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FORAGE ANALYSES

PROCEDURES

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Historical Development of Forage Quality Testing Certification

In 1976, a task force composed of the American Forage and Grassland Council (AFGC) and university and federal research and extension personnel developed a forage evaluation system based on chemical analyses that closely approached actual feeding trials in determining feeding value of hay. Using these analyses, the task force developed proposed hay standards, including five grades and one sample grade for all legumes and grasses. Included in the standards was a relative feed value rating for ranking the various grades of both legumes and grasses. The proposed standards were submitted to the USDA Federal Grain Inspection Service (FGIS) by the AFGC. Due to mixed industry support in 1979, these proposed standards were not adopted by the FGIS.

In 1980, a group of western scientists and, later in 1982, a national group met and a U.S. hay quality steering committee was established. This group included research, extension, and commercial interests. In 1983, the group accepted a common set of analyses which included acid detergent fiber (ADF) used for estimated digestible dry matter and crude protein (CP).

In 1984, the National Alfalfa Hay Testing Association was formed as a joint effort among the American Forage and Grassland Council, the National Hay Association, and commercial forage testing laboratories to improve the repeatability and accuracy of forage testing among laboratories. This effort began with the sending of check samples and reporting of results relative to other laboratories, first for information and eventually for laboratory certification of acid detergent fiber and crude protein analysis. Dry matter was added for laboratory certification in 1992. Neutral detergent fiber results are being reported for information and will be certified at some time in the future.

The organizational name was changed to National Forage Testing Association (NFTA) in 1990 to reflect a broader range of forage types. The association sponsored national forage testing workshops for laboratory personnel in 1991 and 1992. A proceeding from each is available from the National Forage Testing Association.

Interested forage quality testing laboratories may contact the Secretary of the NFTA at the address on the front cover to participate in the check sample program. For a fee, four check samples of alfalfa will be sent at quarterly intervals and results (for DM, CP, ADF, and NDF) reported relative to median results of other participating laboratories. Laboratories will be certified based on accuracy of DM, CP and ADF determinations. Corn silage check samples are also available. Survey information will also be available on frequency of procedures used nationally.

Section A - Laboratory Sample Preparation

Subsampling or sample reduction of an unground sample in the laboratory is frequently the largest single source of variation during the analysis procedure and should be avoided whenever possible. Samples too large to be ground in their entirety are first ground through a large mill (Wiley or equivalent) to pass a 4 to 6 mm sieve. The coarse ground sample is then reduced in a gated riffle splitter and ground again to the fineness desired for analysis.

1. Sample Preparation

Reference:

Animal Feed: Sample Preparation. (950.02) Official Methods of Analysis. 1990. Association of Official Analytical Chemists, 15th edition.

Plants: Preparation of Sample. (922.02) Official Methods of Analysis. 1990. Association of Official Analytical Chemists, 15th edition.

Scope:

This method is applicable to the preparation of forages for laboratory analysis.

Basic Principle:

Laboratory sample preparation is the process of converting the sample received at the laboratory into a homogeneous material suitable for analysis. This process generally involves drying and/or grinding.

Most forage samples received at a laboratory fall into one of the following categories:

1) those dry enough to grind and analyze immediately (sample 90 to 95% or more dry matter).
2) those dry enough to be coarsely ground (to pass a 4 to 6 mm sieve) but too wet to be finely ground.
3) those samples which need to be partially dried before the sample can be coarsely ground (sample dry matter less than approximately 85%).

These sample types must be handled differently. See the following flow chart (Figure 1). The sample type, the type of grinder to be used and the fineness of the grind will determine how "wet" a sample can be ground. Most forages can easily be ground to pass a 4 to 6 mm sieve using a cutting mill (Wiley or equivalent) at 80 to 85% dry matter without problems (sticking in the mill, moisture loss, etc.). However, when using a cyclone mill to grind forage samples to pass a 1 mm sieve, most samples need to be 90 to 95% dry matter to grind properly.

Samples at approximately 85% or greater dry matter that are too large to grind in their entirety to the fineness desired for analysis are first ground through a large mill to pass a 4 to 6 mm sieve. The coarse ground sample is then reduced in a gated riffle splitter. When (Insert Figure 1) necessary, the reduced sample is partially dried (method 2.2.1) and the sample is

ground again to the fineness desired for analysis.

Precautions are taken during sample preparation to avoid sample contamination and retain sample identity. All equipment is kept clean and maintained on a regular schedule.

Prepared samples are stored in airtight containers away from heat and light.

Equipment:

Laboratory forage chopper (Hege 44[1], Wiley No 1, or equivalent)
Cyclone pulverizing mill (Udy or Cyclotec are required for samples using NIR)
Cutting mill, Wiley or equivalent
Riffle sample splitter, gated with 2 pans, 1-inch riffles
Sample containers
Hedge clippers, hand
Dishpans, plastic
Plastic sheet, 3 by 3 feet
Flat-blade spatula, stainless, 12-inch
Plastic bags, Ziplock or Whirlpak or equivalent
Freezer and refrigerator

Reagents:

None.

Safety precautions:

- Wear hearing protectors when operating a grinder. The noise level is on the threshold of being hazardous.
- Wear a dust mask and avoid inhaling dust when handling samples.
- Observe all safety and operating instructions supplied by the manufacturer of the grinding equipment.
- Do not insert fingers or objects into the grinding mill.

Procedure:

*Preparing samples greater than approximately 85% dry matter for grinding****Error! Bookmark not defined.***

1) Remove sample from shipping container and discard any roots from plants and brush off dirt particles. Note and report removed material and any other sample manipulation.
2) Chop samples of whole plants into about half-inch pieces using either hand clippers or the laboratory forage chopper. Include any ears attached to corn plants. When using the laboratory chopper, be sure to brush any sample adhering to the sides of the chopper into the receiving tray. Cored hay samples do not require chopping.

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[1] Mention of a product name is for information only and does not imply endorsement by the National Forage Testing Association or any of the associated public institutions.

3) Grind entire sample to pass 4 to 6 mm sieve in Wiley or another large mill.
4) Reduce the sample size in a gated riffle splitter to amount desired for laboratory subsample. Transfer the remainder to a plastic bag, seal and label.
5) If the sample is too wet to grind (less than 90% dry matter), dry the reduced sample using the forced-air oven (method 2.2.1.1) or microwave oven (method 2.2.1.2).
6) Grind the reduced sample to fineness desired for analysis in appropriate grinder (see section 1.1.1 and 1.1.2).
7) Thoroughly mix the ground sample. Transfer to an airtight container and label immediately.

Preparing samples less than approximately 85% dry matter for grinding

1) Remove sample from shipping container and discard any roots from plants and brush off dirt particles. Note and report removed material and any other sample manipulation.
2) Chop samples of whole plants into about half-inch pieces using either hand clippers or a laboratory forage chopper. Cut open stalk or corn cob pieces to facilitate drying. Include any ears attached to corn plants. When using the laboratory chopper, be sure to brush any sample adhering to the sides of the chopper into the receiving tray. Silages and haylages generally have average particle lengths less than 1 inch and do not require chopping.
3) Place the chopped sample into a clean dishpan or on a clean plastic sheet. Mix thoroughly.
4) If the entire sample cannot be dried, reduce the sample size by making a cone of sample and quartering. Save opposite quarters. Repeat mixing, coning and quartering until the volume is reduced to an appropriate size. Make certain that representative ratios of stem and leaf occur in each pile.
5) Transfer reduced sample to a tared container for drying.
6) Transfer the remainder to a plastic bag, seal, label and refrigerate.
7) Dry the reduced sample using either the forced-air oven (method 2.2.1.1) or microwave oven (method 2.2.1.2).
8) Grind the partially dried sample to fineness desired for analyses in appropriate grinder (see method 1.1.1 and 1.1.2).
9) Thoroughly mix the ground sample. Transfer to an airtight container and label immediately.

Comments:

- Samples to be scanned by NIR must be dried by the same method used to develop the calibration equation.
- Keep all equipment clean to avoid contaminating one sample with another.
- Care should be taken with the cyclone grinder not to heat the sample during the grinding process. Heating may affect subsequent chemical analysis.
- Equipment should be maintained on a regular schedule. The condition of the sieves, rotors, blades or other grinding surfaces should be monitored and regularly recorded in a designated log book.
- Transfer samples quantitatively. Do not leave sample portions in grinder or splitter.
- Store ground samples in airtight containers away from heat and light.

Calculations:

See appropriate dry matter method for dry matter calculations.

1.1 Grinding with a Cutting Mill (Wiley or equivalent)

Safety Precautions:

- Wear a dust mask and avoid inhaling dust.
- Do not insert fingers or objects into the grinding mill.
- Observe all safety and operating instructions supplied by the manufacturer.

Procedure:

1) Inspect the mill for cleanliness.
2) Insert the appropriate screen into the mill. If a fine grind is desired, most samples are ground with the 1 mm screen. If large samples are to be ground, it may be faster to grind them using a 4 to 6 mm screen and then regrind using the 1 mm screen.
3) Latch the door in place. Do not over tighten to avoid the rotor binding against the door.
4) Insert the sample receiving container into place.
5) Close the feeding gate at the top of the mill.
6) Turn on the switch.
7) Add sample to the feeding chute. Partially open the feeding gate to allow sample to enter, then shut the gate. If necessary, push sample into the mill with a spatula or wooden plunger with stop to prevent entry into grinder knives.
8) After the first portion has been ground (listen for the "empty" higher pitched sound), feed another portion into the mill.
9) Allow the mill to run for at least two min after the last portion has been added to ensure that the entire sample has passed through. Listen for the "empty" higher pitched sound, then shut off switch.
10) Hold the sample container beneath the grinding chamber and open the mill. Use a brush or spatula to transfer incompletely-ground residue from mill to the container with ground sample. Remove the screen and transfer residue from it into the sample container.
11) Clean the entire mill using air and/or a brush. A fine, pointed spatula works best to dislodge particles caught behind the knives. The mill may need washing with certain samples. Towel dry or blow dry to prevent rust formation.

Comments:

- The knives need periodic sharpening. Proper alignment of the knives on re-installation is critical to ensure efficient grinding.
- Dust will accumulate in the motor housing compartment and should to be cleaned out periodically.
- Condition of mill, maintenance and repairs should be monitored and regularly recorded in a designated log book.

1.2 Grinding with a Cyclone Mill (Udy, Cyclotec or equivalent)

Safety Precautions:

- Wear hearing protectors and monitor the noise (decibel) level produced by the grinder.
- Wear a dust mask and avoid inhaling dust.
- Observe all safety and operating instructions supplied by the manufacturer.

Procedure

1) Inspect the mill for cleanliness before using. Insert appropriate screen (1 mm required for NIR).
2) Clamp on the appropriate lid or head. Use the forage head for any sample that was oven dried.
3) Insert a clean sample bottle beneath the clear plastic cyclone and turn on the mill.
4) Slowly feed the sample into the mill. Do not overload the mill. A 15 cm powder funnel aids in feeding forages into the mill.
5) If the sample bottle becomes full before all the sample has been ground, shut off mill and empty the bottle into a clean aluminum pan. Then continue grinding the sample.
6) After the last of the sample has passed through, press the palm of your hand against the opening of the forage head. Keep the opening closed for about 5 sec, then release. This forms a vacuum inside the grinding chamber which helps clean out residual sample.
7) Shut off mill.
8) Thoroughly mix the sample and transfer to sample container.
9) Remove the head and screen and clean the mill using a brush and air. The impeller can be cleaned by wiping with a towel.

