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Laboratory Procedures for Feed Evaluation

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Technical Manual 19

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Introduction

Among the technical constraints limiting livestock productivity, issues related to feed are the major ones owing to biological and economic reasons. Feed cost usually accounts for 60-70% of the total cost of production and the feasibility of livestock enterprise is largely the reflection of the type, quality of feed and the strategy of feeding. Removing/reducing nutritional constraints basically lead to dramatic improvement in livestock production and productivity. Information on feed quality is one of the decision, the support tools, required to provide rational basis to optimize utilization of feed resources, to improve animal production and productivity and ultimately to increase financial return to the producer. It serves as decision support tools to overcome nutritional deficiencies. The takeoff point for efficient utilization of feed resources is the description of the resources with respect to their ability to sustain different types and levels of animal performance. It is important to establish the potential of major feedstuffs and the need for appropriate supplements in order to overcome nutritional deficiencies and raise the level of performance. For reliable feed quality information, use of appropriate feed evaluation techniques and procedures is essential. Among various feed evaluation techniques, laboratory based evaluations have special merit in reducing costs and time to arrive at practical solutions for filed level problems. Several chemical, biological and physical methods of feed evaluation are in use globally.

Adequate dietary analyses of any sort require that the methods employed are relevant to a nutritional classification of the dietary chemical components. For many animal studies the problem of nutrient availability is dominant. Nutrients in feed have been traditionally classified according to chemical composition and presumed structure. The underlying principle of all classifications is the desire to categorize component substances into groups possessing similar nutritional effects and properties. The most common classification is based on nutritive availability or expected digestibility. This procedure attempts to classify substances into groups related to their nutritional character and availability. In this manual, laboratory procedures for some chemical and biological methods of feed evaluation are compiled to facilitate daily routines of animal nutrition laboratories at various levels.

Sampling to Analysis

Sampling of Dry Feed

Digestion trial

- Chop hay to about 3.5cm through 2.5cm screen,
- Weigh and bag hay for individual feeding before digestion trial but after intake level has been established,
- Composite samplings from the bags to comprise not less than 5 kg,
- Dry sampled forage if necessary, but at less than 65^0C,
- Grind in a large (Wiley) mill through 2mm (10 mesh) screen,
- Return all contents of mill to the sample,
- Collect in a large plastic bag and mix, by rolling partially filled bag on floor,
- Sub sample from all parts of the bag to an amount of at least 2 kg. and grind through 1mm (20 mesh) screen, and
- Allow ground material to equilibrate with air overnight before placing in enclosed containers.

Sub sampling

- Spread material on a smooth surface, preferably metal, and divide the pile into quarters,
- Select at least eight increments from all parts equally to obtain sub sample, and
- Sample for collaborative work should be obtained from existing stocks in this manner.

Sub sampling for chemical analyses

- Roll bottle thoroughly to obtain mixing, and
- Insert spatula into different places to obtain sample for analytical work.

Sampling of wet material

Gross sampling

Cattle faeces:

- Mix (by hand or with a mechanical mixer) daily collection thoroughly on a clean surface, quarter, and sub sample 1 kg or 10% of wet weight,
- Store in a plastic container in a freezer, and
- Daily samples may be thawed, compostited, mixed, and sub sampled later.

Sheep faeces:

- Collect cumulative faeces from at least 5 days' collection, and pass through a meat grinder with a 6mm plate,
- Clean grinder, add all contents to be ground mass, mix thoroughly, and sub sample 1 kg,
- A salad copper can be used in place of meat grinder, and
- Store in a plastic container in a freezer.

Silages and fresh forages:

- Pass not less than 2 kg frozen silage through meat grinder with 6mm plate,
- Tie large plastic bag over the end of the grinder to collect ground material,
- Clean grinder and add all contents to groundmass, and mix thoroughly,
- A salad chopper can be used in place of meat grinder, and
- Store bag and contents in a freezer.

Sub sampling and handling of wet samples for laboratory analyses:

- Thaw and empty contents of forage sub samples from the gross sampling onto a clean surface and cover with a large sheet of plastic,
- Mix material with hands under the plastic,
- Quarter and sub sample forage and fill 250ml wide-mouth plastic polyethylene bottle not more than two-thirds full,
- Close bottle with a plastic cap (puncture the cap with a spatula allowing the spatula to remain in place primarily within the bottle),
- Weigh closed container to 1mg and remove an amount of material with spatula equivalent to 0.5 to 2g of DM to a requisite sample container,
- Replace cap-spatula assembly and reweigh bottle,
- Take difference between first and second weights as weight of sample taken,
- Weigh samples for DM determination in conjunction with matter weighed for other determinations, and
- Mix sample contents of bottle between weighing by stirring with spatula.

  - Technique is devised so that the loss of weight in wet sampled material and material adhering to the spatula does not effect the determination of net sample weight,
  - Weighing the cap-spatula assembly as well as the sample bottle and contents before and after removing the sample eliminates this source of error,
  - The cap-spatula assembly is removed after weighing is completed and then replaced with an ordinary cap, and
  - Bottles are refrozen; they may be rethawed , mixed, and sub sampled for further analyses at a later time.

Alternate techniques for handling wet samples

Wet grinding with a Wiley mill

- Grind frozen material through an intermediate Wiley mill with a 2mm (10 mesh) screen, and
- Add charges of dry ice to keep sample frozen.

This procedure is equivalent to the grinding in a meat grinder, but it is not amenable to the handling of large sample.

Oven drying

- Weigh 500g sample into a 18 x 30cm tarred pan and dry at 65°C in a forced draft oven,
- Remove pan and allow to equilibrate with air at room temperature for 24 hours,
- Weigh and calculate yield, and
- Grind dried material through 1mm (about 25 mesh) screen in a Wiley mill.

Grinding through fine screen (30-40 mesh) may tend to induce filtering problems.

Caution:

- *In faeces and silages, loss of nitrogen as ammonia results from oven drying.*
- *In silages, there is also a serious loss of volatile organic acids and caloric value.*
- *Damage of lignin, protein, and carbohydrates can occur in all oven-dried materials, so that true values of individual components may not be obtained.*

Freeze-drying

- Wet sample can be freeze-dried and ground through a Wiley mill.
  - Heat damage is avoided, but losses of nitrogen in the form of ammonia occur in faeces and silages, and
  - Volatile fatty acids are lost in freeze-drying.

Acetone drying (suitable for lignin and other components of fiber and cell wall)

- Weigh 100g of wet ingesta into a 500ml wide-mouth Erlenmeyer flask and add 400ml of reagent grade acetone,
- Shake thoroughly and allow standing with occasional shaking for 1 hour,
- Shake and pour mixed contents into a 10cm fritted-glass, coarse-porosity, Büchner funnel previously tarred to 0.1g,
- Allow to settle before applying suction,
- Suck off excess acetone with vacuum,

- Remove vacuum and add 400ml fresh acetone, while stirring to wash any remaining fiber from Erlenmeyer flask. One washing is sufficient,
- Preparation need not be washed free of pigment,
- Preparation is sucked dry on the filter,
- Allow to air-dry for 24 hours at room temperature,
- If humidity is high, funnels containing fiber may be dried at 40°C for 4 hours in a forced draft oven,
- Weigh funnel plus contents and calculate yield of acetone-dried powder,
- DM determinations should be made on original wet ingesta,
- Dried acetone powders are ground in an intermediate Wiley mill.
- Store in a tightly stoppered container, and
- Calculate oven dry sample weight by multiplying acetone dry sample weight with the following correction factor:

$$\text{OD sample factor} = \frac{\text{DM of wet ingesta}}{\text{Acetone powder yield}}$$

Sample Representatives

If yield is to be measured under field conditions, a minimum of 2% of the area has to be harvested. Aspects of botanical purity, soil contamination, leaf dropping, dead material, etc. will have to be considered both in term of their effect on yield and composition. The most problematic thing of all is to sample what the animals will consume. Under tropical conditions, this will rarely be 100%. It may be less than 60% of the total DM, unless selection is prevented and/then intake not much above maintenance will be expected—often below maintenance. Sampling of the forage to stimulate a certain feeding condition, i.e., stocking rate or level of refusals allowed may include:

Separate analysis of different botanical fractions, varying stubble height, for example, from canopy to ground level, sampling stems and/or "branches" not exceeding a certain diameter, following a docile animal around and sample similar material to what animal eats or having animals fistulated at the oesophagus.

