Chapter 2
Collecting, Processing and Storage of Plant Materials for Nutritional Analysis

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Introduction

A solid sampling strategy for plant material is the first step in screening forages for nutritional analysis and extra-nutritional attributes to determine if potential forage species, with good adaptation and biomass production are suitable for use as livestock feeds. Since the morphological phenotype is rarely a good indicator of nutritional traits, nutritional analysis is essential when selecting plants as feeds. It is not possible to select forages based solely on biomass production without taking into account the nutritional and anti-nutritional attributes. Some species with leafy and high productivity may contain plant secondary metabolites that may be toxic and make them unsuitable for use as feeds. A good example of this is *Leucaena*, which is fast growing and yields up to 15 tons/ha of forage dry matter per year, but because of the mimosine in the leaves could initially only be fed in quantities up to 30% of the diet without causing toxicity symptoms. This was not apparent from looking at the plant and emphasizes the need to do a thorough nutritional evaluation before introducing new species as livestock feeds. However, identification of mimosine degrading rumen microbes now allows livestock to consume larger quantities [5] and makes this both a productive and nutritionally useful forage plant in many tropical livestock systems.

Sample Collection

Sampling strategies for assessment of nutritional attributes must consider plant diversity and replication. Not all plants are identical and considerable diversity occurs even within species in nutritional traits, giving the potential to select superior genotypes with both high yield and good nutritional attributes. In addition,

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some nutritional traits are also influenced by environment, plant age, sampling environment and time of sampling causing variation between samples from the same genotype or even within the plant. A good sampling strategy considers all these factors and aims for uniformity in sampling protocols so that environmental effects can be minimised and the true nutritional and extra-nutritional traits can be analysed. Several issues need to be taken into consideration when designing sampling strategies.

Diversity Within a Species

A large amount of diversity in nutritional traits and level and type of plant secondary metabolites has been observed within samples of genotypes from the same species whether grown at one location or collected from different geographical locations. These differences can be quite substantial and therefore it is important to account for the diversity and test samples from different genotypes within a species before drawing conclusions about its nutritional attributes. The tendency is to provide information at the species level, while in fact it would be more useful to provide this information at the variety or genotype level. A study on *Sesbania sesban* to determine influence of accession, environment and individual tree within an accession on nutritive value concluded that nitrogen, neutral detergent fibre, in vitro true digestibility, lignin content and polyphenolic compounds all differed significantly between accessions and sites [4].

Genotypic diversity is often seen within an accession of forage germplasm because sampling is either random or representative individuals showing phenotypic diversity are sampled from within the population at the time of plant collection to capture maximum diversity within the accession. Such accessions can include mixed genotypes. Some mixtures may show differences in agro-morphology while diversity in other traits may only show during laboratory analysis. The optimum way to ensure that all diversity within the accession is represented is to use large numbers of plants so that there is a high probability that genes in low frequency will be maintained [1]. However, using large numbers of plants will make sampling more time consuming and expensive and usually a balance has to be struck between capturing maximum diversity within the sample and practical issues involved in the screening programme. In order to capture diversity within the sample, it is recommended that leaf material be collected from a minimum of 10 plants and preferably 25 plants within each accession.

Physiological Age of Plants and Leaves

The chemical composition of leaves and pods of many forage types is transient owing to rapid biochemical changes occurring during the maturation process. Therefore, physiological age of the plant or plant part will often have a major effect on nutritional and extra-nutritional attributes. Nutritional quality deteriorates as the leaf to stem ratio reduces and the plant ages. Comparison of nutritional quality

among accessions should be undertaken at the same physiological age to provide meaningful data. Taking Napier grass as an example, trebling the time interval between cuts doubled yield, but halved the crude protein and leaf to stem ratio. The same is true for fodder trees where older leaves are less nutritionally useful. Genotypic differences can be clearly seen when age differences are controlled [7].

Juvenile stages tend to have higher levels of plant secondary metabolites. This ecological adaptation confers a competitive advantage when plants are young and more susceptible to grazing animals. It is well documented that polyphenolic compounds such as tannins are a common defence mechanism in plants [3]. Younger tissue on the same plant also shows differences in levels of these compounds. For example, the highest levels of alkaloids occur in young pods in lupins. Concentrations of 4-N-acetyl-2,4-diaminobutyric acid (ADAB), a toxic non-protein amino acid present in *Acacia angustissima* was tripled when ADAB was extracted from young leaves [10]. In order to make valid comparisons between plant material harvested from different plants or accessions, it is recommended to always harvest leaves of a similar physiological age from plants.

Position on the Plant

As well as age, micro-environmental differences may also result in chemical differences in leaf material depending on the leaf position on the plant. This is not very significant in small herbaceous legumes or grasses due to their size, but is relevant when considering fodder trees. This may be due to enhanced respiration or water balance in leaves in direct sunlight with elevated temperatures and light intensity compared to leaves growing in shade. Higher light intensity and temperatures are known to increase amounts of ascorbic acid in tomatoes, with the result that fruits harvested from different locations on the same vine have differing levels of ascorbic acid [8]. The same is true for other micronutrients and anti-nutritional factors, including plant secondary metabolites. Research has shown that there are significant differences in tannin content from leaves growing in shade and in direct sunshine in *Sesbania* (unpublished information). In order to ensure a representative sample, it is recommended that leaves be harvested at a similar stage of maturity from all around the plant.