Comments:

- Equipment should be maintained on a regular schedule. The condition of the sieves, rotors, blades or other grinding surfaces should be monitored and regularly recorded in a designated log book.
- When a belt breaks, replace all belts at the same time. Proper tension of the belts is critical.
- Replace impeller when blades become worn or broken.
- Replace the grinding ring when the surface starts to become smooth.
- Replace the screen when it becomes dented, corroded, or begins to clog.
- Some samples may need to be drier than 85% dry matter to grind properly to pass a 1 mm screen in a cyclone mill.
- Samples to be scanned by NIR must be dried by the same method used to develop the calibration equation.
- Keep all equipment clean to avoid contaminating one sample with another.
- Care should be taken with the cyclone grinder not to heat the sample during the grinding process. Heating will change the chemical analysis.
- Transfer samples quantitatively. Do not leave sample portions in grinder.
- Ground samples must be stored in airtight, properly labeled containers away from heat and light.

Section B - Procedures for Certification

Introduction

Clients have a right to expect that feed testing laboratories: (1) provide true measures of chemical composition and nutritive value, (2) guarantee that results are consistent (repeatable) within a lab, (3) assure that results are comparable (reproducible) among labs and (4) insure that lab variation is not a major source of error in feed evaluation. Research labs have an obligation to insure that their results are comparable to other research and to information used in the field. Regulatory labs must be certain that their results are accurate and repeatable. Participation in the NFTA Certification Program provides some documentation that results from a feed analysis laboratory are acceptable. However, to validate the accuracy of routine analyses by a lab, certification samples must be analyzed by methods that are routinely used in that laboratory. Part of the value of the NFTA is to provide a mechanism whereby clients, researchers and regulators can be assured that laboratory results are valid. The current certification program is designed to evaluate the acceptability of a laboratory's results based solely on an allowable maximum deviation (bias) from reference values obtained on several samples.

Deviations from reference values can be due either to systematic or random errors. Systematic errors among laboratories arise from differences in methods or in-house modifications of methods, but can also be due to poor method design or description, improper analytical techniques, instrumentation malfunctions, or biased calculations. Systematic errors should be identified and corrected to improve agreement among laboratories. Random errors are associated with unavoidable sampling variation, uncontrollable deviations in equipment function, environmental fluctuations, and variations in replicating timing and performance of procedural steps among and within technicians.

Total accuracy is the closeness of repeated results to the true, expected or accepted value. Accuracy is the sum of variation associated with bias and precision. Bias is the systematic deviation of the average of repeated results from an accepted reference value.
Precision is the agreement or repeatability among repeated observations made under the same conditions. Bias and precision are completely independent measures of accuracy. Close agreement of duplicate or triplicate analysis within a laboratory indicates only that the method is precise (can be repeated with little variation), not that it is unbiased or accurate. It is possible, and quite common, to be able to replicate an inaccurate result. Accuracy can only be determined by comparing results to an accepted reference value that is obtained using an accepted reference method.

While many factors cause variation in analyses among laboratories, the greatest source of error (variation) among laboratories is probably associated with differences in routine methods. Routine methods often vary because they are poorly written and difficult to follow by less experienced technicians, instructions are passed from one technician to the next during training resulting in gradual modification of the original method, and methods are changed to suit the operation of the lab. These changes in methods, whether unintentional or not, often result in biases (differences) among labs that erode confidence in the value of feed analysis. Although

modifications can cause errors in all methods, they are especially harmful when the component being measured is defined by the method (such as fiber). Any method that defines the component that is being measured must be followed exactly to guarantee accurate results.

Since the method can affect results, it is evident that the reference value used to determine accuracy (and certification) among laboratories must be based on a reference method. These reference methods must: (1) have established reliability based on inter-laboratory collaborative studies, (2) be adopted by relevant organizations, such as AOAC, (3) be validated within runs using appropriate standards and quality control samples, and (4) be measured directly in accordance with known chemical/physical principles. Based on these criteria, the following methods should be adopted for routine use when appropriate or be used to validate any method that is routinely used in the lab:

Dry matter. The Karl Fischer method is the most accurate procedure for measuring true moisture (and therefore dry matter) in all types of samples when properly performed. This method is based on a chemical reaction of Karl Fisher reagent with water in the sample and is applicable to samples of moisture contents ranging from 0.1 to 100% water. Unfortunately, there is no AOAC approved method for the use of the Karl Fischer methods on feeds and forages. However, for samples >85% dry matter, oven drying at 135^{o}C for 2 hr results in estimates of dry matter that are repeatable and closely agree with Karl Fischer methods. For this reason, the reference method suggested for dry matter is:

Method 2.1.1 Laboratory Dry Matter by Oven Drying for 2 hr at 135^{o}C
(AOAC 930.15)

Crude Protein. Based on long-standing tradition and numerous inter-laboratory collaborative studies, Kjeldahl nitrogen methods are suggested as reference methods for crude protein if crude protein equals nitrogen times 6.25. Combustion methods have been validated against Kjeldahl methods in several studies. For this reason, the suggested reference methods for crude protein are:

Method 3.1 Nitrogen Determination by Kjeldahl (Rack)
(AOAC 984.13)
Method 3.2 Nitrogen Determination by Kjeldahl (Block Digestion)
(AOAC 981.10)
Method 3.3 Nitrogen Determination by Combustion Method
(AOAC 990.03)

Acid Detergent Fiber. Acid detergent fiber (ADF) is literally defined by the procedure used to measure it. Therefore, ADF can only be measured accurately if the AOAC method (973.18) is followed exactly. Any modification of the method that results in

different values for feeds defines a new and different type of fiber that should not be called ADF. For this reason, the suggested reference for acid detergent fiber is:

Method 4.1 Determination of Acid Detergent Fiber by Refluxing (AOAC 973.18)

Neutral Detergent Fiber. There is no AOAC approved method for neutral detergent fiber (NDF). The method for amylase-neutral detergent fiber (aNDF) described in this manual has been thoroughly tested on a diverse set of feeds and solves many of the problems associated with filtering residues. This method will be submitted to AOAC for a collaborative study soon. For this reason, the suggested reference method for neutral detergent fiber is:

Method 5.1 Determination of Amylase Neutral Detergent Fiber by Refluxing.

2. Dry Matter Determination

Dry matter can be determined either by residual weight following drying or with a near infrared reflectance (NIR) spectrophotometer using equations based on such reference techniques. Errors arise if the sample is insufficiently dried prior to taking the final weight or if the sample is overheated and additional compounds are volatilized (e.g. volatile acids and alcohols from haylages and silages). Room humidity for areas where dry matter is being determined should be below 60% RH.

Definition of Terms:

"as fed," "as is," or "as received" refers to the forage or feed as it is consumed by the animal. On a laboratory analysis report, these terms refer to the moisture content of the feed at the time of analysis. Moisture content of a forage at analysis vary from the true dry matter of the original lot if drying occurred between sampling and analysis.

"air dry" refers to a sample that has been allowed to dry in air, without aid of an oven or another drying device. In air of less than 60% relative humidity most air-dry samples will contain about 90% ± 2% dry matter.

"partially dry" refers to the initial dry matter of a sample that has been dried in an oven, usually at 55 to 60^{o}C or in a microwave oven to less than complete dryness. These samples, with 3 to 15% moisture, can be easily ground in most laboratory mills to produce a homogeneous sample for further analysis.

"laboratory dry matter" refers to the (final) dry matter content of the partially dry sample.

dry, dry matter, total dry matter, moisture free refer to a sample that has all the moisture removed. Forage and feedstuff analyses should be compared on a dry matter basis because varying moisture contents of "as is" results can alter analysis of other constituents.

2.1 Single Step Total Dry Matter Determinations

2.1.1 Total Dry Matter by Oven Drying for 2 hr at 135°C

Reference:

Drying of feeds at 135°C for 2 hr. (930.15) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This procedure is applicable for the determination of dry matter on ground air-dry or partially-dried (≥85% dry matter) forages with low volatile acid content. Samples dried by this procedure are not appropriate for subsequent fiber, lignin, or acid detergent insoluble nitrogen analysis. Volatile acids and alcohols may be lost from fermented samples.

This procedure is recommended for developing forage dry matter calibration for NIR.

Basic Principle:

Moisture is evaporated from the sample by oven drying. Dry matter is determined gravimetrically as the residue remaining after drying.

Equipment:

Forced-air drying oven at 135° ± 2°C. Oven should be equipped with a wire rod shelf to allow the circulation of air. It should be vented and operated with vents open.
Analytical electronic balance, accurate to 0.1 mg
Aluminum dish (pan), ≥50 mm diameter, ≤40 mm deep, covered
Desiccator

Reagents:

None.

Safety Precautions:

- Use standard precautions when working around electrical equipment or glassware.
- Make sure that all electrical equipment is properly grounded and installed and maintained by qualified electricians.

Procedure:

1) Dry aluminum dish with cover at 135° C ±2° C for at least 2 hr.
2) Cover dishes and move to desiccator.
3) Immediately cover desiccator and allow covered dishes to cool to room temperature. Do not allow dishes to remain in desiccator more than 2 to 3 hr.
4) Weigh dishes with cover (W_4) to nearest 0.1 mg, removing one at a time from desiccator and keeping desiccator closed between dish removals.

5) Add approximately 2 g ground sample to each dish. Record weight of dish with cover and sample (W_5) to nearest 0.1 mg.
6) Shake dish gently to uniformly distribute the sample and expose the maximum area for drying.
7) Insert samples with lids removed to the side into preheated oven at 135^{o}C and dry for 2 hr after oven has returned to temperature.
8) Move samples to desiccator, place cover on each dish, seal desiccator and allow to cool to room temperature. Do not allow samples to remain in desiccator for more than 2 to 3 hr.
9) Weigh dish with cover and dried sample (W_6), recording weight to nearest 0.1 mg.

For NIR calibration replace step 5 above with:

5.1) Load NIR sample cup placing one scoop of forage (previously dried to 90 to 95% dry matter or greater, ground with cyclone mill to pass 1 mm screen, thoroughly mixed) on each third of the glass surface to ensure that portions of different subsamples are scanned. Overfill the sample holder and scrape off any excess. Press back into holder until tight and level.
5.2) Scan sample on NIR instrument and store spectra.
5.3) Immediately remove sample from NIR instrument and weigh 2 g forage from sample cup to aluminum dishes. Record weight of dish with cover and sample (W_5) to nearest 0.1 mg.

Comments:

- Time and temperature must be adhered to closely.
- Samples should be placed in drying oven so that air can circulate freely. Containers should not touch.
- Slide the desiccator lid open. Do not place the lid on the countertop with the grease side down. The grease will pick up dirt, preventing formation of a seal.
- Seals should be kept clean and well greased and the lid should always slide easily on or off. If the lid "grabs," it is time to remove the old grease and apply fresh lubricant.
- If a lid can be directly lifted off the desiccator, either the desiccator was not properly sealed or, more likely, it needs fresh lubricant.
- Rubber stoppers in the lid should always be pliable.
- Sample dishes should not be packed excessively tight in a desiccator. Air movement is necessary to cool sample dishes. Dishes should not touch each other.
- Open a loaded desiccator very slowly after samples have cooled. A vacuum forms during cooling and abrupt opening results in turbulence which can blow samples out of uncovered containers.
- Desiccator lid should be slid open for the removal of each container and closed during weighing. Leaving the lid open allows samples to absorb moisture.

- Desiccant should be checked and dried periodically. Replace at least twice annually. Use of desiccant with color indicator for moisture is recommended.

Calculation: Percent Total Dry Matter (Total DM)

$$\% \text{ Total DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

Where W_4 = tare weight of dish in grams
W_5 = initial weight of sample and dish in grams
W_6 = dry weight of sample and dish in grams

Calculation: Percent Total Moisture:

$$\% \text{ Total Moisture} = 100 - \% \text{ Total DM}$$

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of ±0.20 and a control limit (3s) of ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.1.2 Total Dry Matter by Oven Drying at 100°C for 24 hr or 105°C for 16 hr

References:

Moisture in Peat. (967.03) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Faichney, G.J. and G.A. White. 1983. Methods for the analysis of feeds eaten by ruminants. Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia.

Windham, W.R., J.A. Robertson, and R.G. Leffler. 1987. A comparison of methods for moisture determination of forages for near infrared reflectance spectroscopy calibration and validation. Crop Sci. 27:777-783.

Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). ARS/USDA Handbook No. 379, Superintendent of Documents, US Government Printing Office, Washington, D.C. 20402

Scope:

This procedure may be used for determination of dry matter on forage samples or for dry weight determinations of fiber residues following fiber extraction. Samples dried by this procedure are not appropriate for subsequent fiber, lignin, or acid detergent insoluble nitrogen analysis. Volatile acids and alcohols may be lost from fermented samples.

Basic Principle:

Moisture is evaporated from sample by oven drying. Total dry matter is determined gravimetrically as residue remaining after drying. Weighing's may be made on hot sample or after cooling in desiccator.

Equipment:

Forced-air drying oven at 100°C (or 105°C), capable of maintaining temperature at ±2°C. Oven should be equipped with a wire rod shelf to allow the circulation of air. It should be vented and operated with vents open.

Aluminum dish (pan), ≥50 mm diameter, ≤40 mm deep, covered if desiccator used

Crucibles, porcelain, low wide form, 50 mL, Coors #1, covered if desiccator used

Top loading electronic balance, accurate to 0.1 mg

Reagents:

None.

Safety Precautions:

- Use standard precautions when working around electrical equipment or glassware.
- Make sure that all electrical equipment is properly grounded and installed and maintained by qualified electricians.

Procedure:

Hot Weigh Method

1) If only moisture is to be determined on the sample, use an aluminum dish. If ash determination is to follow on the dry matter residue, use a porcelain crucible. Dry the appropriate containers and three empty crucibles or dishes to be used for warming balance at 100°C (or 105°C) for at least 2 hr.
2) Warm balance by sequentially placing the three empty crucibles or dishes on balance for 20 sec each.
3) Removing one at a time from the oven, weigh container (W_4), recording weight to nearest 0.1 mg. Weigh rapidly, recording minimum weight (as soon as balance has stabilized, usually within 15 secs after removing from oven). Whenever weighing is interrupted, balance should be re-warmed per step (2).
4) After all containers have been weighed, allow balance and sample containers to cool.
5) Tare empty container to zero and weigh (W_7) approximately 2 g ground sample into each container or weigh approximately 2 g into each container and record weight of sample and container (W_5) to nearest 0.1 mg.
6) Shake container gently to uniformly distribute the sample and expose the maximum area for drying.
7) Place samples into an oven which has been preheated to 100°C (or 105°C) for at least 3 hr. Oven should return to temperature within 1 hr after samples in containers have been placed into it.
8) Leave uncovered samples in oven for 24 hr at 100°C or 16 hr (or overnight) at 105°C.
9) Individually remove containers from oven and hot weigh containers with dried sample as described in steps (2) and (3). Record weight (W_6) to nearest 0.1 mg.

Comments:

- Use a forced-air oven so that drying is more rapid and uniform and temperature drop is minimized during weighing.
- Samples should be placed in drying oven so that air can circulate freely. Containers should not touch each other.
- The balance must be located next to the oven; carrying samples any distance will allow cooling and absorption of moisture.
- Containers should be removed from oven one at a time and immediately weighed.
- Use of computer software to electronically record weight can reduce variance in weights due to operator differences in determining minimum weight.

Procedure:

Cold Weigh Method

1) If only moisture is to be determined on the sample, use an aluminum dish with cover. If ash determination is to follow on the dry matter residue, use a porcelain crucible with cover. Dry the appropriate container at 100°C (or 105°C) for at least 2 hr.

2) Cover containers and rapidly move to desiccator. Immediately cover desiccator and allow containers to cool to room temperature. Do not allow containers to remain in desiccator more than 2 to 3 hr.
3) Weigh container with cover (W_4) to nearest 0.1 mg, removing one at a time from desiccator and keeping desiccator closed between container removals.
4) Tare balance and weigh (W_7) approximately 2 g ground sample into each container or weigh approximately 2 g ground sample into each container and record weight of container with cover and sample (W_5) to nearest 0.1 mg.
5) Shake dish or crucible gently to uniformly distribute the sample and expose the maximum area for drying.
6) Place samples (with covers removed to the side) into oven that has been preheated to 100°C (or 105°C) at least 3 hr prior to use. Oven should return to temperature within 1 hr after samples have been placed into it.
7) Leave uncovered samples in oven for 24 hr at 100°C or 16 hr (or overnight) at 105°C.
8) Move samples to desiccator and place cover on each container as it is transferred. Seal desiccator and allow to cool for at least 1 hr but not more than 2 to 3 hr.
9) Weigh container with cover and dried sample (W_6), recording weight to nearest 0.1 mg.

Comments:

- Samples should be placed in drying oven so that air can circulate freely. Containers should not touch each other.
- Desiccator seals should be kept clean and well greased and the lid should always slide easily on or off. If the lid "grabs," it is time to remove the old grease and apply fresh lubricant.
- Open a loaded desiccator very slowly after samples have cooled. A vacuum forms during cooling and abrupt opening results in turbulence which can blow samples out of uncovered containers.
- Slide the desiccator lid open. Do not place the lid on the countertop with the grease side down. The grease will pick up dirt, preventing formation of a seal.
- If a lid can be directly lifted off the desiccator, either the desiccator was not properly sealed or, more likely, it needs fresh lubricant.
- Rubber stoppers in the lid should always be pliable.
- Sample dishes should not be packed excessively tight in a desiccator. Air movement is necessary to cool sample dishes. Dishes should not touch each other
- Desiccator lid should be left open for minimal amount of time.
- Desiccant should be checked periodically and dried. Replace twice annually. Use of desiccant with color indicator for moisture is recommended.

Calculation: Percent Total Dry Matter (Total DM)

If empty container is tared to zero in step 5 (hot weigh) or step 4 (cold weigh)

$$\% \text{ Total DM} = \frac{W_6 - W_4}{W_7} \times 100$$

Where W_4 = tare weight of container (with cover) in grams
W_7 = dry weight of sample in grams
W_6 = dry weight of sample and container (with cover) in grams

If empty container is not tared to zero in step 5 (hot weigh) or step 4 (cold weigh)

$$\% \text{ Total DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

Where W_4 = tare weight of container (with cover) in grams
W_5 = dry weight of sample and container (with cover) in grams
W_6 = dry weight of sample and container (with cover) in grams

Calculation: Percent Total Moisture:

$$\% \text{ Total Moisture} = 100 - \% \text{ Total DM}$$

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of ±0.20 and a control limit (3s) of ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.1.3 Total Dry Matter by Microwave Drying to Constant Weight

Reference:

None.

Scope:

This procedure is applicable for hay and other samples with low volatile acid content. Samples dried by this procedure are not appropriate for subsequent fiber, lignin, acid detergent insoluble nitrogen analysis or NIR analysis.

Basic Principle:

Moisture is evaporated from sample by microwave radiation. Total dry matter is determined gravimetrically as residue remaining after drying.

Equipment:

Microwave oven with minimum of 600 watts, turntable preferred but not essential
Top loading electronic balance, accurate to 0.01 g
Containers, paper or microwavable glass or plastic, sufficient in size to hold 100 to 250 g coarse forage with maximum sample depth of 1.5 inches

Reagents:

None.

Safety Precautions:

- Periodically test the oven for radiation leaks. Microwaves are absorbed by the body and can produce damaging heat effects, especially on the lens of the eye.
- Cardiac pacemakers may fail in presence of microwave radiation.
- Do not place any metal item or aluminum foil into the oven. Sparking or crackling in the oven indicates the presence of metal.

Procedure:

1) Dry empty container in microwave for 3 min at full power.
2) Weigh empty container on top loading balance and record weight (W_4) to nearest 0.01 g.
3) Tare empty container to zero and weigh forage (100 to 200 g) into container, recording weight to nearest 0.01 g (W_7), or add 100 to 200 g forage to the container and record weight of container and sample to nearest 0.01 g (W_5).
4) Place sample in oven and microwave at full power for about 3 min.
5) Remove sample and mix.
6) Repeat steps 4 and 5 twice for haylage (50% DM) and three times for silage (35% DM).
7) Weigh sample and container recording weight to 0.01 g.

8) Return sample to microwave at 50% power for 1 minute and re-weigh sample and container, recording weight to nearest 0.01 g.
9) Repeat steps 7 and 8 until no weight loss occurs during drying interval. Final weight is W_6. Do not allow sample to char or burn.

Comments:

- Do not place any metal item or aluminum foil into the oven. Sparking or crackling in the oven indicates the presence of metal.
- Be careful not to char or burn samples. As samples approach dryness reduce drying time and/or power setting. Discard darkened or burnt smelling samples and start over. Charring causes weight loss other than moisture and results in low dry matter values.
- Samples with higher moisture contents require longer drying times. Increase the number of drying intervals, not the time per drying interval or microwave power level, as these may lead to charred or burned samples.
- A glass of water placed in the microwave will reduce possibility of charring samples.

Calculation: Percent Total Dry Matter (Total DM)

If empty container is tared to zero in step 3

$$\% \text{ Total DM} = \frac{W_6 - W_4}{W_7} \times 100$$

Where W_4 = tare weight of container in grams
W_7 = dry weight of sample in grams
W_6 = dry weight of sample and container in grams

If empty container is not tared to zero in step 3

$$\% \text{ Total DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

Where W_4 = tare weight of container in grams
W_5 = dry weight of sample and container in grams
W_6 = dry weight of sample and container in grams

Calculation: Percent Total Moisture:

$$\% \text{ Total Moisture} = 100 - \% \text{ Total DM}$$

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of ±0.20 and a control limit (3s) of ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.2 Two Step Total Dry Matter Determination of Wet Samples

For wet samples (less than approximately 85% dry matter) it is necessary to partially dry the sample in forced-air or microwave oven prior to grinding. The goal is to dry the unground sample while keeping sample temperature below 55 to 60^{o}C so that chemical composition is affected minimally. Drying at low temperature (less than 60^{o}C) does not remove all water from the sample; therefore (initial) partial drying does not represent the dry matter of the sample.

Following drying, the sample is ground and analyzed for (final) laboratory dry matter (the remaining 3 to 15% moisture) when other chemical constituents are determined.

Therefore, a two-step procedure for determining dry matter is recommended:

Step 1: Determine partial dry matter of sample (if less than 90% dry matter).

Step 2: Determine laboratory dry matter on ground sample and multiply partial dry matter times laboratory dry matter to determine total dry matter.

Step (1) may be skipped if dry matter ≥90% and the sample can be ground without drying.

Drying method for a sample to be analyzed on NIR should be consistent with the drying method used for samples in calibration of the NIR.

2.2.1 Partial Drying of Wet Samples

2.2.1.1 Partial Dry Matter Using Forced-air Drying Ovens

Reference:

Adapted from Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). ARS/USDA Handbook No. 379, Superintendent of Documents, US Government Printing Office, Washington, D.C. 20402

Scope:

This procedure is applicable to all types of forages and is intended for initial, partial drying of wet samples (less than 90% dry matter, greater than 10% moisture). The procedure has minimal effect on chemical composition, allowing samples to be subsequently analyzed for fiber, lignin, or acid detergent insoluble nitrogen analysis.

Basic Principle:

Moisture is evaporated from sample and partial dry matter is determined gravimetrically as the residue remaining after oven drying. Some moisture remains in the sample because drying at this temperature does not remove all water. Sample should be equilibrated at room temperature for 2 to 4 hrs before measuring partial dry matter to minimize the potential change in moisture that can occur during grinding and storage. Drying at higher temperatures (greater than 60^{o}C) causes chemical changes in the sample that affect subsequent fiber, lignin, or acid detergent insoluble nitrogen analysis.

Equipment:

Forced-air drying oven set at 55^{o} C
Top loading electronic balance, accurate to 0.01 g
Pans, sufficient in size to hold 100 to 250 g coarse forage with maximum sample depth of 1.5 inches

Reagents:

None.

Safety Precautions:

- Use standard precautions when working with electrical equipment or glassware.

