absorbing humidity and molding. Monitor the temperature at the ceiling. Alternatively, if you are very confident you have thoroughly dried the sample, seal it in a plastic bag so it is airtight. It would still be best to store these samples in the driest place possible, like the ceiling above a heat source. Silica gel should not be necessary. Never add silica gel directly into a bag with a sample!!!

If you have used alcohol, the samples will be dried in the lab and weighed. Dry samples in the original jar and scrape out any residue, to be included with the rest of the sample before grinding. Unfortunately alcohol extracts sugars, protein, fats, tannins, practically everything except insoluble fibers, so the residue left after evaporating the alcohol is very important not to lose.

C. Grinding in the lab:

1. grind the dried sample in a Wiley mill, or similar electric grinder, mesh #20 (1 mm screen).
Vacuum and use small brushes (alcohol swabs after sticky samples) to clean mill & mill pieces between samples.
2. grinding a fresh or wet sample is accomplished with a mortar and pestle or a homogenizer/blender. Unfortunately these samples will not have as uniform a particle size as will mechanically ground samples. Therefore this is not the best method.

3. store dry, ground sample in a ziplock bag, twirltop bag, or anything that is air-tight. Some high fat samples will damage plastic bags, dark glass jars are recommended for them. Label bags with permanent marker and put any original (usually paper/box board) labels inside with the dried sample.

4. Problem samples - Grinding with a Wiley mill is a simple procedure for leaves and low fat grains and pulses. For fruits however, high fat samples and high sugar samples can be difficult.
   a. High sugar content occasionally prevents them from completely drying and when you grind them, they turn into stiff taffy, which has to be scraped or chiseled out of the grinding chamber and the chamber washed with ethanol and dried before the next sample can be ground. Usually it helps to grind them along with dry ice, to keep the chamber and the sample cold during the grinding process. Sometimes liquid nitrogen is necessary to freeze the sample and then the frozen sample is ground with dry ice to keep the grinding chamber cool. Do not put liquid N into the grinder, potentially you can crack the chamber.
   b. High fat fruit pulps or seeds turn into butter in the grinder. Generally the high fat items will grind but not filter through the screen. So, grind small quantities at a time, scrap the “butter” out of the chamber and into a bag, clean the screen or use another, clean one and repeat until the whole sample is ground. Try to prevent over-grinding. Alcohol and cotton-tipped applicators are needed to clean the chamber. Liquid nitrogen may help but not always – as described above, freeze the sample in the nitrogen, then grind using dry ice to keep the grinding chamber cool. Do not put liquid N into the grinder.
   c. Very hard fruits or seeds may be cracked by wrapping in heavy paper and hitting with a hammer. All samples have to be broken down small enough to go through the hopper of the Wiley mill, and the smaller the better so that the mill is not stressed too much. Turn the grinder off immediately if the blades stop spinning and remove contents in the chamber. Slightly spin the driveshaft to make sure nothing is wedged. Restart, adding smaller amounts of sample to the hopper.

When grinding leaves, petioles and midribs may be quite fibrous and may sheer into long, thin strands instead of chopping up into tiny pieces. Let the grinder run until 95% of the particles fall through the screen, then turn off the machine and brush what is left in the grinder into the jar along with the rest of the ground sample. You do not want to throw this part out just because it won't pass the screen. They are important chemically and to maintain a representative sample, must be included with the finer ground sample.

SUBSAMPLING FOR EACH ANALYSIS

When weighing subsamples smaller than 0.1 g (100 mg) there can be major problems with static electricity, which leads to sampling error. The analysis procedures are generally done in duplicate, but if you are worried about the error this problem gives, you can increase to triplicates.
Whatever weight the subsample, uniform sub-sampling of the whole sample is extremely important. Static electricity will cause fine particles to stick to the sides of the bag or jar. Also, heavier particles will tend to settle to the bottom and lighter particles stay on top. Mix the whole ground sample before sub-sampling and as you sub-sample, take a little from the top, some from the center, and some from the sides of the bag or jar. This is harder to do when only sampling 0.1g, so be sure to mix well before sampling.