Sample Preparation

Grinding and sub sampling is greatly facilitated if the forages are chopped. This can easily be done with the fresh material either in the laboratory with the chopper or in the field using e.g. a hedge trimmer or big scissors over a sheet. After sub sampling of the chopped material, which has been weighed first, the

sub sample may be put in a paper bag of known weight and dried at a maximum temperature of 60°C—alternatively sun cured. After equilibration in the laboratory and weighing, a 'pre-DM' can be recorded this is necessary when DM yield are computed.

Storage of Samples

During the time between drying and grinding and bottling, samples DM will change with humidity. The time between recording of weight after first drying and grinding should therefore be kept at a minimum for accurate determination of DM yield. Samples may also be affected by mould and insect if stored improperly or be subjected to material loss if stored in loose mesh sacks.

Number of Analysis

Standard practice is often duplications with reruns if duplicate difference exceeds a predetermined value. In some cases, e.g. for DM and ash or when the required degree of accuracy is lower, single determinations may be used. Decisions to pool samples may be made in many instances after a preliminary screening procedure, involving the analysis of only a few samples. This can often greatly reduce the number of samples analysed and the number of different analysis performed.

Laboratory Analysis

Dry Matter (DM) and Moisture

Equipment

- Silica crucibles (or dry bottles),
- Oven set at 100°C, and
- Weighing balance

Procedure

- Dry between 2-5g of (ground) sample, in a crucible, to constant weight at 95-100°C,
- Report loss in weight as moisture on a percentage basis, and
- Moisture determination of feed samples (e.g. wet material such as silage can also be carried out by distillation with toluene.

Ash

Equipment

- Silica or porcelain crucibles,
- Muffle furnace set to reach 600°C,
- Weighing balance, and
- Desiccators

Procedure

- Weigh 2g of sample into a crucible and place in temperature-controlled furnace heated to 600°C for 2 hours,
- Transfer crucible directly to desiccators, cool and weigh immediately reporting % ash to first decimal place, and
- Material whose moisture content has been previously determined can be used in ash determination.
    - Follow the same procedure, and and express as a percentage of DM.

Insoluble and Soluble Ash

Equipment

- Shaking water bath set at 39°C,
- Weighing balance,
- Beakers, Crucible, and
- Oven, Furnace

Procedure

- Weigh accurately no more than 2g of sample into a 250ml beaker,
- Add 50ml water at about 39°C,
- Cover with small plastic bag held in place with a rubber band,
- Incubate at 39°Cfor 1hour with shaking bath at 100 cycles/minute,
- Filter through No. 541 ash less filter paper and wash three times with 50ml water,
- Fold paper to enclose extracted sample, place in weighed silica crucible and dry in oven overnight,
- Place crucible at random in furnace and proceed as for total ash, and
- Calculate insoluble ash as a proportion of the sample DM and soluble assay by difference from total ash.

Note:
For samples that contain a significant proportion of volatile components (e.g. silage some browse materials), DM content must be determined by freeze-drying

Acid Insoluble Ash

Equipment

- Ashing furnace,
- porcelain-crucible, beaker, pH meter, and
- Weighing balance

Procedure

- Weigh in the sample (feed 15g, faeces 5g) to a porcelain-crucible,
- Put them in for ashing at 450°C over night,
- Transfer the ash to a 600ml beaker and add 100ml of 2M HCl,
- Boil for 5 minutes; make sure that you shake the beaker a couple of times,
- Cool down for some minutes,
- Filter through an ash-less filter paper (diameter. 15cm),
- Rinse the beaker with hot deionised water several times to transfer the content to the filter,
- Rinse the sample and the paper (especially the age of the paper) with at least 500ml of deionised water,
- Check pH of the water running through (with a pH-paper or pH meter) to make sure that all of the acid has been washed away,
- Allow the paper to dry out a bit and then transfer the paper to a crucible. If the paper is still wet they should be dried for a while in an oven,
- Ash for about 1 hour at 600°C,
- Remove the ash and take the tare weight of the crucible,

- The difference between the two weights is the acid insoluble ash, and
- Divide with the sample weight and multiply with 100 to get in %.

Crude Protein

Reagents

- Sulphuric acid (concentrated, technical grade, N-free),
- Sodium hydroxide 40% NaOH solution (400 - 450g/lit),
- Methyl red indicator (1g of methyl red in 200ml alcohol) or mixed indicator/boric acid solution,
  - Mixed indicator/boric acid solution,
    - Dissolve 400g of boric acid in about 6lit. of boiled but still very hot deionised water,
    - Make up to 9literand cool.,
    - Add 100ml of bromocresol green solution (100mg in 100ml ethanol) and 70ml of methyl red solution (100mg in 100ml ethanol),
    - Dilute to 10lit. with deionised water and mix carefully.
    - Calculate a blank value by titrating 25ml of boric acid solution with 0.1N sodium hydroxide until a neutral grey colour is obtained,
    - Adjust the boric acid solution accordingly, and
    - Calculate amount NaOH required as: ml 1.0N alkali = ml titre x 40
- Sulphuric acid standard solution (0.1N standard H_2SO_4),
- Sodium hydroxide standard solution (0.1N or 0.5N), and
- Copper sulphate/potassium sulphate mixture (1:5 ratios). Instead of cooper sulphate/potassium sulphate mixture Kjeldahl tablets can be used.

Equipment

- Digestion rack,
- Heating rack and condensers,
- Ventilation system,
- Weighing balance,
- Kjeldahl digestion flasks, 500ml - 800ml capacity,
- Distillation apparatus, and
- Magnetic stirrer

Procedure

- Weigh 0.7 - 2.2g of (ground) samples (at least sufficient so that the amount of N contained is more than 15mg) in Kjeldahl digestion flasks, 500ml - 800ml capacity (always use eye protection),
- Add 10g of mixture of $CuSO_4$ and powdered potasium sulphate (1:5 ratio) or 2 Kjeldahl tablets (called Kjeltabs) and 25ml of sulphuric acid.

Note: *If sample is greater than 2.2g increase H_2SO_4 by 10ml for each g of sample*

- Place flask in inclined position and heat gently until frothing ceases,
- Boil briskly until solution clears and then for about 30 minutes longer,
- Allow to cool (for around 15 minutes), add 250ml of distilled water and then 90ml of 40% NaOH solution without agitation,
- Immediately, connect flask to distilling bulb on condenser of the distillation apparatus with tips of the condenser immersed in standard acid and 5-7 drops of indicator (or mixed indicator/boric acid solution) in receiver, i.e., connect up the distillation apparatus with loops and the 300ml E-flask containing 30ml boric acid indicator solution (methyl red plus methyl blue). (i.e. distill over around 150ml, and titrate this with 0.1N HCl until the colourless end-point is reached. Correct the titre with a blank),
- Rotate flask to mix contents thoroughly then heat until all NH_3 has been distilled (until approximately 150ml of distillate has been obtained),
- Remove receiver, wash tips of the condenser with a little distilled water and titrate excess standard acid in distillate with a standard NaOH solution,
    - If you use mixed indicator/boric acid solution titrate distillate against standard acid solution - see the following calculation:

Calculations:

a. For standard acid and indicator

$$\%N = \frac{[(\text{ml acid} \times \text{N of acid}) - (\text{ml NaOH} \times \text{N of NaOH})] \times 1.4}{\text{weight of sample}}$$

b. For boric acid/mixed indicator

$$\%N = \frac{1.4 \times (\text{ml sample titrant} - \text{ml blank titrant}) \times \text{N of std. acid}}{\text{weight of sample}}$$

$$\%CP = N \times 6.25$$

- Correct for blank determinations on reagents,
- Include standard sample (sample of known N content), and

> Note: *Samples containing volatile nitrogen e.g. faeces should not be dried before N determination.*

- Express as a % of DM.

Ammonia – Nitrogen

Equipment

- Digestion rack,
- Heating rack and condensers,
- Ventilation system,
- Weighing balance,
- Kjeldahl flask, and
- Distillation apparatus

Procedure

- Weigh out around 5g of sample to 3 decimal places,
- Transfer the sample to a kjeldahl flask with the help of a funnel and rinse with de-ionized water,
- Add a couple of squirts (0.4ml) of liquid paraffin and a measure of MgO (around 1.4g),
- Make up to 250ml with de-ionized water,
- Distil over into a beaker containing around 30ml of boric acid indicator solution, shaking the flask carefully a couple of times to dissolve material that may have stock to the side. Check first that the condenser water is turned on,
- Continue the distillation until around 200ml have distilled over,
- Titrate against HCl,
- Multiply the titre by the N-factor (6.25), and divide by the weight of sample, and
- The result is given in %.