Procedure:

1) Dry empty pans at 55^{o}C for 2 hr if non-absorbent (glass, metal) or 8 hr if absorbent (paper products).
2) Weigh empty pans on a top loading balance and record weight (W_1) to nearest 0.01 g.

3) Tare empty container to zero and weigh constant volume of coarse forage into pans recording weight to nearest 0.01 g (W_2) filling pans to maximum sample depth of 1.5 inches. Use following approximate weights:

Low (less than 50%) moisture haylage	70 to 130 g
High moisture hay crop silage	150 to 250 g
Corn silage	150 to 250 g
High moisture corn grain	150 to 250 g

4) Dry in forced-air drying oven at 55^{o} C for 16 to 24 hr.
5) Air-equilibrate samples for 2 to 4 hr and weigh the sample and pan recording weight to nearest 0.01 g (W_3).

Comments:

- Samples must not be stacked or tightly packed in oven to prevent free movement of air.
- The oven temperature must not exceed 60^{o}C or heat-damaged protein will be formed that will affect fiber values.
- This weight approximates a toluene determination moisture content of silages and other products with significant levels of volatile compounds.

Calculation: Partial Dry Matter (Partial DM), expressed as ratio of (w/w) of dry matter to total weight.

$$\text{Partial DM} = \frac{W_3 - W_1}{W_2}$$

Where W_1 = tare weight of pan in grams
W_2 = initial weight of sample in grams
W_3 = dry weight of sample and pan in grams

Quality Control:

Laboratory dry matter of partially dried samples should be between 85 and 95%.

2.2.1.2 Partial Dry Matter Using Microwave Oven

Reference:

None.

Scope:

This procedure is applicable to all types of forages and is intended for an initial, partial drying for wet samples (less than 90% dry matter, greater than 10% moisture). The procedure has minimal effect on chemical composition, allowing sample to be analyzed subsequently for fiber, lignin, or acid detergent insoluble nitrogen analysis.

Basic Principle:

Moisture is evaporated from sample using microwave energy, and partial dry matter is determined gravimetrically as the residue remaining after oven drying.

Equipment:

Microwave oven with minimum of 600 watts, turntable preferred but not essential
Top loading electronic balance, accurate to 0.01 g
Containers, paper or microwavable glass or plastic, sufficient in size to hold 100 to 250 g coarse forage with maximum sample depth of 1.5 inches

Reagents:

None.

Safety Precautions:

- Periodically test the oven for radiation leaks. Microwaves are absorbed by the body and can produce damaging heat effects, especially on the lens of the eye.
- Cardiac pacemakers may fail in presence of microwave radiation.
- Do not place any metal item or aluminum foil into the oven. Sparking or crackling in the oven indicates the presence of metal.

Procedure:

1) Dry empty containers in microwave for 3 min at full power.
2) Weigh empty container on top loading balance and record weight (W_1) to nearest 0.01 g.
3) Tare empty container to zero and weigh forage (100 to 200 g) into container, recording weight to nearest 0.01 g (W_7), or add 100 to 200 g forage to the container and record weight of sample and container to nearest 0.01 g (W_2).
4) Place sample in oven and microwave for about 3 min at full power.
5) Remove sample and mix.
6) Repeat steps 4 and 5 twice for haylage (50% DM) and three times for silage (35% DM).

7) If sufficiently dry (sample crisp, not damp to touch), stop microwave drying. If not, return sample to microwave at 50% power for 1 minute intervals until not damp to the touch. The objective is to dry the sample to 90 to 95% dry matter.
8) Equilibrate sample for 2 hrs at room temperature and weigh sample and container (W_3), recording weight to nearest 0.01g.

Comments:

- Be careful not to char or burn samples. As samples approach dryness, reduce drying time and/or power setting. Discard darkened or burnt smelling samples and start over. Charring affects dry matter determination and results of subsequent analysis of fiber, ADIN, lignin analysis and NIR analysis. Be especially careful when drying the entire sample received (no reserve sample).
- Samples with higher moisture contents require longer drying times. Increase the number of drying intervals, not the time per drying interval or microwave power level, as these may lead to charred or burned samples.
- A glass of water placed in the microwave oven will reduce the possibility of charring samples.

Calculation: Partial Dry Matter (Partial DM), expressed as ratio of (w/w) of dry matter to total weight.

If container is tared to zero in step 3

$$\text{Partial DM} = \frac{W_3 - W_1}{W_7}$$

Where W_1 = tare weight of container in grams
W_7 = initial weight of sample in grams
W_3 = dry weight of sample and container in grams

If container is not tared to zero in step 3

$$\text{Partial DM} = \frac{W_3 - W_1}{W_2 - W_1}$$

Where W_1 = tare weight of container in grams
W_2 = initial weight of sample and container in grams
W_3 = dry weight of sample and container in grams

Quality Control:

Laboratory dry matter of partially dried samples should be between 85 and 95%.

2.2.2 Laboratory Dry Matter of Partially-Dried Samples

2.2.2.1 Laboratory Dry Matter by Oven Drying for 2 hr at 135°C

Reference:

Drying of feeds at 135°C for 2 hr. (930.15) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This procedure is applicable for the determination of dry matter on ground air-dry or partially-dried (≥85% dry matter) forages with low volatile acid or alcohol content. Samples dried by this procedure are not appropriate for further chemical analyses.

This procedure is recommended for developing forage dry matter calibration for NIR.

Basic Principle:

Moisture is evaporated from the sample by oven drying. Dry matter is determined gravimetrically as the residue remaining after drying.

Equipment:

Forced-air drying oven at 135° ±2°C. Oven should be equipped with a wire rod shelf to allow the circulation of air. It should be vented and operated with vents open.

Analytical electronic balance, accurate to 0.1 mg

Aluminum dish (pan), ≥50 mm diameter, ≤40 mm deep, covered

Desiccator

Reagents:

None.

Safety Precautions:

- Use standard precautions when working around electrical equipment or glassware.
- Make sure that all electrical equipment is properly grounded and installed and maintained by qualified electricians.

Procedure:

1) Dry aluminum dish with cover at 135° C ±2° C for at least 2 hr.
2) Cover dish and move to desiccator. Immediately cover desiccator and allow dishes to cool to room temperature. Do not allow dishes to remain in desiccator more than 2 to 3 hr.
3) Weigh dish with cover (W_4) to nearest 0.1 mg, removing one at a time from desiccator and keeping desiccator closed between container removals.
4) Add approximately 2 g ground sample to each dish. Record weight of dish with cover and sample (W_5) to nearest 0.1 mg.

5) Shake dish gently to uniformly distribute the sample and expose the maximum area for drying.
6) Dry samples in oven preheated to 135°C for 2 hr.
7) Move samples to desiccator, placing cover on each dish as it is transferred. Seal desiccator and allow to cool to room temperature. Do not allow samples to remain in desiccator for more than 2 to 3 hr.
8) Weigh dish with cover and dried sample (W_6), recording weight to nearest 0.1 mg.

For NIR calibration replace step 4 above with:

4.1) Load NIR sample cup placing one scoop of forage (previously dried to 90-95% dry matter or greater, ground with cyclone mill to pass 1 mm screen, thoroughly mixed) on each third of the glass surface. Fill to leave adequate space for cell backing.
4.2) Scan sample on NIR instrument and store spectra.
4.3) Immediately remove sample from NIR instrument and weigh 2 g forage from sample cup to an aluminum dish. Record weight of dish with cover and sample (W_5) to nearest 0.1 mg.

Comments:

- Time and temperature described in procedures must be adhered to closely.
- Samples should be placed in drying oven so that air can circulate freely. Containers should not touch each other.
- Do not overload oven.
- Slide the desiccator lid open. Do not place the lid on the counter top with the grease side down. The grease will pick up dirt, preventing formation of a seal.
- Seals should be kept clean and well greased and the lid should always slide easily on or off. If the lid "grabs," it is time to remove the old grease and apply fresh lubricant.
- If a lid can be directly lifted off the desiccator, either the desiccator was not properly sealed or, more likely, it needs fresh lubricant.
- Rubber stoppers in the lid should always be pliable.
- Sample dishes should not be packed excessively tight in a desiccator. Air movement is necessary to cool sample dishes. Dishes should not touch each other.
- The desiccator lid should be left open for minimal amount of time.
- Desiccant should be checked and dried periodically. Replace desiccant twice annually or more often depending on use. Use of desiccant with color indicator for moisture is recommended.
- Open a loaded desiccator very slowly after samples have cooled. A vacuum forms during cooling, and abrupt opening results in turbulence which can blow samples out of uncovered containers.

Calculation: Percent Laboratory Dry Matter (Lab DM)

$$\% \text{ Lab DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

Where W_4 = tare weight of dish (with cover) in grams
W_5 = initial weight of sample and dish (with cover) in grams
W_6 = dry weight of sample and dish (with cover) in grams

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of about ±0.20 and a control limit (3s) of about ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.2.2.2 Laboratory Dry Matter by Oven Drying at 100°C for 24 hr or 105°C for 16 hr

References:

Moisture in Peat. (967.03) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Faichney, G.J. and G.A. White. 1983. Methods for the analysis of feeds eaten by ruminants. Commonwealth Scientific and Industrial Research Organization, Melbourne, Australia.

Windham, W.R., J.A. Robertson, and R.G. Leffler. 1987. A comparison of methods for moisture determination of forages for near infrared reflectance spectroscopy calibration and validation. Crop Sci. 27:777-783.

Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analyses (apparatus, reagents, procedures, and some applications). ARS/USDA Handbook No. 379, Superintendent of Documents, US Government Printing Office, Washington, D.C. 20402

Scope:

This procedure may be used for determination of laboratory dry matter on ground air-dry or partially dried (≥ 85% dry matter) forage samples. This procedure is also applicable for dry weight determinations of fiber residues following acid detergent or neutral detergent extraction.

Basic Principle:

Moisture is evaporated from the sample by oven drying. Laboratory dry matter is determined gravimetrically as residue remaining after drying.

Equipment:

Forced-air drying oven at 100°C (or 105°C), capable of maintaining temperature at ±1°C. Oven should be equipped with a wire rod shelf to allow the circulation of air. It should be vented and operated with vents open.

Aluminum dish (pan), ≥50 mm diameter, ≤40 mm deep, covered if desiccator used

Crucibles, porcelain, low wide form, 50 mL, Coors #1, covered if desiccator used

Top loading electronic balance, accurate to 0.1 mg

Reagents:

None.

Safety Precautions:

- Use standard precautions when working with electrical equipment or glassware.
- Make sure that all electrical equipment is properly grounded and installed and maintained by qualified electricians.

Procedure:

Hot Weigh Method

1) If only moisture is to be determined on the sample, use an aluminum dish. If ash determination is to follow on the dry matter residue, use a porcelain crucible. Dry the appropriate container and three crucibles to be used to warm the balance at 100°C (or 105°C) for at least 2 hr.
2) Warm balance by sequentially placing three empty crucibles on balance for 20 sec each.
3) Removing one at a time from the oven, weigh container (W_4), recording weight to nearest 0.1 mg. Weigh rapidly, recording minimum weight (as soon as balance has stabilized, usually within 15 sec after removing from oven). Whenever weighing is interrupted, balance should be re-warmed according to step (2).
4) After all containers have been weighed, allow balance and sample containers to cool.
5) Tare container to zero and weigh (W_7) approximately 2 g ground sample into each container or weigh approximately 2 g into each container and record weight of sample and container (W_5) to nearest 0.1 mg.
6) Shake container gently to uniformly distribute the sample and expose the maximum area for drying.
7) Place samples into an oven which has been preheated to 100°C (or 105°C) for at least 3 hr. Oven should return to temperature within 1 hr after samples in containers have been placed into it.
8) Leave uncovered samples in oven for 24 hr at 100°C or 16 hr (or overnight) at 105°C.
9) Individually remove containers from oven and hot weigh containers with dried sample as described in steps (2) and (3). Record weight (W_6) to nearest 0.1 mg.