For procedures that call for 1 g, weigh out 1.0xxx, in other words, the tenths position should be zero, the last three digits can be anything, as long as you write down what they were. If the procedure calls for 0.2 g, weigh out 0.20xx, if 0.5 g, weigh out 0.50xx = the 1/100 place should be a zero, the last two places can be anything. Always record the exact weights.

100°C or 100% DRY MATTER (DM)

A. 100°C DM has been officially declared 100% dry by the Association of Official Analytical Chemists (AOAC) (some use 105°C). It is used to standardize sample values obtained from different labs so that comparisons can be made among labs and in the literature, eliminating variability due to humidity and moisture content changes.

B. Subsample 0.5 to 0.2 g of ground sample (most common = 0.3g), recording the exact weight, and place it in a tared porcelain crucible. The tare should be determined by hot weighing the empty container, see hot-weighing section for directions. Record the empty weight on the data sheet. This is not the same as pushing the tare button on the machine. The empty porcelain crucibles should be put in the 100°C forced convection oven the night before, hot-weighed in the morning, that empty weight recorded and then the subsamples added.

C. Place crucibles in the 100°C forced convection oven, leaving them there for at least 8 hours. When people are actively hot weighing during the day, in order to not change the temperature equilibration inside the oven, new samples should be put in the oven after 4 pm, and will be ready for weighing the next day. During the day, leave the tray with your samples on top of the oven until 4 pm (and don’t plan on hot weighing after 4 pm).

D. 100°C DM = 100°C weight / subsample weight x 100

E. This is the %DM that is used to standardize/correct all values obtained in the lab. The field dry matter for the ground sample used in each analysis was previous determined (see above). The 100% dry DM corrects for the water that remains in the sample at room or field temperature. There are additional, finicky methods for correcting for daily fluctutations in humidity but when surveying hundreds of samples from wild populations, this procedure is accurate enough. Unfortunately, in the ecology and primatology literature (but rarely in the nutrition literature) values are sometimes published without correcting them to 100% DM. If you are comparing your values to literature values, read the methods carefully to determine whether the authors corrected their numbers.
If very small samples have been collected, the 100% dry matter procedure is skipped because there isn’t enough sample. Nutrient analyses can be reported as a percentage of field dry matter, or using the mean 100% DM value for similar samples.

F. Total Ash%

a. After hot weighing the DM and recording the weight, place the same crucible with the DM sample in the muffle furnace and ash at 500-550°C overnight.

b. turn the furnace off first thing in the morning and allow to cool during the day. Do not open the door of the furnace until it has cooled to 200°C, at which point you could then remove the beakers with tongs, or you can wait until they become cooler and remove them with gloves.

c. Place in the 100°C oven and hot weigh the next day.

d. % total ash = ash weight / (subsample sample wt. x DM%) x 100

e. % organic matter = (100 - %ash) x %DM

Equipment for these preliminary procedures:
1. balance accurate to 0.1 or 0.01 g - for FDM%
2. some sort of drying rack and heat source
3. aluminium foil sheets or trays – aluminium or box board,
4. paper envelopes or plastic bags or brown glass bottles
5. forced air oven that goes to 100°C at least.
6. drying trays for oven – aluminium or glass
7. Wiley mill
8. porcelain crucibles
5. 500°C muffle furnace
6. tongs.
LIPID EXTRACTION

The reagent used in this procedure is very flammable and has health hazards (carcinogenic). All lipid extraction steps are performed under a hood and wearing latex or nitrile gloves - see step 2c below.

Use the hood that does not have the Kjeldhal equipment, it is dangerous to mix acids and solvents. Glassware used should be left in the hood so the solvent can evaporate off (usually overnight) before placing in the sink for washing.

Reagent:
petroleum ether - for example: case of 6, 1L bottles, boiling range 35°C to 60°C, residue after evap. \( \leq 1 \text{ ppm.} \)

Calibrate both Mettler balances first thing in the morning before use.