Seasonality

Seasonal differences in nutritional compounds and plant secondary metabolites have been reported in several species. Many of these differences are compounded by physiological age effects, but there are also effects of environment involved in these changes. This is related to day length, temperature and amount of water available that will determine metabolism and growth rate within the plant. Studies have shown that samples of leaves of several fodder tree species with high moisture content collected during the rapid growing season showed different nutritional attributes to those collected in the dry season [9]. It is recommended that when plant sampling one should always record the sampling date and that the collection of samples

for comparative purposes should be carried out over a short time period within the same season to minimise seasonal effects when collecting leaf material for plant proximate analyses.

Methodology

Collection Method for Leaf Material for Proximate Analysis

Materials Required

- Secateurs or Cutters
- Scissors
- Strong paper bags of 80–100 g paper of size 200 × 400 mm
- Pencil, notebook and marker
- Balance (range 0–1600 g)

Procedure

1. Determine how many samples to take by observing the plants. If variable, sample more plants to obtain a representative sample of the population.

Note: A good representative sample is needed to ensure accurate results. If the plants look uniform, then randomly take samples from 10 plants per population. If the plants show variation, randomly take samples from 25 plants per population.

2. Determine how much to sample. Take approximately 6 times the weight you need for analysis/storage.

Note: Assume that plants will loose about 80–90% of their weight as water during drying. Use this as a guide to calculate the fresh weight you need to harvest to have the required amount of plant material after drying.

3. Cut leaf material of a uniform maturity stage from all sides of each plant. Cut material into small pieces with scissors or secateurs and mix well.
4. Place into a weighed and labelled paper bag. Weigh the fresh material plus bag and record the weight.

Collection Method to Freeze Dry Leaf Material for Analysis of Plant Secondary Metabolites/Bioactive Compounds

Materials Required

- Secateurs or cutters
- Scissors
- Balance (range 0–1600 g)
- Plastics bags (generally of size 150 × 300 mm)
- Marker

- Stapler
- Cooler box
- Ice flakes

Procedure

The procedure to determine number and quantity of sample, and the cutting and weighing are the same as for proximate analysis. Then:

1. Place the weighed fresh material into a labelled plastic bag and close.
2. Immediately place the bagged sample into ice in a cooler box. Transfer to a freezer (–20°C) as soon as possible for storage before freeze-drying.

Note: Work as quickly as possible to harvest the sample and place the bagged sample immediately on ice in the cold box to avoid changes in composition of extra-nutritional compounds during the sampling procedure.

Sample Processing

Results of nutritional analyses are usually reported as a percentage of dry matter. Plant samples commonly contain from 80 to 90% water and should be dried as soon as possible after sampling to reduce respiration and metabolism, and to prevent deterioration. Leaf material is most commonly air dried in a well-ventilated oven at 60°C to avoid deterioration during the drying process. Freeze-drying or lyophilization is a process where water from frozen materials is removed by converting frozen water directly to water vapour without passing the liquid phase. A vacuum is created in the drier to remove water vapour from the surface of the plant sample.

Selection of the drying method, temperature and time should be done with care to avoid substantial qualitative and quantitative changes in the nutritive and extra-nutritive attributes of samples. Many studies have been conducted to evaluate the effect of oven drying or freeze drying on the nutritional components of forages and have concluded that the drying method can have considerable effect on nutritional value. Freeze-drying usually preserves the quality of the sample and avoids heating, which can cause degradation of some nutritional attributes and inactivation of bioactive compounds. Studies with willow have shown that leaves that were put into a freeze-dryer without being prefrozen or subjected to room drying with desiccation had concentrations of most secondary compounds comparable to those found in fresh leaves [6]. Tannins may undergo oxidative polymerisation with heat, which reduces their solubility and leads to subsequently underestimation of tannin content during analysis.

Dzowela et al. [2] and Papachristou and Nastis [9] reported that oven drying at 40°C artificially increased the fibre and lignin concentration of leaves of a range of fodder trees when compared to air and freeze-drying. There was also a reduction in soluble tannins, total nitrogen and in vitro organic matter digestibility due to oven drying at 65°C in some common fodder trees [2, 11]. It is recommended

that drying temperatures should not exceed 60°C to reduce degradation changes during processing and that freeze-drying is the preferred method when assessing secondary plant compounds or for screening plants for bioactive compounds. In dry environments without access to oven drying, plant material may also be air dried when spread in a thin layer and a shady environment to avoid direct sunlight that can cause overheating and deterioration.