Comments:

- Use a forced-air oven so that drying is more rapid and uniform and temperature drop is minimized during weighing.
- Samples should be placed in the drying oven so that air can circulate freely. Containers should not touch each other
- The balance must be located next to the oven; carrying samples any distance will allow cooling and addition of moisture.
- Containers should be removed from oven one at a time and immediately weighed.
- Use of computer software to electronically record weight is recommended to reduce variance in weights due to operator differences in determining minimum weight.

Procedure:

Cold Weigh Method

1) If only moisture is to be determined on the sample, use an aluminum dish with cover. If ash determination is to follow on the dry matter residue, use a porcelain crucible with cover. Dry the appropriate containers at 100°C (or 105°C) for at least 2 hr.

2) Cover containers and move to desiccator. Immediately cover desiccator and allow containers to cool to room temperature. Do not allow containers to remain in desiccator more than 2 to 3 hr.
3) Weigh container with cover (W_4) to nearest 0.1 mg, removing one at a time from desiccator and keeping desiccator closed between container removals.
4) Tare container and weigh (W_7) approximately 2 g ground sample into container with cover or add approximately 2 g ground sample to each container and record weight of container with cover and sample (W_5) to nearest 0.1 mg.
5) Shake container gently to uniformly distribute the sample and expose the maximum area for drying.
6) Place samples with covers removed to side into oven that has been preheated to 100°C (or 105°C) at least 3 hr prior to use. Oven should return to temperature within 1 hr after samples have been placed into it.
7) Leave uncovered samples in oven for 24 hr at 100°C or 16 hr (or overnight) at 105°C.
8) Move samples to desiccator, placing cover on each container as it is transferred. Seal desiccator and allow to cool for at least 1 hr but not more than 2 to 3 hr.
9) Weigh container with cover and dried sample (W_6), recording weight to nearest 0.1 mg.

Comments:

- Samples should be placed in the drying oven so that air can circulate freely. Containers should not touch each other. Air movement is necessary to cool sample dishes.
- Desiccator seals should be kept clean and well greased and the lid should always slide easily on or off. If the lid "grabs," it is time to remove the old grease and apply fresh lubricant.
- Do not place the lid on the counter top with the grease side down. The grease will pick up dirt, preventing formation of a seal.
- If a lid can be directly lifted off the desiccator, either the desiccator was not properly sealed or, more likely, it needs fresh lubricant.
- Rubber stoppers in the lid should always be pliable.
- Open a loaded desiccator very slowly after samples have cooled. A vacuum forms during cooling and abrupt opening results in turbulence which can blow samples out of uncovered containers.
- Desiccator lid should be slid open for the removal of each container and reclosed before weighing. Leaving the lid open allows samples to absorb moisture.
- Desiccant should be checked and dried periodically. It should be replaced twice annually. Use of desiccant with color indicator for moisture is recommended.

Calculation: Percent Laboratory Dry Matter (Lab DM)

If empty container is tared to zero in step 3 (hot weigh) or step 4 (cold weigh)

$$\% \text{ Lab DM} = \frac{W_6 - W_4}{W_7} \times 100$$

Where W_4 = tare weight of container (with cover) in grams
W_7 = initial weight of sample in grams
W_6 = dry weight of sample and container (with cover) in grams

If empty container is not tared to zero in step 3 (hot weigh) or step 4 (cold weigh)

$$\% \text{ Lab DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

Where W_4 = tare weight of container (with cover) in grams
W_5 = initial weight of sample and container (with cover) in grams
W_6 = dry weight of sample and container (with cover) in grams

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of about ±0.20 and a control limit (3s) of about ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.2.2.3 Laboratory Dry Matter by Microwave Drying to Constant Weight

Reference:

None.

Scope:

This procedure is applicable for the determination of laboratory dry matter in ground, air-dry or partially dried (≥ 85% dry matter) forages. Samples dried by this procedure are not appropriate for subsequent fiber, lignin, or acid detergent insoluble nitrogen analysis.

Basic Principle:

Moisture is evaporated from sample by microwave radiation. Total dry matter is determined gravimetrically as residue remaining after drying.

Equipment:

Microwave oven with minimum of 600 watts, turntable preferred but not essential
Top loading electronic balance, accurate to 0.01 g
Pans, paper or microwavable glass or plastic

Reagents:

None.

Safety Precautions:

- Periodically test the oven for radiation leaks. Microwaves are absorbed by the body and can produce damaging heat effects, especially on the lens of the eye.
- Cardiac pacemakers may fail in presence of microwave radiation.
- Do not place any metal item or aluminum foil into the oven. Sparking or crackling in the oven indicates the presence of metal.

Procedure:

1) Dry empty pans in microwave for 3 min at full power.
2) Weigh empty pans on top loading balance and record weight (W_4) to nearest 0.01 g.
3) Weigh forage (100 to 200 g) into pans, recording weight of pan and sample to nearest 0.01 g (W_5).
4) Place sample in oven and microwave for about 3 min at full power.
5) Remove sample and mix.
6) Weigh sample and container recording weight to 0.01 g.
7) Return sample to microwave at 50% power for 1 min and re-weigh container recording weight to nearest 0.01 g.
8) Repeat steps 6 and 7 until no weight loss occurs during drying interval. Do not allow sample to char. Record final weight of pan and dried sample (W_6) to nearest 0.01g.

Comments:

- Be careful not to char or burn samples. As samples approach dryness, reduce drying time and/or power setting. Discard darkened or burnt smelling samples and start over. Charring affects dry matter determination.
- Samples with higher moisture contents require longer drying times. Increase the number of drying intervals, not the time per drying interval or microwave power level, as these may lead to charred or burned samples.
- A glass of water placed in the microwave oven during drying will reduce the possibility of charring samples.

Calculation: Percent Laboratory Dry Matter (Lab DM)

$$\% \text{ Lab DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

Where W_4 = tare weight of pan in grams
W_5 = initial weight of sample and pan in grams
W_6 = dry weight of sample and pan in grams

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of about ±0.20 and a control limit (3s) of about ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.2.2.4 Dry Matter by Near Infrared Reflectance Spectroscopy

Reference:

Windham, W.R., F.E. Barton II and J.A. Robertson. 1988. Moisture analysis of forage by near infrared reflectance spectroscopy: Preliminary collaborative study and comparison between Karl Fischer and oven drying reference methods. Journal of the Association of Official Analytical Chemists 71:256-262.

Martin, G.C., J.S. Shenk, and F.E. Barton II. 1989. Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality. United States Department of Agriculture, Agricultural Research Service. Agricultural Handbook No. 643.

Scope:

This procedure is applicable for determining laboratory dry matter of ground, air-dry or partially dried (90 to 95% dry matter) forage. Samples must be ground through cyclone grinder with 1 mm screen and be 90 to 95% dry matter.

Basic Principle:

Random portions of a sample are loaded into a NIR sample holder and reflected light from the ground sample is measured in the infrared region (generally 1100 to 2500 nm). Instrument is part of a system that has been calibrated using representative samples from population to be tested. Equations selected based on calibration statistics, which have been validated, are used to calculate dry matter content of feed and forage samples.

Equipment:

Near infrared reflectance spectrophotometer, wavelength range at least 1100 to 2500 nm
Sample holders with infrared transmitting quartz window
Computer with software for collecting, storing and processing spectra

Reagents:

None.

Safety Precautions:

- Follow manufacturer's recommendation for safe operation of instrument.

Procedure:

1) Prepare samples by the same method as the calibration samples were prepared.
2) For best results run instrument (but not lamp) continuously. If instrument is cold, warm-up time should be 1 hr.
3) Clean sample holder with a camel hair brush or vacuum. Additional cleaning may be done with soft tissue or lint-free cloth. Glass should be free of finger prints and foreign material.

4) Load NIR sample cup placing one scoop of forage (previously dried to 90-95% dry matter or greater, ground with cyclone mill to pass 1 mm screen, thoroughly mixed) on each third of the glass surface to ensure that portions of different subsamples are scanned. Overfill the sample holder and scrape off any excess.
5) Press back into holder so that sample is firmly pressed against window.
6) If any abnormality appears in window, remove back and repeat process. Consistency in sample handling, preparation, and cell packing is crucial to success.
7) Scan sample, collect spectra, and process data.

Comments:

- NIR instrument should be maintained in a dust-free environment. Clean or change filter monthly. Clean ceramic standard and drawer assembly monthly.
- Never touch or clean grating.
- NIR instrument should be maintained in a stable-temperature (25 ±3^{o}C) room with controlled humidity (60 ±5%). Room should be free from vibration and have stable and dedicated electrical current.
- Sample must be dried and ground by the same methods as those samples used to develop the calibration equation.
- Spectrophotometer reads only a fraction of 1 mm depth of material touching glass of sample container. Therefore container must be loaded carefully with different subsample in each quadrant to make spectral reading more representative of entire sample.

Calculations:

Prediction is made by direct comparison to calibration. No additional calculations.

Quality Control:

Include at least one set of duplicates in each run. These duplicates must be two subsamples each packed in a separate holder. Scanning the same sample twice is not a true replicated analysis using NIR. An acceptable average standard deviation (s) among replicated analyses for moisture or dry matter using reference methods is about ±0.10, which results in a warning limit (2s) of about ±0.20 and a control limit (3s) of about ±0.30.

Quality control for NIR analysis involves monitoring the accuracy of both the instrument and the calibration equation. Instrument diagnostics should be run and recorded weekly, after instrument warm-up, to insure that maximum response, wavelength accuracy and repeatability are within acceptable manufacturer's tolerances. For example, for NIRSystems model 6250 and 6500 instruments, maximum response should be between 55000 and 58000 (adjust if below 51000), suggested wavelength error should be <0.5 times currently observed error (restandardize if observed error is too large) and root mean square corrected (RMSC) repeatability of multiple scans (32 revolution and 30 scans) should be less than 20 to 30 (correct problems if greater). Each manufacturer should

provide acceptable performance specifications for their instrument, and often software is provided to monitor instrument accuracy. However, it is the responsibility of the operator to run the diagnostic software routinely and record the results a minimum of once a week.

In addition to meeting specifications, instrument operation should be monitored by scanning a check sample (that has been sealed in a sample holder) each day and storing spectral data weekly. Daily results for each analysis (DM, CP, ADF, NDF, and minerals) should be plotted on an X-control chart and the chart should be examined for trends. Standard deviation (s) for the check sample can be established after 10 scans and should be substantially lower than acceptable standard deviations of duplicate reference method analyses (.10, .20, .35 and .60 for DM, CP, ADF, and NDF, respectively) because the same sample is being scanned. Results outside of ±2s upper and lower warning limits are evidence of problems with the analytical system. Results outside of ±3s upper and lower control limits are evidence of loss of control and no NIR analyses should be done without detecting and correcting the problem. Two consecutive analyses falling on one side of the mean between warning limits and control limits also indicate a loss of control.

Monitoring the calibration equation consists of two tests that determine the existence of bias and unacceptable increases in standard error of prediction corrected for bias [SEP(C)]. Every 20 to 25th sample predicted by the NIR should be analyzed by the reference method used to develop the calibration equation (Note: Any bias or increased SEP(C) can be due to inaccuracies in NIR or differences in reference method analytical procedure). A continuous time chart of observed bias and SEP(C) should be plotted to observe trends. After nine (N) samples have been accumulated, analyze these samples by the reference method (A sample size of nine is a good compromise between the number of analyses required and the statistical accuracy desired for the confidence limits given below). Calculate the observed bias and SEP(C) using the equations given below. Determine the confidence limits for bias and SEP(C) based on the standard error of the calibration equation (SEC). If the SEP(C) of the nine samples is less than the SEP(C) limit and absolute value of the bias is less than the bias limit, the calibration equation is acceptable. If the SEP(C) of the nine samples is less than the SEP(C) limit but the absolute value of the bias exceeds the bias limit, the calibration equation may be corrected by adding the bias to the intercept of the calibration equation or to each value (bias adjustment) although recalibration is recommended. If both SEP(C) and bias exceed their limits, add samples to the calibration data set and recalibrate.