DAY 1:
1. Weigh out 0.5 - 1.0 g in duplicate of sample directly into a labeled glass jar or Erlenmeyer flask with an airtight glass stopper. The glass jars will require that you label each bottle with a sharpie pen with the sample ID number and an ‘a’ and ‘b’ on the duplicates. The Erlenmeyer flasks have numbers on them already – use them in numerical order.
   a. Use 1 g in duplicate if you have a sample you suspect will be high in fat, and if you will be saving the fat-free residue for the fiber and/or tannin analyses.
2. Under the **FUME HOOD** & **WEARING** latex or nitrile **GLOVES** & **lab coat:**
   a. Add 50 ml of petroleum ether if you weighed out 0.5 g sample, add 100 ml if you weighed out 1.0 g, to the jar/flask with sample.
   b. Cover with a ground glass stopper that is the right fit, and gently swirl. Be careful not to dislodge the stopper but don't press the stopper down either because they can get stuck.
   c. If petroleum ether spills on one of your gloves, remove the glove, throw it out and get a new glove. These gloves are not rated to protect you from petroleum ether (strong organic solvents), but you do have at most 15 minutes to take them off before the solvent penetrates the glove to your skin (and this assumes complete submersion), so don’t panic.
3. Select as many 100-150 ml glass **beakers** as you have jars/flasks with sample in them.
   a. Place the beakers (in numerical order) in the oven at 60°C and leave them for 5-6 hours or overnight.
b. Hot weigh later the same day or the next to obtain their tare. Record them on the data sheet.

4. Leave the jars/flasks containing ether & sample under the hood, stoppered, for at least 4 days, swirling once daily.

DAY 4 OR 5:

Calibrate the Mettler balance used for hot weighing first thing in the morning before use.

5. In the fume hood set up a wooden frame with one large glass funnel with a fritted glass filter, in each hole (6 in total). Place the pre-weighed beakers, in numerical order, from step 3 above, under the stem of each funnel (wait until they have cooled if you have just hot weighed them).

6. Wearing latex or nitrile gloves, pour off the petroleum ether from one jar or flask, through a glass funnel, and into a labeled and pre-weighed beaker.
   a. If petroleum ether spills on one of your gloves, see comments in step ‘2c’ above.
   b. To speed up the filtering, use one of the syringes with big rubber stoppers and a large gauge needle in the rubber stopper to push the liquid through faster.
   c. If you plan on saving the fat free residue, the sample residue should be left in the bottle or flask to be dried in the hood and the next day transferred to small twirl-top bags, clearly labeled.

7. Leave the beakers with the filtrates in the fume hood to allow the filtrates to evaporate over night. Leave the hood turned on all night.
   a. Also leave the glass funnels, jars/flasks with sample residues, stoppers, and any empty petroleum ether bottles in the hood to dry/evaporate over night.

8. The next day, after all solvent is evaporated, place the beakers in a 60°C oven.

9. After 8 hrs minimum (usually the next day), hot weigh the beaker with the lipid residue.
   a. Enter the data into a lab template file on the lab computer and email it to yourself ASAP, or enter the data into your own computer. Usually leave the hand written paper data sheet in the lab.
CLEAN UP:

The extraction bottles: after putting the fat-free residue in small bags (see step ‘6a’), wash the bottles and stoppers with hot, soapy water. Rinse in warm tap water then distilled water.

The lipid beakers: wash in very hot & very soapy water and scrub with a brush. Fat washes off better with hot water. Rinse in hot tap water then room temp. distilled water. Check them as you pull them out of the final rinse or when dry to be sure they are clean. Some residues are very difficult to wash off and you can try rinsing with sulfuric acid (pour the rinses in the ADF rinses carboy), and if that doesn’t work, throw the beaker in the broken glass bin.