Ether Extract

Reagents

- Diethyl ether, anhydrous, and
- Defatted cotton

Equipment

- Fat extraction apparatus,
- Solvent beakers,
- Extraction thimbles,
- Oven,
- Desiccators, and
- Weighing balance

Procedure

- Weigh 1.5-2.0g (W_1) of sample and transfer into a clean thimble previously extracted with ether,
- Cover the sample with defatted cotton,
- Place thimble and sample in sample container and fix under the condenser of the fat extraction apparatus,
- Dry solvent beaker in an oven at 105°C for 30 minutes, cool in a desiccators to room temperature and weigh (W_2),
- Add 30-40ml of diethyl ether to the weighed solvent beaker and place on a condenser with a ring that is hand-tightened as much as possible,
- Turn on the water to cool the condenser,

- Raise the hot plates until they are in contact with the beakers and turn on heaters. Check for ether leaks after the ether starts to boil and condense. The apparatus may be left (with occasional checks) for 8 hours until extraction is complete,
- Lower the heater and allow the thimble to drain empty. Remove the samples and place glass ether reclaiming tubes under the condenser. Replace beaker, raise hot plate and distil ether into the reclaiming tubes. Evaporate the beaker to dryness,
- Remove beakers from hot plates. Allow the beakers to remain in open air under a hood to completely dry the ether, and
- Dry the ether extract in a forced-draft oven at 105°C for 30 minutes. Cool in desiccators to room temperature and weigh (W_3).

Calculation

$$\% \text{ EE (Ether extract) on DM basis} = \frac{(W3\ W2)}{W1 \times DM\%} \times 100$$

where: W_1 = weight of air dried sample
W_2 = weight of empty beaker
W_3 = weight of beaker + ether extract

Neutral Detergent Fiber

Reagents

Prepare 1lit. of neutral detergent solution (NDS) as follows:

- Measure out 1lit. of distilled water,
- Place 18.61g of disodium ethylene diamintetraacetate (EDTA), (dehydrate crystal, reagent grade); and 6.81g sodium borate decahydrate, (reagent grade) in a large beaker,
- Add some distilled water (take from formerly measured 1lit. distilled water) and heat until dissolved,
- Then add to a solution containing 30g sodium lauryl sulphate and 10ml 2-ethoxyethanol (ethylene glycol monoethyl ether), purified grade,
- Place 4.56g of di-sodium hydrogen phosphate (anhydrous, reagent grade) in a beaker,
- Add some distilled water (take from formerly measured 1lit. distilled water) and heat until dissolved,
- Then add to solution containing the other ingredients and add the rest of distilled water
- Use grade acetone that is free from color and leaves no residue upon evaporation, and
- Amylase enzyme solution [2.5% w/v in 60.8ml of 0.1M $NaHPO_4$ and 39.2ml of 0.1M potassium di-hydrogen phosphate (KH_2PO_4)]. Prepare on the day of use.

Equipment

- Fiber digestion apparatus, drying oven, ashing furnace,

- 600ml tall form beakers without spout (or any other suitable beakers), and
- Weighing balance, gooch crucible, vacuum pump, filtering apparatus

Procedure

- Weigh 0.5g of sample ground to pass through 1mm screen (sieve size),
- Place in 600ml refluxing beaker,
- Add in order 50ml NDS, place beaker on refluxing unit and boil,
- Heat to boiling in 5 to 10 minutes,
- Reduce heat as boiling begins, to avoid foaming,
- Adjust boiling to an even level and reflux for 60 minutes, timed from onset of boiling,
- Place hot-weighed Gooch crucible on a filter manifold and rinse with hot water,
- Filter contents into previously weighed Gooch crucible of coarse porosity (porosity 1) using light suction (use low vacuum at first and increase it only as more force is needed),
- Remove vacuum, break up mat, and fill crucible with hot water,
- Filter liquid and repeat washing procedure. If smooth filteration does not occur, add 1-2ml amylase enzyme solution to the crucible and contents, then add about 30ml boiling water. Allow to stand for 5-10 minutes and wash twice with hot water,
- Wash twice with acetone in same manner (for recovery reason acetone washing done separately) and suck dry. Acetone washing can be continued until a clear solution is obtained,
- Dry crucible at 100°C for 8 hours or overnight and weigh. Hot weigh to obtain yield of cell wall,
- Report yield of recovered NDF as percent of cell wall constituent,

$$\%NDF = \frac{(\text{wt crucible + residue}) - \text{wt crucible}}{\text{Oven dry sample wt}} \times 100$$

- Estimate cell soluble material by subtracting the above value from 100.

$$\%\ \text{Cell soluble} = 100 - NDF$$

- Ash residue in the crucible for 3 hours at 500 to 550°C and hot weigh.

Report ash content as ash insoluble in neutral detergent.

$$\%\text{Insoluble ash (NIA)} = \frac{(\text{wt crucible + ash}) - \text{wt crucible}}{\text{Oven dry sample wt}} \times 100$$

Acid Detergent Fiber

Reagent

- Prepare 1lit. of acid detergent solution (ADS) as follows:

Dissolve 20g of cetyl trimethyl ammonium bromide (CTAB) GPR grade or technical grade in 1 litre of 1N H_2SO_4 (1N H_2SO_4 = 49.04g conc. 100% H_2SO_4 for 1 liter).

Equipment

- Fiber digestion apparatus,
- 600ml tall form beakers without spout (or any other suitable beakers),
- Weighing balance,
- Gooch crucible,
- Filtering apparatus, vacuum pump, and
- Drying oven, ashing furnace.

Procedure

- Weigh 0.5g of air dried sample ground to pass through 1mm screen,
- Place in 600ml refluxing beaker,
- Add 50ml of ADS and heat to boiling,
- Heat to boiling in 5 to 10 minutes,
- Reduce heat as boiling begins, to avoid foaming,
- Reflux for 60 minutes from onset of boiling, adjust boiling to a slow even level,
- Place hot-weighed Gooch crucible on a filter manifold and rinse with hot water,
- Filter contents into previously weighed Gooch crucible of coarse porosity (porosity 1) using light suction,
- Break up the filtered mat with a rod, and wash twice with hot water,
- Rinse sides of the crucible in the same manner,
- Repeat wash with acetone until it removes no more colour; break up all lumps with a glass rod so that the solvent comes into contact with all particles of fiber,
- Dry crucible at 100°C for 8 hours or overnight and hot weigh, and
- Calculate ADF

$$\text{ADF\%} = \frac{(\text{wt crucible + dry residue}) - \text{wt crucible}}{\text{Oven dry sample weight}} \times 100$$

Acid Detergent Lignin

Reagent

- 72% Sulphuric acid,
  - Preparation of 72% H_2SO_4 by weight - calculate grams acid and water needed in 1lit. solution by:

$$\text{acid needed (g)} = \frac{100 \times 98.08 \times 12 \text{ moles}}{H_2SO_4 \text{ assay (\%)}}$$

$$\text{water needed (g)} = (1000 \times 1.634) - \text{grams acid}$$

- Weigh amount of water into a 1lit. MCA volumetric flask (with a bulb in the neck) and add the calculated amount of H_2SO_4 slowly with occasional swirling.

> **Caution!** *Flask must be cooled in a water bath (sink) in order to add the required weight of H_2SO_4.*

- Cool to 20°C and check if volume is correct,
  - If volume is too small, take out about 1.5ml and add 2.5ml water. Repeat if necessary.
  - If volume is too large, take out 5ml and add 4.45ml H_2SO_4.
- Meniscus should be within a 0.5cm of calibration mark at 20°C, and
- Hydrobromic acid-reagent grade

Equipment

- Crucible,
- Muffle furnace,
- Drying oven,
- Weighing balance, and
- Filtering apparatus, vacuum pump

Procedure

- Add to the crucible containing ADF sufficient 72% H_2SO_4 (cooled about 15°C) to cover contents,
- Stir, with a glass rod to a smooth paste breaking all lumps (let glass rod remain in crucible), at hourly intervals and refill with H_2SO_4 as acid drains away,
- After three hours filter off remaining acid and wash contents with hot water until free from acid,
- Rinse and remove stirring glass rod,
- Dry crucible at 100°C and hot weigh (W_1), and
- Ignite crucible in muffle furnace at 500°C for 3 hours, and then cool to 100°C and hot weigh again (W_2).

Calculate

$$\% \text{ ADL} = \frac{(W1 - W2) \times 100}{\text{Oven dry sample weight}}$$

$$\% \text{ Cellulose} = \frac{(\text{wt of crucible} + \text{ADF}) - W1 \times 100}{\text{Oven dry sample weight}}$$

$$\% \text{ Acid insoluble ash (AIA)} = \frac{(W2 - \text{wt of crucible}) \times 100}{\text{Oven dry sample weight}}$$

Permanganate Lignin, Cellulose and Silica

Reagent

- Acetone - use grade that is free from color and leaves no residue upon evaporation,

- Acid detergent solution (ADS),
- n-Hexane - technical grade,
- Standard potassium permanganate,
- Dissolve 50g of potassium permanganate (reagent grade) and 0.05g of silver sulphate (reagent grade) in 1lit. of distilled water.