NIR Quality Control Calculations:

$D_i = X_i - Y_i$

$Bias = D_i / N$ Bias Confidence Limit = ±0.55 X (SEC)

Install Equation Editor and double-click here to view equation. SEP(C) Confidence Limit = 1.29 X (SEC);

where D_i = difference
X_i = reference method value
Y_i = NIR value for *i*th sample
N = 9 (number of samples)
Bias = average difference between reference and NIR values
SEP(C) = standard error for prediction corrected for bias
SEC = standard error of the calibration equation.

This monitoring procedure can be used with any NIR instrument and some manufacturers have incorporated this approach into their quality control monitoring software.

2.2.3 Determination of Sample Total Dry Matter using the Two-step Procedure Error! Bookmark not defined.

Reference:

Abrams, Stephen M. 1984. Laboratory procedures for determining dry matter, crude protein and acid detergent fiber. Proceedings National Alfalfa Hay Quality Testing Workshop. Chicago, IL.

Mertens, D. R. 1993. Determining dry matter in diverse types of feeds. Proc. NFTA Forage Analysis Workshop, Denver, CO. pp B1-B10.

Scope:

This procedure is applicable for determining total dry matter on all types of forages.

Basic Principle:

Total dry matter is determined in a two-step process. First, the sample is partially dried by one of the methods described in section 2.2.1. Second, laboratory dry matter is determined by one of the methods described in section 2.2.2. Total dry matter is then calculated by multiplying partial dry matter times laboratory dry matter.

Equipment:

See appropriate dry matter methods.

Reagents:

None.

Safety Precautions:

See appropriate dry matter methods.

Procedure:

1) Determine partial dry matter (Section 2.2.1).
2) Determine laboratory dry matter (Section 2.2.2).
3) Calculate total dry matter by multiplying partial dry matter times laboratory dry matter.

Calculation: Percent Total Dry Matter (Total DM)

$$\text{Partial DM} = \frac{W_3 - W_1}{W_2 - W_1} \qquad \text{Lab DM} = \frac{W_6 - W_4}{W_5 - W_4} \times 100$$

$$\% \text{ Total DM} = \text{Partial DM} \times \text{Lab DM}$$

Where W_1 = tare weight of container (with cover) in grams
W_2 = initial weight of sample and container (with cover) in grams
W_3 = dry weight of sample and container (with cover) in grams
W_4 = tare weight of container (with cover) in grams
W_5 = initial weight of sample and container (with cover) in grams
W_6 = dry weight of sample and container (with cover) in grams

Calculation: Percent Total Moisture:

$$\% \text{ Total Moisture} = 100 - \% \text{ Total DM}$$

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of ±0.20 and a control limit (3s) of ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

2.3 Wet, Fermented Samples

2.3.1 Toluene Determination of Moisture

References:

Moisture in Animal Feed by Distillation with Toluene (925.04) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Dewar, W.A. and P. McDonald. 1961. Determination of dry matter in silage by distillation with toluene. J. Sci. Agric. 12:790-795.

Fenner, Heinrich. 1984. Methods for determining fermentation products in acid preserved feeds and forages. Massachusetts Agricultural Experiment Station. Res. Bull. 691. 14pp.

Scope:

This procedure is recommended for determination of moisture in fermented feeds (haylages and silages) which contain high levels of volatile acids. Volatile acids and alcohols have low vaporization points and are lost when the sample is oven dried.

Basic Principle:

Water is distilled from sample and trapped under a layer of toluene.

Equipment:

250 mL pyrex flask
Bidwell-Sterling Moisture Receiver, calibrated to 0.01 mL by distilling known amounts of wa
from adhering to inner surface.
500 mm Liebig condenser
Toluene distillation hood

Reagents

Toluene, reagent grade

Safety Precautions:

- Use standard precautions when working with electrical equipment or glassware.
- Toluene is flammable. Observe proper precautions for flammable solvents. Avoid inhaling vapors. Avoid skin contact.

Procedure:

1) Weigh sufficient wet sample (at least 25g) to yield a minimum of 5 mL water, record weight (W_1) to nearest 1 mg, and transfer to 500 mL flask.
2) Add sufficient toluene to cover sample completely.
3) Immediately fill receiving tube with toluene, pouring it through top of condenser.

4) Bring to boil and distill slowly, ca. 2 drops/sec, until most of the water passes over, then increase rate of distillation to about 4 drops/sec. Distill 1hr (or longer if necessary for wetter samples) to obtain clearing at the top of the condenser.
5) When all water has apparently been distilled, wash down condenser by pouring toluene in at top and continue distillation for a short time (approximately 15 min) to ensure all water is distilled. If 0.1 mL of additional water is distilled in 15 minutes, repeat this step.
6) If water remains in the condenser after distillation is completed, wash it down with toluene.
7) Let receiving tube come to room temperature and read volume of water in lower layer of receiver and record volume (V) to nearest 0.01 mL.

Comments:

- To be accurate, distilled water should be analyzed for volatile acids and alcohol that can co-distill with water (see references).
- Toluene dry matter is approximately equivalent to partial drying (section 2.2.1.1 or 2.2.1.2) which leaves 3 to 5% moisture in the sample (which is roughly equivalent to volatile acids lost during drying) and is adequate for dry matter adjustment of crude protein and fiber.

Calculation: Percent Total Dry Matter

$$\% \text{ Total DM} = \left[1 - \frac{V}{W_1}\right] \times 100$$

Where V = Volume of water in mL
W_1 = weight of sample in grams

Calculation: Percent Total Moisture:

$$\% \text{ Total Moisture} = 100 - \% \text{ Total DM}$$

Quality Control:

Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for moisture or dry matter is about ±0.10, which results in a warning limit (2s) of about ±0.20 and a control limit (3s) of about ±0.30. Plot the results of the duplicate analyses on an R-control chart (Appendix D) and examine the chart for trends. Results outside the 95 percent confidence limits warn of possible problems with the analytical system. Results outside the 99 percent confidence limits indicate loss of control, and results of the run should be discarded. If more than five or six points in succession fall on one side or the other of the 50 percent line, it is a strong indication that something has changed and is cause for investigation.

3. Nitrogen and Crude Protein Determination

3.1 Nitrogen Determination by Kjeldahl (Rack)

References:

Protein (Crude) Determination in Animal Feed: Copper Catalyst Kjeldahl Method. (984.13) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Protein (Crude) Determination in Animal Feed: $CuSO_4/TiO_2$ Mixed Catalyst Kjeldahl Method. (988.05) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

The methods described are applicable for determination of nitrogen (N) in forages.

Basic Principle:

The Kjeldahl method is the standard method of nitrogen determination dating back to its development in the late 1800's. The method consists of three basic steps: 1) digestion of the sample in sulfuric acid with a catalyst, which results in conversion of nitrogen to ammonia; 2) distillation of the ammonia into a trapping solution; and 3) quantification of the ammonia by titration with a standard solution.

Equipment:

Kjeldahl flasks, 500 to 800 mL
Kjeldahl digestion unit with fume removal manifold
Kjeldahl distillation apparatus - Kjeldahl flask connected to distillation trap by rubber stopper. Distillation trap is connected to condenser with low-sulfur tubing. Outlet of condenser should be less than 4 mm diameter.
Erlenmeyer flask, 500 mL
Analytical balance, sensitive to 0.1 mg

Reagents:

Sulfuric acid, concentrated, 95-98%, reagent grade
Sodium hydroxide, pellets, flakes, or 45% solution with specific gravity ≥1.36 (low N)
dissolve 450 g in cool water and dilute to 1 L
Potassium sulfate (K_2SO_4), anhydrous
Copper sulfate ($CuSO_4$), anhydrous
Titanium dioxide (TiO_2)
Alundum, boiling stones, 8-14 mesh
Pumice
Methyl red indicator
dissolve 1 g methyl red (sodium salt) in 100 mL methanol or ethanol

Tributyl citrate (for antifoam) or paraffin or antifoam A or equivalent
Lysine monohydrochloride, reagent grade, dried at 110^{O}C for four hr
Hydrochloric acid standard solution, 0.5 N
Prepare by diluting 430.1 mL 36.5 to 38% HCl to 10 L with distilled water and standardize by method 3.1.1
Sodium hydroxide standard solution
Prepare 0.1 N sodium hydroxide (NaOH) solution and standardize by method 3.1.2.
After standardizing hydrochloric acid and sodium hydroxide, check one against the other by titrating one with the other and calculating normality.

Safety Precautions:

- Handle acid safely: use acid resistant fumehood. Always add acid to water unless otherwise directed in method. Wear face shield and heavy gloves to protect against splashes. If acids are spilled on skin, immediately wash with large amounts of water.
- Sulfuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated acid or alkali. Use effective fume removal device to protect against acid fumes or alkali dusts or vapors. Always add concentrated sulfuric acid or sodium hydroxide pellets to water, not vice versa. Concentrated sodium hydroxide can quickly and easily cause blindness. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.
- Keep baking soda and vinegar handy in case of chemical spills.
- The sulfur oxide fumes produced during digestion are hazardous to breathe. Do not inhale.
- Digests must be cool before dilution water is added to avoid a violent reaction during which the acid can shoot out of the flask. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

Procedure:

Digestion

1) Weigh approximately 1 g ground sample into digestion flask, recording weight (W) to nearest 0.1 mg. Include reagent blank and high purity lysine HCl as check of correctness of digestion parameters. Weigh a second subsample for laboratory dry matter determination.
2) Add 15 g potassium sulfate, 0.04 g anhydrous copper sulfate, 0.5 to 1.0 g alundum granules, or add 16.7 g K_2SO_4, 0.01 g anhydrous copper sulfate, 0.6 g TiO_2 and 0.3 g pumice. Then add 20 mL sulfuric acid. (Add additional 1.0 mL sulfuric acid for each 0.1 g fat or 0.2 g other organic matter if sample weight is greater than 1 g.)
3) Place flask on preheated burner (adjusted to bring 250 mL water at 25^{o}C to rolling boil in 5 min).

4) Heat until white fumes clear bulb of flask, swirl gently, and continue heating for 90 min for copper catalyst or 40 min for $CuSO_4/TiO_2$ mixed catalyst.
5) Cool, cautiously add 250 mL distilled water and cool to room temperature (<25°C). Note: If bumping occurs during distillation, volume of water may be increased to ca. 275 mL.

Distillation

6) Prepare titration flask by adding appropriate volume (V_{HCl}) accurately measured acid standard solution to amount of water so that condenser tip is immersed (try 15 mL acid and 70 mL water if undecided). For reagent blank, pipet 1 mL of acid and add approximately 85 mL water. Add 3 to 4 drops methyl red indicator solution.
7) Add 2 to 3 drops of tributyl citrate or other antifoam agent to digestion flask to reduce foaming.
8) Add another 0.5 to 1.0 g alundum granules.
9) Slowly down side of flask, add sufficient 45% sodium hydroxide solution (approximately 80 mL) to make mixture strongly alkali. (Do not mix until after flask is connected to distillation apparatus or ammonia will be lost.)
10) Immediately connect flask to distillation apparatus and distill at about 7.5 boil rate (temperature set to bring 250 mL water at 25°C to boil in 7.5 min) until at least 150 mL distillate is collected in titrating flask.
11) Remove digestion flask and titrating flask from unit, rinsing the condenser tube with distilled water as the flask is being removed.

Titration

12) Titrate excess acid with standard sodium hydroxide solution to orange endpoint (color change from red to orange to yellow) and record volume to nearest 0.01 mL (V_{NaOH}). Titrate the reagent blank (B) similarly.