CALCULATION:

% lipid = \frac{\text{wt. crucible with lipid residue} - \text{wt. empty crucible}}{\text{sub-sample wt. x dry matter %}} \times 100

Equipment:
1. hood
2. glass bottles with glass stoppers
3. scintered (or fritted glass) glass funnel w/ coarse porosity
4. 100-150 ml beakers
5. 60°C oven
6. balance accurate to 0.01g
7. Misc.: sharpie pen, spatula, weighing paper, disposable gloves.

Reference:


modification = soxlet equipment not used, instead no heat and extracted at room temp for 4 days.

lipid residue can be stored in DCM (methylene chloride) if you want to test for terpenoids.
Kjeldahl Total Nitrogen or Crude Protein

DIGESTION:

Calibrate both Mettler balances first thing in the morning before use.

Wear gloves and a lab coat:

1. Measure 1.0 - 0.5 g sample into large glass tubes (0.5 preferred), in their stainless steel tube racks. As little as 0.3 g can be used, but not less.
2. Turn on the hood and turn on the hood’s water less than a quarter of a turn so it does not splash.
3. Turn on the digester unit to 400°C (the temperature is usually already set).
4. Add 1 level teaspoon of digestion mix:
   
   Digestion mix = 24.1 g \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \) mixed into 515 g \( \text{Na}_2\text{SO}_4 \) spec. reagent, anhydrous.

Crush all clumps and mix thoroughly. Save the extra in a beaker covered with a Petri lid.

5. Add 25-26 ml concentrated sulfuric acid (\( \text{H}_2\text{SO}_4 \)) to each tube and put each into a digester slot.
6. Place the rack of exhaust caps on top of the tubes. Put the front heat shield into position. Make sure all caps are squarely in place.
7. Pull down the glass window of hood down to the yellow sticker’s line and leave to digest for 45 minutes (set timer).
   a. Check to see whether the solutions have all turned blue-green. The first batch of the day might take 1.5 hr to turn blue-green because the digester heats up slowly. Some samples are also harder to cook than others. Some samples splash more when cooking, sending black bits of incompletely burned sample up on the sides of the tubes. Longer cooking time usually washes them back down into the acid.
   b. After turning blue-green color, cook 30 min more; use a timer.
8. When digestion is complete, remove the front heat shield and pick up the tubes’ rack by the handles. The caps’ rack should stay in place. Shift both racks together to the white stand. Carefully fit the tubes’ rack within the sides of the white stand.
9. Set timer for tubes to cool 20 minutes.
10. After 20 minutes, remove caps and very, very slowly add 75 ml distilled water - you are adding water to acid, which can cause water to shoot up and out of the tube. This is the ‘diluted sample’.
11. After about 20 minutes, swirl the tubes individually to prevent or to break up crystals that have started to form. If crystals form overnight, place the tubes in hot tap water for 5 – 10 minutes, then take them out and swirl - repeat until the crystals dissolve. You may have to let them cool again.

Samples should be allowed to cool completely before distilling. Usually the last digestion of the day should wait until the next day for distillation. The morning digestion might be cool enough to distill in the afternoon. Digested & diluted samples should be covered with Parafilm if not distilled the same day.

**DISTILLATION:**

Wear gloves and a lab coat:
Digested & diluted samples can sit for as long as 2 weeks, covered with Parafilm, but it is recommended to distill as soon as possible.

**Mix up boric acid:**

a. Mixed indicator: 0.208 g methyl red and 0.16 g methylene blue in 167 ml 95% ethyl alcohol. Mix with a stirring bar without heat.
b. Add 240 g boric acid to 6 L hot distilled water; dissolve with stirring bar & plate set on low heat.
c. Add mixed indicator to boric acid & mix well without heat; solution will be purple in color.