Note: *Keep out of direct sunlight.*

- Lignin buffer solution,
  - dissolve 6.0g ferric nitrate nonahydrate and 0.15g of silver nitrate in 0.1lit. (100ml) of distilled water,
  - combine with 500ml of glacial acetic acid and 5.0g of potassium acetate, and
  - add 400ml of tertiary butyl alcohol and mix thoroughly.
- Combine permanganate solution,
  - combine and mix standard potassium permanganate and lignin buffer solutions in the ratio of 2:1, by volume, before use,
  - unused mixed solutions may be kept for about a week in a refrigerator,
  - solution is usable if purple and contains no precipitate, and
  - old solutions assume a reddish colour and should be discarded.
- Demineralising solution, and
  - Dissolve 50g of oxalic acid dihydrate in 700ml of 95% ethanol.
  - Add 50ml of concentrated (12N) HCl and 250ml of water and mix.
    - 80% ethanol, and
    - add 845ml of 95%ethanol to 155ml of distilled water.
- Hydrobromic acid, reagent grade.

Equipment

- Muffle furnace,
- Weighing balance,
- Pan, glass rods, crucible,
- Filtering apparatus, vacuum pump, and
- Drying oven

Procedure

- Determine ADF according to standard procedure (use a 1g sample except on samples containing a high amount of lignin, i.e., $\geq$ 15%, use 0.5g sample),
- Place previously weighed crucibles, with ADF, in a shallow enamel pan containing cold water to a depth of 1cm,
  - Fiber in crucibles should not be wet.
- Add about 25ml of combined saturated potassium permanganate and lignin buffer solution to the crucibles in the enamel pan containing cold water,
- Adjust level of water in the pan (2-3cm) to reduce flow of solution out of crucibles,
- Using a separate short glass rods for each crucible stir contents to break lump and wet all particles,
- Allow crucible to stand at room temperature (20-25°C) for 90 minutes; add more permanganate solution if necessary,

- Purple colour must be present at all time.
- Suck the crucibles dry and place in a clean enamel pan, do not wash.
- Fill crucible no more than half full with demineralising solution. Demineralising solution may be added directly to crucibles in case filtering is difficult,
- Take care to avoid spilling by foaming,
- After about 5 minutes suck the crucibles dry and refill with demineralising solution,
- Repeat again if necessary, i.e., if solution is brown,
- Treat until fiber is white for 20 - 30 minutes,
- Thoroughly wash contents with 80% ethanol,
- Suck dry and repeat twice,
- Similarly wash twice with acetone and suck dry,
- Dry crucibles at 100°C overnight and weigh (W_1),
- Calculate lignin content as loss in weight from ADF

$$\%\text{Lignin} = \frac{(\text{wt of crucible + ADF}) - W1}{\text{Oven dry sample weight}} \times 100$$

- Ash at 500°C for 3 hours, cool and weigh (W_2).
- Calculate residual ash as difference between this weight and original weight of crucible.

$$\%\text{Ash} = \frac{(W2 - \text{wt of crucible})}{\text{Oven dry sample weight}} \times 100$$

- Calculate cellulose by weight loss upon ashing,

$$\%\text{Cellulose} = \frac{(W1 - W2)}{\text{Oven dry sample wt}} \times 100$$

- To determine silica, add sufficient drops of 48% HBr to moisten all particles in the residue ash (the ashed permanganate lignin or ADF residue). The residue ash is preferable if it is greater than 2% for the sake of greatest value,
- Use no more than 4ml of acid,
- Allow to stand for 1-2 hours. Add more drops of HBr if much red colour forms,
- Suck off excess acid on vacuum and wash with acetone. Do not use water,
- Dry and wash briefly at 500°C, cool and weigh (W_3), and
- Report silica as difference between this weight and original weight of crucible.

$$\%\text{Silica} = \frac{W3 - \text{wt of crucible}}{\text{Oven dry sample weight z}} \times 100$$

Estimating Digestibility

In vitro Digestibility: cellulase enzyme technique

Reagent

Acid-pepsin solution:

- Acid: 0.125M hydrochloric acid (10.7ml HCl of specific gravity 1.13 made up to 1000ml with water), and
- Acid-pepsin for one tube: 0.12g pepsin 1:10,000 (ICN Pharmaceuticals Inc. Cat. No. 102598) dissolved in 25ml 0.125M HCl.

Cellulase-Acetate buffer solution:

- Acetate buffer: 6.8g sodium acetate ($CH_3COONa.3H_2O$) and 2.9ml glacial acetic acid (CH_3COOH) per 1000ml H_2O (final pH 4.6), and
- Cellulase-Acetate buffer for one tube: 0.3g cellulase (Onozuka 35) dissolved in 50ml acetate buffer.

Note: Both the acetate buffer and the 0.125M HCl solutions can be made in bulk.

Quantities required (suggested) per number of tubes:

No. tubes	Vol. acid (ml)	wt of pepsin (g)	Vol. of acetate buffer (ml)	wt of cellulase (g)
1	25	0.12	50	0.3
20	500	2.4	1000	6.0
40	1000	4.8	2000	12.0
60	1500	7.2	3000	18.0
100	2500	12.0	5000	30.0
140	3500	16.8	7000	42.0
180	4500	21.6	9000	54.0
200	5000	24.0	10000	60.0

Equipment

- Weighing balance,
- Tubes, rubber bungs, porcelain crucibles (porosity 1 or 2), pH meter,
- Incubator, and
- Drying oven

Procedure

Day 0 (eg: Thursday) Week 1

- Weigh into 120ml plastic centrifuge tubes in duplicate an amount of air-dry sample of feed that would be approximately 0.5g oven-dry,
- In each run include 3 tubes of a feed sample of known *in-vitro* digestibility, or that has had its cellulase digestibility determined several times, and
- Weigh out 1.0g of feed sample in duplicate into porcelain crucibles, and determine the DM content, and the OM (100 - Ash%) if OMD is being determined.

Day 1 (eg: Friday)

- Add 25ml of acid-pepsin solution to each tube,
- Insert rubber bungs, and
- Incubate for 72 hours (i.e. over the weekend) at 50°C. If possible shake gently twice a day.

Day 4 (eg: Monday) Week 2

- Remove bungs,
- Inject 1.5ml 1M sodium carbonate down the side of each tube,
- Add 50ml cellulase-acetate buffer solution to each tube,
- Check that pH is within range of 4.5 to 4.7. Adjust if this is not the case,
- Insert rubber bungs, and
- Incubate for 48 hours at 50°C. If possible shake gently twice a day.

Day 6 (eg: Wednesday)

- Filter the contents of each tube through dried and pre-weighed sintered glass crucibles (Porosity 1 or 2), and wash the residue and
- Dry crucibles overnight at 105°C.

Day 7 (eg: Thursday)

- Weigh dried crucibles, and
- Ash for 3 hours at 500°C and weigh, if OMD is to be determined.

Note:

- *Either a mechanical shaker which operates continuously or hand shaking twice daily on working days should give adequate results.*
- *1.5ml of sodium carbonate keeps the pH close to 4.6 when the buffer is added. However, this amount may need altering.*
- *Most researchers use porosity 1 crucibles an advantage being that these can also be used for fiber analysis.*

Calculations

$$\% \text{ DMD} = \frac{(\text{DM sample} - \text{DM residue})}{\text{DM sample}} \times 100$$

$$\% \text{ OMD} = \frac{(\text{OM sample} - \text{OM residue})}{\text{OM sample}} \times 100$$

$$\% \text{ DOMD or OMD } \% \text{ DM} = \frac{\text{(OM sample - OM residue)}}{\text{DM sample}} \times 100$$

In vitro digestibility: two stage technique of tilley and terry

Reagents

- Phosphate - bicarbonate buffer
  - Solution-1: 3.7g anhydrous Na_2HPO_4 and 9.8g $NaHCO_3$ dissolved in 1 lit. of distilled water, and
  - Solution-2: 47g NaCl, 57g KCl, 4g $CaCl_2$ and 6g $MgCl_2$ dissolved in 1 lit. of distilled water.

Then, the buffer solution is made by including 10ml of solution-2 in each lit. of solution-1.

