DRY AND WET WEIGHT FECAL EXTRACTION – BOILING METHOD

1. Feces can be dried using a lyophilizer, savant rotary evaporator or conventional oven (~50°C to 95°C oven temps have been reported for several hours or until dry; i.e., weight no longer changes). Solar ovens are not recommended due to potential steroid alteration by UV light. Drying times will vary for each technique and even among individual or species samples.

2. Weigh out feces in numbered 16x125 mm glass tubes. Record weight of each sample.

3. The following weights are suggestions:
   Dry:
   Herbivores
   Weigh out 0.1 g (+/- 0.01) of dry powdered feces
   Carnivores
   Weigh out 0.2 g (+/- 0.02) of dry powdered feces

   Wet:
   Herbivores and Carnivores
   Weigh out 0.5 g (+/- 0.01) of wet feces

4. If monitoring extraction efficiency, add appropriate amount of tracer or unlabeled hormone to each tube (see below).

5. Add 4.5 ml of ETOH and 0.5 of dH_2O (90% ETOH) to each tube and vortex briefly.

6. Boil tubes in boiling water bath (96°) for 20 minutes. Keep rack from touching the bottom to prevent splattering of tube contents. Add 100% ETOH as needed to keep from boiling dry.

7. Bring the volume of the extract up to approximately pre-boil levels with 100% ETOH.

8. Centrifuge the samples at 2500 rpm for 20 minutes, making sure the centrifuge is balanced.

9. Pour off the extracts into a second set of identically labeled 16x125 mm tubes.

10. Add 4.5 ml ETOH and 0.5 ml of dH_2O to the original tubes containing the fecal pellets and vortex each tube for 30 seconds.

11. Centrifuge these tubes at 2500 rpm for 20 minutes.

12. Pour off the extract into the second set of tubes containing the first extract.

13. Dry down the second set of tubes under air in a warm water bath.

14. Re-suspend the dried down extracts in 3 ml ETOH, vortex and dry down under air in a warm water bath.

15. Bring up in 1 ml of MEOH and vortex briefly.

16. Pour off extracts into labeled 12x75 mm plastic tubes and store frozen
17. Make dilutions of MeOH extract for sample analysis, unless dilution is <1:10. In those cases, dry down extract under air and reconstitute in 1 ml dilution buffer (this must be done for all parallelism checks).

FECAL EXTRACTION – RACK SHAKER METHOD

1. Steps 1-4 same as above.
2. Add 4.5 ml of ETOH and 0.5 of dH_2O to each tube and vortex briefly.
3. Load samples into a rack shaker. Shake at room temperature for 4 hours or at 4°C overnight (suggested times).
4. Centrifuge at ~2500 rpm for 20 min.

FECAL EXTRACTION – VORTEXING METHOD

1. Steps 1-4 same as above.
2. Load samples onto a multitube vortexer.
3. Vortex each sample at a speed of ~60-70 (fast enough so a good vortex is created; not so fast that the tubes work their way up out of their place), with 1 pulse/sec.
4. Vortex for 30 minutes (suggested time).
5. Centrifuge at ~2500 rpm for 20 min.
6. Follow Steps 9-16 as above.

EXTRACTION EFFICIENCY PROTOCOL

Use of radioactive tracer (most efficient and accurate method)

1. Dilute H_3 or C_{14} labeled hormone to about 2000 dpm/100 µl.

2. Add 100 µl of tracer (H_3 or C_{14}) to all tubes. At the same time add 100 µl to two scintillation vials with same repeater pipet as that used to add tracer to samples.

3. Extract the sample as described previously.

4. Take 100 µl from each sample extract (the 1 ml MeOH extract from step 14 of the extraction protocol) and add to scintillation vials. Do in singlicate, not duplicate.

5. Add 3 ml of scintillation fluid to all vials, including the two vials with tracer. Create 2 blank vials and add only 3 ml of scintillation fluid.

6. Cap all vials firmly and invert to mix the fluid and set aside for an hour.

7. Determine DPM in a Beta Counter.

8. Calculations:
   - Average your blank values (zero values) and subtract this from everything, including your totals.
   - Average totals.
   - Sample x 10 (because is a tenth of the total sample volume)/averaged total value = multiply by 100 to get a percentage.
   - Between 80 and 100 is acceptable. If the percent recovery is over 100 the quench needs to be checked because the color of the extract may be interfering with the counter's ability to read the sample. If the sample is too dark it needs to be diluted out by adding more scintillation cocktail when you run the next recovery.

Use of unlabeled hormone (requires two extractions per sample)

1. Dilute unlabelled steroid in assay buffer to a concentration ~10 times the value of the 50% binding standard concentration at the µl concentration used in each assay (i.e., 50 µl for most assays). This concentration is an estimate. Because samples are diluted before analysis, the concentration added may need to be adjusted. Goal should be to spike samples with enough steroid so that the value falls within the standard range. Best results will be obtained using 'low' concentration samples so that the endogenous concentration falls near the 80% binding portion of the curve.

2. Add cold steroid dilution to all tubes (i.e., 50 µl for most assays) (i.e., spiked sample). Also set aside duplicate sample tubes for each sample with no added hormone (to determine endogenous concentration in unspiked sample) (i.e., unspiked sample).

3. Extract samples as describe previously.

EXTRACTION EFFICIENCY

Definition: A way of defining how efficient or effective the extraction method is at 'extracting' or pulling out metabolites of interest (steroid or protein hormones) from a specific matrix (urine, fecal, blood or saliva). Monitoring extraction efficiency also permits monitoring how consistent the extraction process is from sample to sample.

- Similar to recovery but samples are spiked before extraction.
- Spike each sample with:

  Labeled hormone: ~2000 dpm/100 µl of ^{3}H or ^{14}C radiolabeled steroid

  or

  Unlabeled (cold) hormone: (mass is dependant upon sensitivity of assay system) – add enough mass to read at about 50% binding

- Extract sample as per protocol.

After extraction:

- Determine "Amount Observed":

  Labeled hormone: aliquot 50 µl from the final 1.0 ml extract to scintillation vial and count radioactivity.

  or

  Unlabeled hormone: analyze by RIA or EIA to determine concentration.

- Determine "Amount Expected":

  Labeled hormone: aliquot 100 µl of original stock of radiolabeled steroid to scintillation vial and count radioactivity.

  or

  Unlabeled hormone: dilute standard stock the sample was spiked with to determine concentration.

Results:

Amount Expected = (expected minus background)
Amount Observed = (observed minus background)
% Extraction efficiency = (Amount Observed/Amount Expected)*100