1. **Start up:**

a. Check the level of alkali (40% w/v sodium hydroxide) in the tank on the floor. Fill up if necessary.
b. Make sure that a test tube and a receiving flask are placed in their proper positions and that the safety door is pulled down.
c. Switch on the power
d. Turn on the cold water tap to a flow of about 1.5-2.5 L/min (line up the two black marks).
e. Open the valve for steam by putting the black handle on the front panel in down position.
   - after a couple of minutes steam is generated. Let the distillation continue until some water is collected in the receiving flask (3-4 min). The system is then heated up.
   f. Check that the temperature of the condensate in the receiving flask is below 25-30°C (by touching the flask, it should not be hot). If hot, increase the flow of the cooling water a small amount.
g. Close the steam valve on the front panel, and remove the flask. Remove and empty tube and flask.

When removing the tube, the Teflon pipe through which the steam enters the sample, should be placed in the
front notch of the lower plastic holder. This makes it possible to place the next tube without touching the Teflon pipe, which will have traces of caustic alkali on it.

2. **Sample Distillation:**
   
a. Still wearing gloves, fill the receiving flasks, which have the same number designations as the tubes that will be distilled, with the receiving solution - 25 ml Boric acid solution from above. When measuring out the boric acid, let the blue, neutralized solution in the rubber tube flow out until the color is dark purple. Only dark purple solution should be added to the flasks.

   b. Place the tube with the first diluted sample to be distilled in its position and place the receiving flask containing the receiving solution on the platform. Use flask #1 with tube #1. To avoid contamination don't touch the glass outlet tube with your fingers, hold it by its plastic tubing if necessary.

   c. Pull down the safety door

   d. *Gently and slowly,* pull the alkali handle all the way down to dispense about 50 ml of alkali. If the solution in the test tube does not stay brown, add a small additional amount of alkali. If the solution turns dark blue, that is equivalent to turning brown and is ready to distill.

   f. Open the steam valve and set the timer to the appropriate distilling time, usually 4 min.

   g. After the timer buzzer rings:

      - Push the steam valve up. Let the distillate drain for about 1 minute.

      - Remove the test tube carefully - it is hot and frequently covered with alkali drips. Use two folded paper towels as a hot-mitt. Replace the tube in its stainless steel tube rack.

      - Remove the receiving flask and set aside.

   h. Place the test tube and the receiving flask for the next distillation in place and continue in the same manner until all the samples are done. When removing the tube, the Teflon pipe through which the steam enters the sample, should be placed in the slot of the plastic holder. This makes it possible to replace a tube without touching the Teflon pipe, which will have traces of alkali on it.

3. **Closing down:**
   
a. Place the warm-up test tube, 1/4 filled with water, on the distilling unit, and put an empty receiving flask in place.

   b. Distill for about 3 min., in order to clean out the system.

   c. **Switch off the power.**

   d. **Turn off the water tap**

   e. Remove test tube and receiver flask, pull out the drain trough under the tube holder and the platform for the receiver flask and clean it with water. Wipe the instrument clean from any spillage.
TITRATION:
1. Use 0.1 N HCl to titrate the resulting solutions:
   a. Fill the mounted burette with HCl by rotating the spigot handle back and down. Release the HCl by rotating the spigot handle forward and down.
   b. Put a magnetic stir bar into each flask containing the green boric acid & distillate mixture. Turn the magnetic stirrer to the lowest speed possible.
   c. Slowly add HCl from the buret into the receiving flask containing the boric acid & distillate mixture.
   d. Stop adding HCl when the color goes from green to cobalt blue. Just a drop or two can be enough for it to pass the blue stage and turn purple, which is too much. Read the amount of HCl added from the buret and record that number.
   e. Repeat with the rest of the flasks. Do not bother to re-zero the buret each time, you can calculate by difference how much HCl goes into each flask. If you run through a whole buret and still have more samples, refill the buret.
   f. If you overshoot and the solution turns purple, use a pipette that measures in increments of 0.1 ml and using 0.1N NaOH, count the number of drops that you need to get back to blue. That amount, which should be equivalent to the 0.1N HCl, should be subtracted from the number of HCL mls added in the first place.