> **Note:**
> *Before use, warm to 39°C and bubble CO_2 through the solution using a gas distribution tube until the pH is 6.9.*
> *10 lit. batch can be mixed at one time.*

- Acid - pepsin solution
  - Acid: 28% HCl (280ml concentrated HCl and 720ml H_2O).
  - Pepsin solution: 6g pepsin 1:10,000 (ICN Pharmaceuticals Inc. Cat. No. 102598) dissolved in 250ml of 28% HCl.

> **Note:** *28% HCl can be made as a stck solution.*

Equipment

- Plastic centrifuge tubes, rubber bungs, gas release valves,
- Weighing balance,
- Drying oven,
- Porcelain crucible, beakers, pipette, pH meter, automatic dispenser, thermos flask,
- CO_2 cylinder,
- Laboratory stirrer,
- Incubator, and
- Ashing furnace

Procedure

Day 0 (e.g. Friday) Week-1

- Transfer into 120ml plastic centrifuge tubes in quadruplicate a weighed amount of air dry sample of feed that would be approximately 0.5g oven dry,
- In each run include 4 tubes with no substrate as blanks. Also include 4 tubes of a sample of known *in vitro* digestibility determined several times,

- Weigh out 1.0g of feed sample in duplicate into porcelain crucible, and determine the DM content, and the OM (100 - ash % DM) if OMD is being determined, and
- Warm the phosphate - bicarbonate buffer over the weekend (39°C in a water bath. Use a 5 lit. beaker and keep covered.

Day 1 (e.g. Monday) Week-2

- Ensure all tubes, reagents and containers are warm at 39°C,
- Add 2ml distilled water to each tube with the continuous pipetting outfit to moisten sample (for very finely ground and dense samples, this may need to be done on Monday night using either 2 or 4ml to ensure complete moistening. Otherwise "lumps" may form),
- Adjust the pH of the buffer to 6.9 with CO_2,
- Collect an adequate amount of rumen liquor from two or three sheep (fistulaeted cattle) into a pre-warmed thermos flask. Strain through several layers of muslin into a pre-warmed container. Dilute the strained rumen liquid with the buffer in a ratio of 1:4. Keep the rumen liquid-buffer mixture stirred with a laboratory stirrer and at 39°C, and check that the pH is still 6.9,
- Deliver 50ml of rumen liquor/buffer mixture to each tube with an automatic dispensing machine,
- Insert rubber bungs fitted with gas release valves into each tube, and
- Swirl contents of each tube gently, and then switch on mechanical shaker. Incubation is for 48 hours.

Day 3 (e.g. Wednesday)

- After 48 hours, stop the shaker and remove rubber bungs.
- Inject 1.5ml acid-pepsin solution down the side of each tube with a continuous pipetting outfit. Allow froth to settle and inject a further 1.5ml. Repeat using 2.0ml
- Rinse down sides of tubes with a little distilled water if necessary and replace rubber bungs. Swirl the contents of each tube gently.
- Switch on mechanical shaker and incubate for 48hours.

> **Note:** *Instead of a mechanical shaker/water bath a precision incubator can be used. However, one should ensure that the contents of the tubes are swirled at least twice a day.*

Day 5 (e.g. Friday)

- Remove racks with tubes from water bath trying not to disturb the contents of the tubes,
- Remove the stoppers after scraping and allow to stand still residue has settled (approximately half an hour),
- Scrape the tube walls with a plastic scraper,
- Wash residues into 50ml capacity crucible (silica or porcelain). These should be dried and pre-weighed if DMD is being determined, and
- Dry crucible in oven at 80 - 85°C over the weekend.

Day 1 (e.g. Monday) Week-3

- Weigh the dry crucibles
- Ash at 500°C over night if OMD is being determined and re-weigh.

Calculations

$$\%\text{DM digestibility (DMD)} = \frac{\text{DM sample - (DM residue - DM blank)}}{\text{DM sample}} \times 100$$

$$\%\text{OM digestibility} = \frac{\text{OM sample - (OM residue - OM blank)}}{\text{OM sample}} \times 100$$

$$\%\text{DOMD or OMD\% DM} = \frac{\text{OM sample - (OM residue - OM blank)}}{\text{DM sample}} \times 100$$

No. of tubes	Vol. strained rumen liquid (ml)	Vol. buffer (ml)	wt of pepsin/vol. of 28% HCl (g/ml)
20	200	800	2.4/100
40	400	1600	4.8/200
50	500	2000	6.0/250
60	600	2400	7.2/300
70	700	2800	8.4/350
100	1000	4000	12.0/500

Note: *Always use excess (at least 250ml) of rumen fluid-buffer mixture as this makes dispensing much easier.*

In-sacco Degradability - the Nylon Bag Technique

The nylon bag technique is a simple method of obtaining basic information on degradability of feeds in the rumen. The method gives relative estimates of the proportion of the feed that is rapidly fermented, and the rate of degradation of the relatively insoluble components of the feed.

Note:

- Bags should be prepared from nylon or other synthetic cloth with mesh aperture dimensions of 40 - 50 µm.
- They should be sewn with polyester or nylon thread and should be approximately 140 x 90mm for samples of about 3 - 5g of DM.
- Large bags should be used if large or less dense samples are required; conversely small bags can be used for smaller samples. The size of bag relative to sample size is very important and will depend upon the amount of residue required for analysis.
- The material incubated must be able to move freely within the bags so as to avoid the formation of micro-environments in the bags with resultant poor replication.
- The size of bag and samples may have to be adjusted in accordance with the diameter of the rumen cannulae.
- With bags 140 x 90mm, the diameter of the rumen cannulae should be around 40 - 50mm and within such a cannula it should be possible to incubate 5 - 6bags at once in sheep and to withdraw them at intervals. For cattle larger cannulae and more bags may be used.
- The bags can be washed and reused so long as the holes are not blocked and appreciable deterioration has not occurred.

Pre-requisites

- Use six bags for each feed,
- One bag should be washed under running tap water for 5 minutes to remove soluble and then dried to constant weight at 70°C. The other five bags are placed in the rumen and removed at different times, and
- For each feed, estimates should be obtained by incubating bags in three different animals. Replicates within the animal are not essential. However, under situations where the residue is required for further analysis or as deemed necessary for statistical analysis replication could be essential.

Procedure

- Grind the feed sample (dried) to a standard particle size of 2-3mm (fresh material should be finely chopped),
- An amount of feed prepared in this way (3-5g of DM) is placed in each of six bags which are numbered and identified,
- Close bags using rubber bands,
- Five bags are placed in the rumen so that they are fully immersed in rumen fluid [pre-wetting the bags with water, placing a marble inside the bag, and/or attaching the bags with a nylon cord (about 20cm long) to a flexible rubber hose will ensure that the bags are well immersed in the rumen contents],
- Incubate bags in the rumen (40-60 bags can be incubated at a time in cattle while in sheep, 8 - 10 bags could be incubated at the same time) of a fistulated animal fed roughage supplemented with protein to a level of approximately 8% CP of the diet,
- Remove bags from the rumen at desired time of incubation (4, 6, 12, 24, 36, 48, 72, and 96 hours) after insertion. For forage-based feeds, use above incubation times, but for rapidly digested material more samples may be prepared or removed earlier,.
  - Place the 96-hour sample into the rumen on the morning of day-1 of incubation in each fistulated animal. On the next morning (day-2), insert the 72-hour sample at the same

hour as the day-1 sample. Continue the activity in the same manner until the recommended number of bags to be incubated at a time are reached and then remove all at a time.

- After removing from the rumen, wash gently each bag under running tap water to remove rumen fluid, adhering micro-organisms, and feed particles on the out side. ***Wash until contents are clean,***
- Bags may be further washed with gentle squeezing. However, the procedure must be standardized, and
  - A nylon bag containing a reference material (a feed sample of known degradability) should be included in each animal to facilitate comparisons.
- Dry the bags to constant weight at 70oC and record weight.

Calculations

- Subtract the weight of the nylon bag from the weight of the bag plus residual sample,
- Calculate the loss of material from the bag as a % of the DM placed in the bag, and
- Plot the % loss of material (or material remaining) against time. Then, either by curve fitting or by regression, estimate the intercept. This gives an estimate of the proportion of highly soluble materials. The disappearance from the bag after 48 hours is calculated from degradability at 48 hours.

$$TDMD = 1 - \frac{(Bag + residue) - Bag\ tare\ weight}{Sample\ DM} \times 100$$

hour as the day-1 sample. Continue the activity in the same manner until the recommended number of bags to be incubated at a time are reached and then remove all at a time.