Comments:

- Reagent proportions, heat input and digestion time are critical factors - do not change.
- Ratio of salt to acid (wt:vol) should be 1:1 at end of digestion for proper temperature control. Digestion may be incomplete at lower ratio; nitrogen may be lost at higher ratio. Each gram of fat consumes 10 mL sulfuric acid and each gram of carbohydrate consumes 4 mL sulfuric acid during digestion.
- Catalyst mixtures are commercially available in powdered or tablet form. Dispensers are available for convenient delivery of powdered catalyst mixtures.
- Check with local authorities for proper disposal procedures of copper containing waste solution.
- Include a reagent blank and at least one sample of high purity lysine hydrochloride in each day's run as check of correctness of digestion parameters. If digestion is not complete, make appropriate adjustments. A standard, such as NIST Standard

Reference Material No. 194, ammonium phosphate ($NH_4H_2PO_4$), certified 12.15%N should also be included.
Following is a list of acceptable standards available to include in Kjeldahl runs:

Standard	Theoretical Yield % nitrogen
Ammonium p-toluenesulfonate (Hach 22779-24)	7.402
Glycine p-toluenesulfonate (Hach 22780-24)	5.665
Nicotinic acid p-toluenesulfonate (Hach 22781-24)	4.743
Lysine monohydrochloride (Sigma L-5626 or Aldrich Gold Label)	15.34
Various ammonium salts	
Diammonium hydrogen phosphate (100% assay)	21.21
Ammonium chloride (100% assay)	26.18
Ammonium sulfate (100% assay)	21.20
Ammonium dihydrogen phosphate (NIST SRM 194)	12.15
Citrus leaves (NIST SRM 1572)	2.86
Urea (NIST SRM 2141)	46.63

The ammonium salts and glycine p-toluenesulfonate serve primarily as a check on distillation efficiency and accuracy in titration steps because they are digested very readily. Lysine and nicotinic acid are difficult to digest, therefore serve as a check on digestion efficiency.

Calculation: Percent Nitrogen (N)

$$\%N \text{ (DM basis)} = \frac{[(V_{HCl} \times N_{HCl}) - (V_{BK} \times N_{NaOH}) - (V_{NaOH} \times N_{NaOH})]}{W \times \text{Lab DM}/100} \times 1.4007$$

Where V_{NaOH} = mL standard NaOH needed to titrate sample
V_{HCl} = mL standard HCl pipetted into titrating flask for sample
N_{NaOH} = Normality of NaOH
N_{HCl} = Normality of HCl
V_{BK} = mL standard NaOH needed to titrate 1 mL standard HCl minus B
B = mL standard NaOH needed to titrate reagent blank carried through method and distilled into 1 mL standard HCl

1.4007 = milliequivalent weight of nitrogen x 100
W = sample weight in grams

Calculation: Percent Crude Protein (CP)

$$\text{CP (DM basis)} = \%\ \text{N (DM basis)} \times F$$

Where F = 6.25 for all forages and feeds except wheat grains
F = 5.70 for wheat grains

Quality Control:

Include a reagent blank, one sample of high purity lysine hydrochloride, and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for crude protein ranges from about ±0.10 for samples with 10% CP to ±0.20 for samples with 20% CP, which results in warning limits (2s) ranging from ±0.20 to 0.40 and control limits (3s) ranging from ±0.30 to 0.60. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

3.1A Addendum to Nitrogen Determination by Kjeldahl (Rack): Options for Experienced Laboratories

References:

Protein (Crude) Determination in Animal Feed: Copper Catalyst Kjeldahl Method. (984.13) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Protein (Crude) in Animal Feed: $CuSO_4/TiO_2$ Mixed Catalyst Kjeldahl Method. (988.05) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Nitrogen (Total) in Fertilizers: Kjeldahl Method (Mercury). (955.04) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Nitrogen (Total) in Milk. (991.20) Official Methods of Analysis. 2nd supplement. 1991 Association of Official Analytical Chemists. 15th Edition.

Scope:

A number of alternatives to the original procedure are described; alternatives used will depend on sample type, the user of analytical data, equipment availability, and environmental considerations.

Basic Principle:

A number of alternatives to the method have evolved: several catalysts other than copper are available for digestion. Choice of catalyst will depend on the difficulty of breakdown of the peptides in the sample protein to be analyzed and environmental problems associated with the disposal of the waste containing the catalyst. If necessary, the digestion can also be modified to reduce nitrates to ammonia, which might otherwise not be recovered, by the addition of salicylic acid and a reducing agent.

A distillation/titration alternative utilizes a weak acid (boric acid) as the trapping solution. The ammonia is then quantified by titration with a standard strong acid (hydrochloric acid).

Additional Equipment:

None.

Additional Reagents:

Alternative catalyst

Mercury catalyst

Mercuric oxide (HgO) or metallic mercury (Hg), reagent grade, N-free

Potassium sulfate (K_2SO_4) or anhydrous sodium sulfate (Na_2SO_4), reagent grade, N-free

Alternative trapping solution (with indicator)/titrant

Boric acid solution, 4%

dissolve 400 g boric acid (H_3BO_3) in distilled water containing
70 mL 0.1% alcoholic solution of methyl red and
100 mL 0.1% alcoholic solution of bromocresol green and
dilute to 10 L with distilled water.

Standard hydrochloric acid solution, 0.2 N

Prepare by diluting 172 mL 36.5 to 38% HCl to 10 L with distilled water and standardize by method 3.1.1

Option for nitrate containing samples:

Salicylic acid, reagent grade, N-free

Reducing agent (one of the following)

a) Sodium metabisulfite, ($Na_2S_2O_3 \cdot 5H_20$)

b) Zinc dust, impalpable powder

Potassium nitrate, NIST SRM 193, dried at 110°C for 2 hr (used to check nitrate reduction)

Sulfide or thiosulfate solution (when mercury catalyst is used)

Dissolve 40 g commercial potassium sulfide (K_2S) in 1 L water (solution of 40 g Na_2S or 80 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_20$) in 1 L water may be used)

Safety Precautions:

- Handle acid safely: Use acid-resistant fumehood; always add acid to water unless otherwise directed in method; wear face shield and heavy rubber gloves to protect against splashes; if acids are spilled on skin, immediately wash with large amounts of water
- Sulfuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated acid or alkali. Use effective fume removal device to protect against acid fumes or alkali dusts or vapors. Always add concentrated sulfuric acid or sodium hydroxide pellets to water, not vice versa. Concentrated sodium hydroxide can quickly and easily cause blindness. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.

- Mercury in contact with ammonia, halogens and alkali can produce extremely toxic and cumulative vapors. Regard spills as extremely hazardous and clean up promptly. Powdered sulfur sprinkled over spilled mercury can assist in cleaning up spills. A high degree of personal cleanliness is necessary for persons who use mercury. Use skin and respiratory protection when dry mercuric salts are to be used.
- The sulfur oxide fumes produced during digestion are hazardous to inhale.
- Digests must be cool before dilution water is added to avoid a violent reaction during which the acid can shoot out of the flask. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

Procedure:

Digestion Alternatives

1) Weigh approximately 1 g ground sample into digestion flask, recording weight (W) to nearest 1.0 mg. Weight range should depend on nitrogen content of sample. Weigh a second subsample for laboratory dry matter determination.
2) Add one of the following catalysts:
   Option a) Mercury: add 0.7 g HgO or 0.65 g Hg, 15 g K_2SO_4 or anhydrous Na_2SO_4
   Option b) $CuSO_4/TiO_2$: add 16.7 g K_2SO_4, 0.01 g anhydrous $CuSO_4$, 0.6 g TiO_2
   Option c) Copper: add 15 g K_2SO_4, 0.04 g anhydrous $CuSO_4$
3) Add 3 g pumice or 0.5 to 1.0 g alundum granules, and 20 mL concentrated sulfuric acid. (Add additional 1.0 mL sulfuric acid for each 0.1 g fat or 0.2 g other organic matter if sample weight is >1 g.)
4) Place flask on preheated burner (adjusted to bring 250 mL at 25^{o}C water to rolling boil in 5 min).
5) When white fumes clear bulb of flask swirl gently and continue heating
   (for option a) until 30 min after clearing.
   (for option b) 40 min.
   (for option c) 90 min.
6) Cool, cautiously add 250 mL distilled water and cool to room temperature (<25^{o}C). Note: add water as soon as possible to reduce amount of caking.

NOTE: If recovery of nitrate nitrogen is of concern, replace steps 2 and 3 with 2A and 3A:

2A) Add 40 ml H_2SO_4 containing 2 g dissolved salicylic acid to each flask. Shake until thoroughly mixed and let stand, with occasional shaking, at least 30 min. Transfer to a fume hood and then add either 5 g $Na_2S_2O_3 \cdot 5H_20$ or 2 g zinc dust (as impalpable powder, not granulated zinc or filings). Shake and let stand 5 min, then heat slowly until frothing ceases and white fumes appear (ca 10 min).

3A) Turn off heat and add boiling chips (3 g pumice or 0.5 to 1.0 g alundum granules) and one of the following catalysts:

Option a) Mercury: add 0.7 g H_2O or 0.65 g Hg, 15 g K_2SO_4 or anhydrous Na_2SO_4
Option b) $CuSO_4/TiO_2$: add 16.7 g K_2SO_4, 0.01 g anhydrous $CuSO_4$, 0.6 g TiO_2
Option c) Copper: add 15 g K_2SO_4, 0.04 g anhydrous $CuSO_4$

Alternative Distillation and Titration

7) (For boric acid receiving solution) Place 250 mL titrating flask containing 25 mL boric acid solution with mixed indicator so that tube of condenser is immersed below surface of absorbing solution.
8) Add 2 to 3 drops of tributyl citrate to digestion flask to reduce foaming.
9) Add another 0.5 to 1.0 g alundum granules.
10) If mercury catalyst was used, add 25 mL of the sulfide or thiosulfate solution and mix to precipitate mercury.
11) Slowly down side of flask, add sufficient 45% sodium hydroxide solution (approximately 80 mL) to make mixture strongly alkali. Do not mix.
12) Immediately connect flask to distillation apparatus, swirl to mix contents and distill at about 7.5 boil rate until ≥ 150 mL distillate is collected in titrating flask. If excessive bumping occurs during distillation, increase dilution water from 250 mL to 300 mL.
13) Remove titrating flask from unit, rinsing the condenser tube with distilled water as the flask is being removed.
14) Titrate with 0.2 N HCl to neutral gray endpoint and record volume to nearest 0.1 mL (V_A). Titrate reagent blank (V_B) similarly. (Color change is green to gray to purple.)

Comments:

- Reagent proportions, heat input and digestion time are critical factors - do not change.
- Choice of catalyst will depend on the difficulty of peptide breakdown in the sample protein, environmental problems, and costs associated with disposal of the catalyst.
- Commercial preparations are available for the 4% boric acid indicator solution and HgO/K_2SO_4 , $CuSO_4/TiO_2/K_2SO_4$ and $CuSO_4/K_2SO_4$ catalysts.
- The sulfuric acid/salicylic acid solution is sensitive to light and air and has a very short shelf life (approximately 2 days).
- Include a reagent blank and at least one sample of high purity lysine hydrochloride in each day's run as check of correctness of digestion parameters. If digestion is not complete, make appropriate adjustments. A standard, such as NIST Standard Reference Material No 194, ammonium phosphate ($NH_4H_2PO_4$), certified 12.15% nitrogen should also be included. If nitrate recovery is critical, a sample of potassium nitrate (KNO_3) should also be added.

Following is a list of some standards available to include in Kjeldahl runs:

Standard	Theoretical Yield % nitrogen
Ammonium p-toluenesulfonate (Hach 22779-24)	7.402
Glycine p-toluenesulfonate (Hach 22780-24)	5.665
Nicotinic acid p-toluenesulfonate (Hach 22781-24)	4.743
Lysine monohydrochloride (Sigma L-5626 or Aldrich Gold Label)	15.34
Various ammonium salts	
Diammonium hydrogen phosphate (100% assay)	21.21
Ammonium chloride (100% assay)	26.18
Ammonium sulfate (100% assay)	21.20
Ammonium dihydrogen phosphate (NIST SRM 194)	12.15
Citrus leaves (NIST SRM 1572)	2.86
Urea (NIST SRM 2141)	46.63
Potassium nitrate (NIST SRM 193)	13.85

The ammonium salts and glycine p-toluenesulfonate serve primarily as a check on distillation efficiency and accuracy in titration steps because they are digested very readily. Lysine and nicotinic acid are difficult to digest, therefore serve as a check on digestion efficiency.