CLEANUP!!!!!
1. Wearing gloves pour contents of the tubes down the sink with lots of cold water running. Place the tubes in soapy water and scrub out with a large bottlebrush. Rinse once in tap water and once in distilled water. Hang up on wall rack.
2. Wearing gloves, retrieve the magnetic rods before they go down the sink (and are lost forever down the sink) by using the magnetic rod to remove the magnetic stir bars from the flasks. Put them in soapy water.
3. Pour the liquid contents of the flasks down the drain, with the cold water running, and place the flasks in soapy water. Wash & rinse stir bars and flasks in tap water, then in distilled water. Hang flasks on wall rack.

CRUDE PROTEIN CALCULATIONS:

Normality of acid x .014 = gms N equivalent to 1 ml of acid = 0.1 x 0.014 = 0.0014
MW of N
Protein contains about 16% N; gms N/ml acid x 6.25 = gm protein/ml acid (protein factor) = 0.00875.
% CP = \[ \frac{[(\text{ml HCl} - \text{ml blank}) \times \text{protein factor}]}{\text{sample weight} \times \text{DM\%}} \times 100 \]

gm N = (\text{ml HCL} - \text{ml blank}) \times \text{equivalent of N}.


BSA 0.16 g N = (x) \times 0.00875 \quad x = 18 \text{ ml}

**FREE SIMPLE SUGARS (FSS)**

*formerly known as: WATER SOLUBLE CARBOHYDRATES (WSC)*

*With 12 samples in duplicate, this procedure must be finished within 6 hours*

Reagents:
1. distilled water
2. phenol: \(5 \text{ g phenol}/100 \text{ ml dist. water} – \text{Mix in hood. Can store for months in fridge in a dark-glass bottle.} \)
3. sucrose standard: \(5 \text{ mg purified sucrose}/100 \text{ ml dist. water} = 0.005g = 0.005\% \text{ sucrose} - \text{must mix up fresh each day, just before you will use it (see below)} \)
4. concentrated sulfuric acid - straight out of the bottle.

**I. Extraction:**
1. Weigh out 200 mg (0.2 g) sample, in duplicate, into 125 ml erlenmeyer flasks labeled in numerical order.
2. Add 40 ml distilled H2O - measure with graduated cylinder.
3. Bring to a boil on hot plate, turn down and let simmer 6 min. exactly, swirl several times, gently, while simmering to bring down the particles that go up and get stuck on the sides of the erlenmeyer. Adjust heat or move flasks to the edge of the hot plate – too much heat will cause too vigorous boiling.
4. While waiting for the flasks to boil, prepare filtration system - arrange glass funnels on top of 50 ml graduated cylinders labeled with lab tape (in numerical order of erlenmeyers). Drop a pea-size cotton ball in to funnel and press on hole
4. Filter while still warm - pour into glass funnel aiming carefully for the cotton. Be careful that the particulate residue is trapped by the cotton and does not go into the graduated cylinder.
5. Remove funnels when drained.

6. Add room temperature distilled H2O to filtered solution in graduated cylinder, up to exactly 50 ml.

7. Pour samples into small, plastic bottles. Label the bottles with tape, specifying "FSS" and the sample number. **These extracts must be used within 3 hours** before they ferment and the sugars turned into alcohol and can not be identified by this method.

**CLEAN UP!!!!**

1. The glassware must be set to soak immediately because the particles will cement to the sides of the cylinders and flasks upon drying. Pick out and discard the cotton in the funnels and place the flasks and funnels into a bucket full of warm water to soak. Later wash and scrub them in warm, soapy water. The cylinders can go directly into the soapy water.

**II. Sugar Reaction:**

Using an automatic pipette set for 0.5 ml and the blue pipet tips:

1. Shake each bottle, pipet 0.5 ml sample from the bottles into small, wide-mouthed test tubes (0.3 ml if you suspect high sugar values).

   a. Add 4.5 ml of distilled water to each tube (4.7 ml if you suspect high sugar values).

   b. Vortex using the hand-switch at low speed. There will be one tube per bottle.