- After removing from the rumen, wash gently each bag under running tap water to remove rumen fluid, adhering micro-organisms, and feed particles on the out side. ***Wash until contents are clean,***
- Bags may be further washed with gentle squeezing. However, the procedure must be standardized, and
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$$TDMD = 1 - \frac{(Bag + residue) - Bag\ tare\ weight}{Sample\ DM} \times 100$$

Quantification of Tannins

Extraction of Tannins

Procedure

- Weigh 200mg of finely ground (about 80 mesh) leaf sample in a beaker of 20ml capacity,
- Add 10ml of 70% aqueous acetone to the beaker,
- Put the beaker in an ice bath and subject the suspended leaf sample to ultrasonic treatment for 3 minutes,
- Let the contents of the beaker cool for about 2 minutes (ultrasonic treatment produces heat; tannins may get inactivated at high temperature),
- Repeat the above ultrasonic treatment 3 times for a total period of 12 minutes (In a preliminary study, the ultrasonic treatment was done for 3, 6, 9, 12, 15 and 20 minutes; the recovery of soluble proanthocyanidins did not increase beyond 12 minutes of the treatment),
- Centrifuge the contents (preferably at 4°C) for 20 minutes at about 3000g, and
- Collect the supernatant in a glass tube and keep it on ice.

Notes:

For leaf samples (200mg, 80 mesh in size, in 10ml of 70% aqueous acetone), shaking in water bath (30°C, 139 cycles/minute with 4.2cm amplitude) for 2 hours extracted the same amount of total phenols and proanthocyanidins as 12 minutes of sonication.

1. At 20, 40, and 60 minutes of shaking, the recovery was lower and did not reach a plateau, and at 90 minute the recovery was almost at the plateau.
2. For seed samples (ground to pass a 1mm sieve), the pattern of recovery of both total phenols and proanthocyanidins using the shaker was similar to that for forage samples, but the recoveries of both total phenols and proanthocyanidins using 12 minute sonication were lower as compared to shaking for 2 hours.
3. Lower recovery from seed samples using the sonicator could be due to the larger particle size of seeds, and the relatively low power output of the sonicator.

Conclusion: *For forage samples, the tannins can be extracted using 12 minutes sonication or by using a shaker. For seed samples, tannins should be extracted using a shaker.*

Using 50% aqueous methanol, the recovery of total phenols and proanthocyanidins was 20 to 60% lower than when using 70% aqueous acetone.

Pigments and fat from the sample can be removed by extracting with diethyl ether containing 1% acetic acid before extracting tannins.

Determination of Tannins

Reagents

i. Folin Ciocalteu reagent (1N):

- Dilute 1:1 the commonly available Folin Ciocalteu reagent (2N) with distilled water,
- Keep in refrigerator (4°C),
- It should be golden in color, and
- Do not use it if it turns olive green.

ii. Sodium carbonate (20%):

- Weigh 40g of Na_2CO_3, and
- Dissolve it in about 150ml distilled water and make it up to 200ml with distilled water.

iii. Insoluble polyvinylpyrrolidone (polyvinyl polypyrrolidone, PVPP): *commercially available*

iv. Standard tannic acid solution (0.5mg/ml):

Dissolve 25mg tannic acid (TA) in 50ml of distilled water *(always use a freshly prepared solution).*

Preparation of the calibration curve:

	TA sol. (ml)	Dist. H_2O (ml)	Folin (ml)	Na_2CO_3 (ml)	A_{725nm}	µg TA
B	0	1.00	0.50	2.50	0	0
T1	0.02	0.98	0.50	2.50	0.236	10
T2	0.03	0.97	0.50	2.50	0.365	15
T3	0.04	0.96	0.50	2.50	0.477	20
T4	0.05	0.95	0.50	2.50	0.596	25
T5	0.06	0.94	0.50	2.50	0.698	30

Analysis of total phenols:

- Take suitable aliquots of the tannin containing extract (initially try with 0.05, 0.2 and 0.5ml) in test tubes, make up the volume to 1.0ml with distilled water, add 0.50ml of the Folin reagent and then 2.50ml of the sodium carbonate reagent,
- Vortex the tubes and record absorbance at 725nm after 40 minutes,
- Calculate the amount of total phenols as tannic acid equivalent from the above calibration curve, and
- Express total phenolic content on a DM basis (x%).

Example:

50µl tannin-containing extract in the assay mixiture gives 0.564 absorption = 24µg tannic acid (TA) equivalent (from the standard curve).
Standard curve for tannic acid using Folin-Ciocalteu reagent (sigma):

$$y = 0.023x + 0.012, r = 0.999$$

where: y = absorbance (725nm)
x = tannic acid (µg)
1ml extract = 24 x 20 = 480µg tannic acid (TA) = 0.48mg TA
20mg leaf or seed = 0.48mg TA (since 200mg leaf or seed was extracted in 10ml)
100mg leaf or seed = 0.48 x 5 = 2.4mg TA
x = 2.4/0.95 (since DM of feed or seed = 95%)
x = 2.53%

Removal of tannin from the tannin-containing extract:

The PVPP binds tannins.

- Weigh 100mg of the PVPP in a 100 x 12mm test tube,
- Add 1ml distilled water and then 1ml of the tannin-containing extract (100mg PVPP is sufficient to bind 2mg of total phenols; if total phenolic content of feed is more than 10% on a DM basis, dilute the extract appropriately),
- Vertex it,
- Keep the tube at 4°C for 15 minutes, vertex it again, then centrifuge (3000g for 10 minutes) and collect the supernatant. This supernatant has only simple phenolics other than tannins (tannins would go along with the PVPP),
- Measure the phenolic content of the supernatant as mentioned above (take at least double the volume preferable three times) you used for previous total phenol estimation, because you have already diluted the extract two-fold and expect to lose tannin-phenols through binding with PVPP, and
- Express the content of non-tannin phenols on a DM basis (y%).

Example:

150µl of the supernatant after PVPP treatment in the assay mixture gives 0.274 absorption = 11.34µg tannic acid (TA) equivalent (from the standard curve).

1ml supernatant = 11.34 x 1000/150 = 75.9µg TA = 0.076mg TA

10mg leaf or seed = 0.076mg TA (since the extract is diluted two-fold)

100mg leaf or seed = 0.076 x 10 = 0.76mg TA

y = 0.76/0.95 (since DM of feed or seed = 95%)
y = 0.80%

(x - y) is the percentage of tannins as tannic acid equivalent of a DM basis.

Tannins (as tannic acid equivalent) = 2.53 - 0.80 = 1.73% in the DM

Determination of Condensed Tannins

Reagent

- Butanol - HCl reagent (butanol - HCl 95:5 v/v),
  - Mix 950ml of n-butanol with 50ml concentrated HCl (37%).
- Ferric reagent (2% ferric ammonium sulphate in 2N HCl),
  - Bring 16.6ml of concentrated HCl up to 100ml with distilled water to make 2N HCl,

- Dissolve 2g ferric ammonium sulphate in this volume of 2N HCl, and
- Store in a dark bottle.

Procedure

- Add 0.50ml of the tannin-containing solution that contain a suitable amount of tannins (absorbance at 550nm should not exceed 0.6 after addition of the reagents and heating; if absorbance is >0.6 dilute the solution with 70% acetone, record the dilution factor and repeat the analysis) to a 100 x 12mm glass test tube pipette,
- Add 3.0ml of the butanol-HCl reagent and 0.1ml of the ferric reagent,
- Vortex the tubes,
- Cover the mouth of the tube with a glass marble and put the tubes in a heating block adjusted at 97-100°C for 60 minutes,
- Cool the tube and record absorbance at 550nm against a suitable blank (unheated mixture; a pink colour is produced in the blank if the extract has flavan-4-ols. In such a case, use a blank comprised of 0.5ml of the extract, 3ml of butanol and 0.1ml of the ferric reagent), and
- Condensed tannins (% in DM) as leucocyanidin equivalent is calculated by the formula:

$$\frac{(A550nm \times 78.26 \times \text{Dilution factor})}{(\%DM)}$$

where, Dilution factor = the additional dilution used if the absorbance exceeded 0.6 (e.g. when the extract is from 200mg sample in 10ml solvent, the dilution factor is 1)

Note:
Vanillin-HCl is also used for determination of condensed tannins but Vanillin-HCl method is not specific. It measures condensed tannins as well as simple flavonoids.