At this stage in the processing, when samples reach the laboratory for either oven or freeze-drying, they are usually assigned a sequential laboratory number. The details of accession number, trial entry number, replicate, collection site, plot number, plant part, maturity, date of harvest and any unique identifier provided by the collectors are usually entered into the register and/or computer file so that each sample can be linked back to its source through the laboratory number. Although some of these details appear unnecessary, it is always better to have all information that can be used to verify sample identification in any cases of errors in recording. When the collection is made from outside of the research station, it is important to have an exact record of the collection site to link the collection with environmental data. In these cases, a global positioning system (GPS) can be used to record the exact site (longitude, latitude and altitude) and the data recorded on the collection sheet and in the registry. Codes may be used for sites and full information kept in a separate code file (Table 2.1).

Table 2.1 An example of the recording system used in our laboratory

Lab no.	Unique identifier	Site code	Trial	Trial entry no.	Plot no.	Harvest date	Maturity	Plant part	Replicate

Oven Drying of Leaf Material for Proximate Analysis

Materials Required

- Pencil and notebook
- Strong paper bags of 80–100 g paper of size 200 × 400 mm
- Balance (range 0–1600 g)
- Large well ventilated or forced air oven capable of maintaining temperatures of 60°C

Procedure

1. Place the weighed and open paper bags with leaf material into a ventilated oven at 60°C for 3 days.

Note: Ensure bags are sufficiently well spaced for good air circulation to avoid uneven drying.

2. After 3 days, weigh the dry material plus bag and record the weight.
3. Calculate the dry weight of the leaf material. Percent dry matter is calculated by weight loss during oven drying:

$$\text{Percent dry matter by weight (\%w)} = (\text{weight of oven dried sample} \times 100)/(\text{weight of fresh sample})$$

Freeze Drying Plant Material for Analysis of Plant Secondary Metabolites/Bioactive Compounds

Materials Required

- Balance (range 0–1600 g)
- Sample bags
- Marker
- Stapler
- Freeze dryer

Procedure

1. A tray freeze dryer is most commonly used for drying plant samples.
2. In your notebook record the tray number where each sample will be dried.
3. Arrange the samples in thin layers for rapid drying in the numbered trays in the freeze dryer.
4. Follow the manufacturer's instructions for your freeze drier for creating the vacuum and setting the temperatures.
5. Freeze dry the material at –30 to –50°C for 60 h.
6. Turn off the freeze drier and allow the material to reach room temperature.
7. Empty each tray into a numbered sample bag, checking the sample and tray numbers carefully against the list and sample bag.

Grinding Plant Samples

After drying, most plant samples are ground to small particles to ensure homogenous samples for the analysis. Oven dried, freeze-dried and air-dried samples are all ground in the same way. A range of grinder types can be used for grinding plant samples including hammer mills; Wiley mills and cross-beater mills are all suitable machines, providing they have a range of sieves to ensure uniform particle size. Thomas-Wiley, Laboratory Mill, Model 4 mills are often used in our laboratory. The particle size of the ground material is important to ensure reproducible results in the nutritional analyses. Different analyses require samples ground to different particle size. In some cases where several analyses are carried out on the same sample, it is important to grind sub-samples to a specified size, as required for that analysis. Samples that pass through a 1 mm mesh sieve are suitable for proximate analysis while samples should not be ground through a screen smaller than 2 mm for

nylon bag degradability studies. For quantification of plant secondary metabolites, a screen size of 0.5 mm should be used.

Materials Required

- Grinder with 2, 1 and 0.5 mm sieves
- Stiff brush for cleaning the grinder
- Notepad and pencil
- Marker pen
- Sample cups or plastic bags

Procedure

1. Arrange all bags with dried samples in the order of the list and check that all samples are present.
2. Open the first bag and mix the sample well in the bag.
3. Pass the sample through a clean grinder with the required size of screen for the analysis selected.

Note: Ensure a uniform particle size and avoid fine grinding to reduce differences in analysis from coarse and fine ground samples. Where very fine particles of a 0.5 mm screen is required, it is possible to first grind the entire sample through a larger screen size of 1 mm or 2 mm. After careful mixing, a sub-sample can then be taken and ground to the smaller screen size.

4. Collect the ground sample in a labelled plastic bag or sample cup and seal to prevent absorption of moisture.
5. Clean the grinder thoroughly and carefully after each sample.

Storage of Dried Plant Samples

Dried plant samples will not deteriorate during storage for several years if stored in good storage conditions. It is important to store samples until all analysis and experiments are completed and you have verified that there is no need to repeat any laboratory work. It is common to store samples for at least 2 years and possibly longer if there is a likelihood of continuing research that requires returning to earlier samples for additional analysis. Ground leaf materials should be stored in cool, dry and dark environments in sealed containers to maintain quality during storage.

Note: Remember to make a list and arrange containers in order of the list for easy access to samples later.

Materials Required

- Balance (range 0–1600 g)
- Labels and permanent pen
- Plastic containers with airtight lids

Procedure

1. Prepare labels for inside and outside each container.
2. Pack weighed ground samples in airtight sealed and well-labelled containers.
3. Arrange in numeric order in cartons or on shelves and prepare a list of samples and storage containers so that you can easily locate samples later.
4. Store in a cool place out of direct sunlight.

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