2. Then prepare 4" screw cap test tubes in rows of 3, i.e. 3 tubes/bottle.

   For example - for 6 samples in duplicate (12 bottles), a total of 43, 4" tubes will be used = 6 per sample (three per bottle),
   and 4 sucrose standards
   and 3 reagent blanks.

   Label the tubes accordingly. See diagram of a rack of test tubes below.

3. a. Pipet 0.5 ml diluted sample into each designated sample 4" tubes.

   b. Sucrose standard (recipe above): pipet 0.5 ml sucrose solution into 4 tubes.

   c. Reagent blank = pipet 0.5 ml distilled water into 3 tubes.

**4. IN THE HOOD, WEARING GLOVES & lab coat:** TURN FAN ON and add the following reagents:

   a. Add 0.5 ml phenol to two sample tubes using a hand pipetter.

   b. Into the third sample tube, add 0.5 ml distilled water instead of phenol. This will be the sample blank.
c. In addition:
   i.  sucrose standards = add 0.5 ml phenol
   ii. reagent blanks = add 0.5 ml phenol

5. Add 2.5 ml concentrated sulfuric acid with automatic pipetter, to all tubes.
6. Cap and vortex using the hand switch at medium speed.
7. **Wait 1 hour** and then **read at 490 nm** - turn the spectrophotometer on **5 min. before use**
   a. Zero at the start and re-zero every 10-20 samples using concentrated sulfuric acid in a tube.
   b. The sucrose standard should read in the range of **0.4-0.6** on the spec.
   c. **Do your readings within the next hour.** You cannot leave these to be read the next day.
8. If a reading is >0.9, these samples will have to be rerun, starting with a greater dilution above at Sugar Reaction, Step 1 – dilute 0.3 ml sample with 4.7 ml distilled water and continue with steps 2-7. If the readings are still high, start over completely, weighing out only 0.1 g instead of 0.2 g, and possibly use 0.3 ml instead of 0.5 ml as well. **RECORD THE DILUTIONS THAT YOU USE!!!!!!!** and you might want to run samples in triplicate.

**CLEAN UP:**
1. While waiting the one hour for color to develop - scrub all flasks, funnels, and cylinders etc. that are soaking with bottle brushes. Rinse once in tap water, once in distilled water. Hang on wall racks.
2. The contents of the test tubes should be poured into a waste bottle to be taken to Bio. Labs. for disposal. Labeled bottles are in plastic basins under the muffle furnace. Put one bottle in the hood, place a medium sized funnel in the bottle, dumping the tube contents into bottle and place the empty tubes in a bucket full of soapy water. Since most of the tubes you have used are narrow-mouthed for screw-caps, they will not easily fill with water. Hold them under water until they fill with water and sink. When finished dumping, take the bucket to the sink and rinse the tubes in a basin full of tap water and twice in distilled water. Place the tubes, mouth **down**, in the special test tube drying racks.

**III. Calculation:**

\[
\frac{\text{sample abs.} - \text{sample blank} - \text{reagent blank}}{\text{sucrose abs.} - \text{reagent blank}} \times 0.005 \times 100 \text{ (x dilution if done)} = \% \text{ sugar}
\]

references:


V. EQUIPMENT:
   a. balance accurate to 0.0001g
   b. large hot plate
   c. timer
   d. 125 ml erlenmeyer flasks
   e. 50 ml graduated cylinders
   f. small glass funnels
   g. cotton wool
   i. open mouth 3-4" long test tubes for performing dilutions
   j. 4" long screw cap test tubes
   k. test tube racks
   l. automatic pipet adjustable to 0.5 ml, & medium-sized pipet tips
   m. automatic pipetter with appropriate jars for H2SO4
   n. vortex
   o. Spatula, weighing paper, sharpie pen, lab tape, kim-wipes

FREE SIMPLE SUGARS (FSS)