Protein Precipitation Methods for Determination of Tannins

The above methods for quantification of tannins are based on chemical properties of tannins. The method for total tannins is based on oxidation-reduction principles (tannins are reducing agents) and that for condensed tannins is based on oxidative de-polymerization of condensed tannins (proanthocyanidins) to anthocyanidins in butanol-HCl-Fe^{+++} mixture. Tannins are also determined in terms of their protein binding property. The advantage of protein binding/precipitation methods is that they measure potential biological activity of tannins in feeds. Whereas, the methods that based on chemical properties only indicate the chemical nature of tannins present. In order to get meaningful results both the chemical and protein binding/precipitation methods should be employed.

Extraction of tannins from plants for determination of protein precipitation capacity

The method is similar to that described above in "Extraction of tannins" section except that 50% aqueous methanol is used in place of 70% aqueous acetone, as acetone interferes in protein precipitation assays.

Bovine serum albumin-dye method for determination of protein precipitation capacity

Preparation of BSA-dye complex

Reagents:

- Prepare 5 litres of acetate buffer (0.2M, pH 4.8 - 4.9) by diluting 57ml of glacial acetic acid with about 4 litres of distilled water.
    - Adjust its pH to 4.8 - 4.9 by adding approximately 2M NaOH (8g in 100ml), and make the volume to 5 litres with distilled water.
- Dissolve 1g of sodium bicarbonate in 100ml distilled water,
- Dissolve 2g of BSA (Sigma) in 40ml of the 1% sodium bicarbonate solution. Add 150mg of Remazol brilliant blue die (Sigma R 8001) to the BSA solution. Stir gently for 30 minutes at room temperature,
- Transfer the BSA-dye into a dialysis bag (12-14,000MW cut off), and dialyze against 1 litre of the acetate buffer (prepared in step 1) overnight in a cold room or refrigerator (4°C). Discard the acetate buffer, re-dialyze overnight with fresh buffer. Discard the buffer again, and dialyze for a third time with fresh acetate buffer overnight. Dilute the 40ml of the dialyzed blue BSA to 1 litre (exact) with the acetate buffer (2mg BSA/ml). Store this in the cold at 4°C, and
- Isopropanol-SDS-TEA reagent. Mix 200ml of isopropanol with 50ml of triethanolamine and 10g sodium lauryl sulphate (sodium dodecyl sulphate, SDS), and make the volume to 1 litre with distilled water. Store it at room temparature.

Conversion of the blue colour to the BSA precipitated:

- This conversion is needed for calculating the amount of protein precipitated by tannins. A standard curve is needed for this conversion. The protein precipitated by tannins is dissolved in isopropanol-SDS-TEA reagent (IST), therefore this calibration is also done in this solution.

ml Dye	mg BSA	ml IST	A_{590nm}*
0.1	0.2	2.9	0.063
0.2	0.4	2.8	0.128
0.3	0.6	2.7	0.188
0.4	0.8	2.6	0.247
0.6	1.2	2.4	0.362
0.8	1.6	2.2	0.478
1.0	2.0	2.0	0.591
1.5	3.0	1.5	0.858

**for a typical batch (it could vary from batch to batch)*

Relationship between the blue colour of BSA-dye complex and its BSA content:

$$y = 0.284x + 0.016,\ r = 1.000$$

where, y = absorbance (590nm)
x = BSA (mg)

Protein precipitation capacity using the BSA-dye complex:

To the BSA-dye (2ml) containing 2mg/mlBSA,

- Add 50% methanol and increasing levels of the tannin-containing extract (see section "Extraction of tannins from plants for determination of protein precipitation capacity" above) to make 3ml,
  - For example: use 0.95, 0.90, 0.85, 0.80, 0.75, 0.70ml of 50% methanol and then 0.05, 0.10, 0.15, 0.20, 0.25, 0.30ml of the extract; this may vary depending on the amount of tannins in the sample.
- Vortex the contents,
- Allow the mixture to stand in the refrigerator (4°C) overnight,
- Centrifuge at about 3000g for about 10 minutes,
- Remove the supernatant carefully without disturbing the blue precipitate,
- Add 3ml of isopropyl alcohol-SDS-TEA reagent to the precipitate and vortex it to dissolve the precipitate,
- Record the absorbance at 590nm,
- Calculate the amount of protein precipitated from the calibration curve,
- Draw a linear regression between BSA precipitated and mg leaf in the aliquot taken for the assay,
- The slope of the curve (mg BSA precipitated/mg leaf)represents the protein precipitation capacity, and
- Avoid expressing the results as tannic acid equivalent, as tannic acid from different commercial sources behave differently towards tannin assay.

Example (*Acacia cyanophylla*)

µl taken for assay	mg leaf[1]	A_{590nm}[2]	mg BSA pptd
25	0.5	0.090	0.26
50	1.0	0.153	0.48
75	1.5	0.219	0.72
100	2.0	0.290	0.97
150	3.0	0.425	1.44
175	3.5	0.522	1.78
200	4.0	0.572	1.96
250	5.0	0.698	2.40
300	6.0	0.804	2.77

[1] *200mg leaf is extracted in 10ml 50% aqueous methanol*
[2] *conversion of absorbance at 590nm to mg BSA pptd*

Protein precipitation of *Acacia cyanophylla* using the BSA-dye complex method:

Protein precipitation capacity = 0.469mg BSA pptd/mg leaf

$$y = 0.469x + 0.038,\ r = 0.998$$

where, y = BSA pptd (mg)
x = leaf weight (mg)

Simultaneous determination of protein precipitation capacity of tannins and protein-perceptible phenolics

Reagents:

- Acetate buffer (pH 4.8 - 4.9, 0.2M). Its preparation is described above in " Bovine Serum Albumin-Dye method for determination of protein precipitation capacity" section,
  - Add NaCl to it to make its concentration 0.17M.
- Sodium dodecyl sulphate (SDS) 1% (w/v) in distilled water,
- SDA-triethanolamine solution [1% SDS (w/v) and 7% (v/v) triethanolamine in distilled water],
- Ferric chloride reagent (0.01M ferric chloride in 0.1M HCl). For making 0.1M HCl, dilute 4.2ml concentrated HCl up to 500ml water. Dissolve 0.81g ferric chloride in 500ml of 0.1M HCl. Filter and store the contents in a brown bottle,
- NaOH (13.5N). Mix 54g of NaOH in about 80ml of distilled water and make the volume to 100ml with distilled water,
- Glacial acetic acid,
- BSA standard solution (1mg/ml in distilled water). For protein determination in the tannin-protein complex by ninhydrin assay,
- Ninhydrin reagent,
  - Dissolve 0.16g $SnCl_2.2H_2O$ in 100ml of the citrate buffer [0.2M, pH 5 (i.e. dissolve 4.2g of citric acid monohydrate in about 80ml of distilled water, adjust the pH to 5.0 with concentrated NaOH, make the volume 100ml with distilled water)].To this solution add 4g of ninhydrin dissolved in 100ml of methyl cellosolve. This reagent can be stored in a dark coloured bottle in the cold (4°C) for about a month.
- Dilute solvent. Mix equal volume of distilled water and normal-propanol (propan-1-ol). Distilled water can also be used as a diluent solvent if ninhydrin is dissolved in cellosolve instead of methyl cellosolve in the above step.

Formation of tannin-protein complex:

To the BSA-dye (2ml) containing 1mg/mlBSA

- Add 50% methanol and increasing levels of the tannin-containing extract (see section "Extraction of tannins from plants for determination of protein precipitation capacity" above) to make 3ml,
  - For example: use 0.95, 0.90, 0.85, 0.80, 0.75, 0.70ml of 50% methanol and then 0.05, 0.10, 0.15, 0.20, 0.25, 0.30ml of the extract; this may vary depending on the amount of tannins in the sample.
- Vortex the contents,
- Allow the mixture to stand in the refrigerator (4°C) overnight,
- Centrifuge at about 3000g for about 10 minutes,
- Remove the supernatant carefully without disturbing the blue precipitate, and
- Add 1.5ml of 1% SDS solution to the precipitate and cortex it to dissolve the precipitate.

Determination of tannin in the complex:

- Take an aliquot (1ml) of the above dissolved complex, and to it add 3ml of SDS-triethanolamine solution,
- Then add a 1ml portion of the ferric chloride reagent,
- Record absorbance at 510nm after 15 - 30 minutes, and
- Convert the absorbance to tannic acid equivalent using a standard curve.

Standard curve for tannic acid using $FeCl_3$ reagent:

$$y = 2.085x + 0.008, r = 0.999$$

where, y = absorbance (510nm)
x = tannic acid (mg)

- Multiply the value obtained by 1.5 to obtain tannins in the complex,
- Draw a linear regression between tannins precipitated as tannic acid equivalne and mg leaf (in aliquot taken for the assay), and
- The slope of the curve (mg tannic acid precipitated/mg leaf; let it be x) represents the protein precipitable phenolics.

Example (*Acacia cyanophylla*)

µl taken for assay	mg leaf[1]	A_{510nm}	mg TA[2]	mg TA in complex[3]
100	2	0.121	0.054	0.081
150	3	0.167	0.077	0.116
200	4	0.234	0.109	0.164
250	5	0.292	0.136	0.204
300	6	0.341	0.160	0.240
350	7	0.422	0.199	0.299
400	8	0.472	0.222	0.333
500	10	0.591	0.280	0.420

[1] *200mg leaf is extracted in 10ml 50% aqueous methanol (see section "Extraction of tannins from plants for determination of protein precipitation capacity")*
[2] *conversion of absorbance at 510nm to mg tannic acid by the standard curve (see below)*
[3] *obtain by multiplying values in the previous column (which correspond to 1ml of the soluble tannin-protein complex) by 1.5, because the tannin-protein complex is dissolved in 1.5ml of 1% SDS*

Protein perceptible phenolics (x, mg tannic acid equivalents pptd/mg leaf DM) for the above example = 0.043/0.9535 = 0.045
since DM of the leaves was 95.35%

Protein perceptible phenolics = 0.043mg tannic acid equivalents pptd/mg leaf

$$y = 0.043x - 0.009, r = 0.999$$

where, y = tannic acid pptd (mg)
x = leaf weight (mg)

Determination of total phenolics:

- Take different aliquots (generally 0.05, 0.10, 0.15, 0.20, 0.25, 0.30ml, but this may vary depending on the amount of phenolics in the sample) of the extract (see section "Extraction of tannins from plants for determination of protein precipitation capacity"), make it up to 1ml with 1% SDS, and add to it 3ml SDS-triethanolamine solution and 1ml ferric chloride reagent,
- Record absorbance at 510nm as described above,
- Convert the absorbance to tannic acid equivalent using the standard curve,
- Draw a linear regression between tannic acid equivalent and mg leaf (in the aliquot taken), and
- The slope of the curve (mg tannic acid equivalent/mg leaf; let it be y) represents total phenolics.

Example (*Acacia cyanophylla*)

µl taken for assay	mg leaf[1]	A_{510nm}	mg tannic acid equivalent
50	1	0.145	0.066
100	2	0.280	0.131
150	3	0.404	0.190
200	4	0.532	0.251
250	5	0.674	0.319
300	6	0.824	0.391

[1] *200mg leaf is extracted in 10ml 50% aqueous methanol (see section "Extraction of tannins from plants for determination of protein precipitation capacity")*

Total phenolics (y; mg tannic acid equivalent/mg leaf DM) = 0.064/0.9535 = 0.067
since DM of the leaves was 95.35%
Therefore, protein precipitation phenolics as percent of total phenolics = (x/y)100 = (0.045/0.067) x 100 = 67.2%.

Total phenolics = 0.064mg tannic acid equivalent/mg leaf
$y = 0.064x - 0.000$, $r = 0.999$
where, y = tannic acid (mg)
x = leaf weight (mg)

Preparation of calibration curve:

	TA sol* (ml)	1% SDS (ml)	SDS_TEA (ml)	$FeCl_3$ (ml)	A_{510nm}	TA (mg)
B	0	1.0	3.0	1.0	0	0
T1	0.1	0.9	3.0	1.0	0.107	0.05
T2	0.2	0.8	3.0	1.0	0.225	0.10
T3	0.3	0.7	3.0	1.0	0.319	0.15
T4	0.4	0.6	3.0	1.0	0.426	0.20
T5	0.5	0.5	3.0	1.0	0.527	0.25

** tannic acid solution (TA sol), 0.5mg/ml in 1% SDS*
Note: Mention the commercial source of tannic acid used, as tannic acid from different commercial sources behave differently towards tannin assay (Makker and Becker, 1993)

Determination of protein in the complex:

The method is based on hydrolysis of protein-tannin complex under alkaline conditions and determination of released amino acids by the ninhydrin reaction. The prtein in the complex can not be determined by the Lowry or Bradford assay as the presence of tannins/phenolics interfere in the determination of protein in these assays.

- Take an aliquot (0.1ml) of the dissolved complex (seé above) in a test tube and dry in an oven at about 80°C,
- Add to it 0.3ml of NaOH (13.5N), and hydrolyze the protein at 120°C for 20 minutes in an oven,
- Allow the tubes to cool, and add to each tube 0.5ml of glacial acetic acid very slowly to neutralize the alkali,
- Add 1ml of the ninhydrin reagent, cover the mouths of the tubes with marbles, and heat the mixture in a boiling water bath for 20 minutes,
- Cool the tubes, and add 10ml of distilled water or 50% propanol,
- Read the absorbance at 570nm, and
- Determine the protein content from the standard curve prepared againest BSA (20 - 100µg, obtained by taking 20-100µl of a 1mg/ml solution of BSA in distilled water) which had been dried and hydrolyzed along with the samples.

The protein precipitating capacity is determined from the regression coefficients (slope) of linear regression fitted to measurements performed at different concentrations. The results are expressed as mg BSA precipitated/mg leaf (z)

Example (*Acacia cyanophylla*)

µl taken for assay	mg leaf[1]	A_{570nm}	mg BSA[2]	mg BSA in complex[3]
50	1	0.140	0.0128	0.192
100	2	0.277	0.0265	0.398
150	3	0.460	0.0451	0.677
200	4	0.640	0.0649	0.974
250	5	0.797	0.0812	1.218
300	6	0.944	0.0965	1.447
350	7	1.10	0.110	1.65

[1] *200mg leaf is extracted in 10ml 50% aqueous methanol (see section "Extraction of tannins from plants for determination of protein precipitation capacity")*

[2] *conversion of absorbance at 570nm to mg BSA by the standard*

[3] *obtained by multiplying values in the previous column (which correspond to 0.1ml of the soluble tannin-protein complex) by 15, because the tannin-protein complex is dissolved in 1.5ml of 1% SDS*

Protein precipitation capacity (z= mg BSA pptd/mg leaf) for the above example = 0.252/09535 = 0.264, *since DM of the leaves was 95.35%*

Specific activity of tannins (mg BSA precipitated/mg tannin) = z/x
= 0.264/0.045
= 5.87

Protein precipitation capacity = 0.252 mg BSA pptd/mg leaf

$$y = 0.252x - 0.070, r = 0.999$$

where, y = BSA pptd (mg)
x = leaf wt (mg)

Determination of condensed tannins (proanthocyanidins) bound to feed and their fiber fractions

Reagents

- Butanol-HCl reagent (butanol-HCl 95:5 v/v): mix 950ml of n-butanol with 50ml concentrated HCl (37%), and
- Ferric reagent (2% ferric ammonium sulphate in 2N HCl): Bring 16.6ml of concentrated HCl up to 100ml with distilled water to make 2N HCl. Dissolve 2g ferric ammonium sulphate in 100ml of 2N HCl. This reagent should be stored in a dark bottle.

Determination of condensed tannins in NDF and ADF

Procedure:

Prepare NDF and ADF

- In a 100 x 12mm glass test tube weigh 10 - 60mg of NDF or ADF,
- Add 0.5ml of 70% aqueous acetone to the tubes followed by 3.0ml of the butanol-HCl reagent and 0.1ml of the ferric reagent,
- Vortex the tubes,
- Cover the mouth of the tube with a glass marble and put the tubes in a heating block adjusted to 100°C for 60 minutes,
- Swirl the tubes gently after about every 15 minutes,
- Cool the tubes,
- Centrifuge the contents, and
- Record the absorbance of the supernatant at 550nm against a suitable blank (unheated mixture).

Condensed tannins (% in NDF or ADF on DM basis) as leucocyanidin equivalent is calculated by the formula:

$$\frac{A550nm \times 782.6}{(\text{weight of NDF or ADF in mg})(\%\text{DM of NDF or ADF})}.$$

If A550 is > 0.6, dilute and record the absorbance.

Determination of bound condensed tannins

The extractable tannins are removed following ultrasonic and centrifugation. The remaining tannins in the residue (bound tannins) are measured using the

following procedure, in which the residue is washed twice with aqueous acetone and freeze-dried.

- To the residue add about 5ml of 70% aqueous acetone,
- Vortex and centrifuge (3000g) for 10 minutes,
- Discard the supernatant,
- Again add about 5ml of 70% aqueous acetone,
- Vortex and centrifuge (3000g) for 10 minutes and discard the supernatant,
- Lyophilize the residue, and
- Weigh 10-60mg of the residue, and measure the condensed tannins in the residue as described above for condensed tannins in NDF and ADF.

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