Diluting at the initial extraction step

I. Extraction:
1. Weigh out **200 mg (0.2 g)** sample, in duplicate, into 125 ml erlenmeyer flask labeled in numerical order.
2. Add **80 ml** distilled H2O - measure with graduated cylinder.
3. Bring to a boil on hot plate, turn down and let simmer 6 min. **exactly**, swirl several times, gently, while simmering to bring down the particles that come up and get stuck on the sides of the erlenmeyer. Adjust heat or move flasks to the edge of the hot plate – too much heat will cause too vigorous boiling.
4. While waiting for the flasks to boil, prepare filtration system - arrange glass funnels with a pea-size cotton ball, on top of **100 ml** graduated cylinders labeled with lab tape (in numerical order).
5. Filter while still warm - pour into glass funnel aiming carefully for the cotton. Be careful that the particulate residue is trapped by the cotton and does not go into the graduated cylinder.
6. Remove funnels
7. Add room temperature distilled H2O to filtered solution in graduated cylinder, up to exactly **100 ml**
8. Pour samples into medium-sized, plastic bottles. Label the bottles with tape, specifying "FSS" and the sample number.

9. Proceed with the sugar reaction step above.
**Disposal of Hazardous Material**

The effluent of the building’s sinks is monitored for its pH. We cannot pour strong acids or bases down the sink.

Neutral enough: Neutral Detergent Reagent;
- Contents of the big protein analysis test tubes;
- Contents of the Erlenmeyer flasks resulting from protein analysis;
- Contents of soapy washbasin after use
- Contents of rinse basin after use
- Contents of bottles rinsed for recycling

Too acid or alkaline:
- Full strength 40% NaOH used in protein analysis
- Acid Detergent Reagent
- Concentrated Na2SO4 (sulfuric acid)
- 72% Na2SO4 used in lignin analysis
- Contents of the test tubes from sugar analysis and starch analysis
- Contents of the test tubes after condensed tannins analysis

Each needs to be put in separate bottles with caps and a Hazardous Waste ticket attached, and be placed in one of the three plastic basins on the floor under the muffle furnaces’ counter. This is one Satellite Accumulation Areas (SAA) in the lab. Bottles with acids go in one basin, alcohols or organic solvents in a different basin, and alkali in a third basin. The second SAA is under the fiber analysis machines and is a five-gallon tank for the Acid Detergent effluent and Lignin-used sulfuric acid. Wastes can also be kept, in a bottle in a plastic tray or basin, in one of the hoods, while they are being filled, for a few days.

No matter where a waste is being collected, immediately put on a ticket and immediately enter the contents and check off the hazards. Once a given bottle is full, fill out the **online request pick-up** form on the lab computer:

```
User = \Nancy, password = Welcome!
Open Explorer -> Favorites – select ‘newHazMatPickup’
Name – enter ‘Na’ and then select the full name,
email – enter nc
Select campus Cambridge etc.
Select building Peabody Museum
Principal Investigator – enter Ri
Department – enter H
Waste Pickup – enter 56H
Select Chemical Waste Pickup Request
Type in total number of containers of the same size
Type in the TOTAL volume from the containers of the same size
```
Select container size
After submit, repeat for bottles/containers of a different size, that is, start from the top again.

**EQUIPMENT**

1. Calibrate the Mettler balance each morning before use.
2. Keep the hood window closed to within a few inches of the sash when not working in the hood.
3. The cute little pipettes that look so small and nifty, cost $100-$200 each, depending on their size. Be careful with them, do not over-tighten and do not drop.
4. The small, fritted glass crucibles used in the fiber procedure cost $75 each – please handle with care!
5. Don't throw gloves out with only one use, but don’t use them until they have holes either.

Reagents:

6. Bovine Serum Albumin used in the Radial Diffusion tannin analysis, is not equipment but it needs reminding that it must be kept in the freezer except for the brief time it takes to weigh out the standards.
7. Check the labels of reagents used to see whether they need to be in the freezer, fridge, or room temp.
8. Label all reagents made in the lab with the date they were first mixed up.