Calculation: Percent Nitrogen (N)

For boric acid trapping solution/standard HCl titrant:

$$\% \text{ N (DM basis)} = \frac{(V_A - V_B) \times N_{HCl} \times 1.4007}{W \times \text{Lab DM}/100}$$

Where V_A = Volume, in mL, of standard HCl required for sample
V_B = Volume, in mL, of standard HCl required for blank
N_{HCl} = Normality of standard HCl
1.4007 = milliequivalent weight of N X 100
W = sample weight in grams

Calculation: Percent Crude Protein (CP)

$$CP\ (DM\ basis) = \%\ N\ (DM\ basis)\ X\ F$$

Where F = 6.25 for all forages and feeds except wheat grains
F = 5.70 for wheat grains

Quality Control:

Include a reagent blank, one sample of high purity lysine hydrochloride, and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for crude protein ranges from about ±0.10 for samples with 10% CP to ±0.20 for samples with 20% CP, which results in warning limits (2s) ranging from ±0.20 to 0.40 and control limits (3s) ranging from ±0.30 to 0.60. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

3.1.1 Standardization of Hydrochloric Acid Standard Solution

Reference:

Standard Solution of Hydrochloric Acid. (936.15) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This method is applicable for the preparation and standardization of hydrochloric acid solution.

Basic Principle:

An acidic solution is titrated with a standardized base solution to determine normality.

Equipment:

Buret, 50 mL, graduated to 0.1 mL
Illuminated magnetic stirrer
Volumetric pipet, 40 mL, class A

Reagents:

Hydrochloric acid, concentrated, 36.5 to 38%, reagent grade
Sodium hydroxide, standard solution (see method 3.1.2)
Distilled water, carbon dioxide (CO_2) free prepared either 1) by boiling for 20 min and cooling with soda-lime protection or 2) by bubbling air, freed from CO_2 by passing through tower of soda lime, through water for 12 hr.
Indicator, same as used in Kjeldahl method (3.1)

Safety Precautions:

- Handle acid safely: Use acid-resistant fume hood; always add acid to water unless otherwise directed in method; wear face shield and heavy rubber gloves to protect against splashes; if acids are spilled on skin, immediately wash with large amounts of water.

Procedure:

Preparation

1. Dilute appropriate volume of 36.5 to 38% HCl to 10 L with carbonate-free distilled water as indicated below and mix well:

Desired Normality	mL HCl to dilute to 10 L
0.01	8.6
0.02	17.2

0.10	86.0
0.20	172.0
0.50	430.1
1.0	860.1

Standardization

1. Fill a 40 mL volumetric pipet with the acid to be standardized and discard to rinse the pipet.
2. Withdraw a 40 mL aliquot of the HCl into the volumetric pipet. Wipe the tip of the pipet before transferring the acid into a 250 or 300 mL Erlenmeyer that has been rinsed with CO_2-free H_2O. Do this in triplicate.
3. Rinse a Schellbach buret with standardized NaOH of approximately same concentration as acid to be standardized. Fill the buret and wait for at least a minute (to allow the solution to drain down the inside walls) before taking an initial reading. Always read the buret at eye level and at the tip of the meniscus.
4. Add indicator used in Kjeldahl method to the acid solution and titrate, a drop at a time, with NaOH while stirring.
5. Titrate to orange endpoint (color change from red to orange to yellow). Use of an illuminated background is helpful. When the endpoint is reached, remove any drops from the buret tip (by touching the flask to the tip) and wash down the sides of the Erlenmeyer with CO_2-free H_2O to see if the color persists. If it doesn't, add another partial drop of NaOH and check the color before reading the buret.
6. Calculate normality and adjust as necessary with HCl or water. Mix thoroughly and recheck standardization as described above.
7. Record standardization in log book.

Comments

- The titration values of replicates should be within 0.05 mL of each other.
- Normality will be exact only if the same indicator is used in determination as in standardization.
- Several other standardization procedures are available. See AOAC. 1990. Official Methods of Analysis. 15th Ed. 936.15.
- Tris-hydroxymethyl-aminomethane (THAM), primary base standard, may be substituted for standardized NaOH in steps 3 and 4.

Calculations:

$$\text{Normality of HCL} = \frac{\text{mL of Base Std} \times \text{Normality of Base Std}}{\text{mL HCl in the flask}}$$

Base Std = NaOH or THAM primary base standard

If the normality is too high, dilute solution to required normality by following formula:

$$V_1 = V_2 \times N_2 / N_1$$

Where N_2 and V_2 represent normality and volume of stock solution and V_1 equals volume to which stock solution should be diluted to obtain desired normality, N_1.

3.1.2 Standardization of Sodium Hydroxide Standard Solution

Reference:

Standard Solution of Sodium Hydroxide (936.16) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This method is applicable for the preparation and standardization of standard sodium hydroxide solution.

Basic Principle:

A basic solution is titrated with a standardized acidic solution to determine normality.

Equipment:

Buret, 50 mL, graduated to 0.1 mL
Illuminated magnetic stirrer
Volumetric pipet, 40 mL, class A
Analytical balance, sensitive to 0.1 mg
Container of alkali resistant glass
pH meter with glass electrode (alternate to using phenolphthalein indicator)

Reagents:

Distilled water, carbon dioxide (CO_2) free prepared either 1) by boiling for 20 min and cooling with soda-lime protection or 2) by bubbling air, freed from CO_2 by passing through tower of soda lime, through water for 12 hr.

Sodium hydroxide solution, to 1 part reagent grade NaOH add 1 part distilled, carbon dioxide-free water by weight.

Acid potassium phthalate, NIST SRM for Acidimetry 84, dry for 2 hr at 120°C and cool in desiccator.

Buffer solution, pH 8.6, 12.00 mL 0.2 N NaOH added to 50 mL 0.2M boric acid/potassium chloride solution made as follows:

Boric acid-potassium chloride solution - dry boric acid (H_3BO_3) to constant weight in desiccator over $CaCl_2$. Dry potassium chloride (KCl) 2 days in oven at 115 to 120°C. Dissolve 12.405 g H_3BO_3 and 14.912 g KCl in water and dilute to 1 L.

Phenolphthalein indicator, 1%,
dissolve 1 g phenolphthalein in 100 mL 95% ethanol.

Safety Precautions:

- Alkalis can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated alkali; if spilled on skin wash with copius amounts of water. Use effective fume removal device to protect against alkali dusts or vapors. Always add sodium hydroxide pellets to water, not vice versa.

Procedure:

Preparation

1) Add appropriate volume of NaOH solution (1 to 1) to CO_2-free distilled water necessary to make 10 L of solution:

Desired Normality	mL NaOH to be diluted to 10 L
0.01	5.4
0.02	10.8
0.10	54.0
0.20	108.0
0.50	270.0
1.0	540.0

Standardize

1) Accurately weigh enough dried acid potassium phthalate (ca. 0.4 g) to titrate about 40 mL of NaOH solution and transfer to 300 mL flask.
2) Add 50 mL CO_2-free water, stopper flask and swirl until sample dissolves.
3) Titrate to pH 8.6 with solution being standardized, taking precautions to exclude CO_2 and using as indicator either glass-electrode or phenolphthalein. If using indicator, add 3 drops phenolphthalein to a flask containing 50 mL of pH 8.6 buffer and stopper. This flask is used as the reference endpoint for a pH 8.6 titration.
4) Determine volume NaOH required to produce endpoint of blank by matching color in another flask containing 3 drops phenolphthalein and same volume (50 mL) CO_2-free water.
5) Subtract volume required to titrate blank from that used to titrate the potassium acid phthalate and calculate normality. Normality should be slightly high.
6) Adjust to desired concentration, mix well, and recheck standardization.
7) Record final standardization in logbook.

Calculations:

$$\text{Normality} = \frac{\text{g } KHC_8H_4O_4 \text{ X } 1000}{\text{mL NaOH X } 204.229}$$

Adjust to desired concentration by following formula: $V_1 = V_2 \text{ X } N_2 / N_1$

Where N_2 and V_2 represent normality and volume of stock solution and V_1 equals volume to which stock solution should be diluted to obtain desired normality, N_1.

3.2 Nitrogen Determination by Kjeldahl (Block Digestion)

Reference:

Protein (Crude) in Animal Feed: Semiautomated Method. (976.06) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Protein (Crude) Determination in Animal Feed: Copper Catalyst Kjeldahl Method. (984.13) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Crude Protein in Meat: Block Digestion Method. (981.10) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Protein (Crude) in Animal: Semiautomated Method - Alternative System. (990.02) Official Methods of Analysis. 1st Supplement. 1990. Association of Official Analytical Chemists. 15th Edition.

Nitrogen (Total) in Milk. (991.20) Official Methods of Analysis. 2nd supplement. 1991 Association of Official Analytical Chemists. 15th Edition.

Scope:

This method is applicable for the determination of nitrogen (N) in all types of forages and feeds.

Basic Principle:

The Kjeldahl method (macro) is the standard method of nitrogen determination. The original "rack" method was improved in 1970 by the introduction of aluminum block heaters which greatly increased efficiency of the digestion and further improved in 1974 by the introduction of steam distillation.

The "block" method consists of:

1) digestion of the sample in sulfuric acid with a catalyst, which results in conversion
2) determination of ammonia, either
    a) colorimetrically on an autoanalyzer or
    b) by steam distillation and titration.

Several catalysts are available for digestion, including mercury, copper, and copper/titanium. Choice of catalyst will depend on the difficulty of breakdown of the peptides in the sample protein to be analyzed and environmental problems associated with the disposal of the waste containing the catalyst.

The ammonia can be determined colorimetrically by heating with salicylate and hypochlorite to produce blue color which is proportional to the ammonia concentration. The color is intensified by adding sodium nitroprusside. Tartrate is added to the buffer to prevent precipitation of calcium and magnesium.

If the ammonia is determined by titration, it can be distilled into either of two types of trapping solutions which require different titrants:

1) the ammonia can be trapped in a known amount of standard, strong acid (HCl); the excess acid is back-titrated with a standard base (NaOH)

2) the ammonia can be trapped in a weak acid (boric acid) and titrated with a standard, strong acid (HCl).

Whatever alternatives are selected, method checks should be performed to assure applicability of the method for samples to be analyzed and that the analytical system is functioning to provide reliable data.

Equipment:

Block digester, capable of maintaining 410°C and digesting 20 samples at a time in 250 mL calibrated volumetric tubes constricted at the top. Block must be equipped with removable shields to enclose exposed area of tubes completely at or above height of constriction.
Fume hood, acid
Weighing paper, nitrogen-free, 7 cm (optional)
Analytical balance, sensitive to 0.1 mg
Steam distillation apparatus - digestion tube connected to distillation trap by rubber stopper. Distillation trap is connected to condenser with low-sulfur tubing. Outlet of condenser should be less than 4 mm diameter.
Automatic analyzer (for colorimetric quantification)

Reagents:

Digestion

Sulfuric acid, 95-98%, reagent grade
Mercury catalyst tablets (or alternative catalyst)
Lysine monohydrochloride, reagent grade, dried at 110°C for four hr

Colorimetric quantification

Sodium hypochlorite solution, Dilute 6 mL commercial bleach solution containing 5.25% available Cl (Chlorox or equivalent) to 100 mL with water and mix. Prepare fresh daily.
Wetting agent for Traacs 800 Autoanalyzer, add 50 mL methanol to 50 mL Triton X-100.
Wetting agent for (Braun & Leubbe) AAII Autoanalyzer, Brij-35
Sodium chloride-sulfuric acid solution, dissolve 100 g NaCl in water, add 7.5 mL H_2SO_4, and 1 mL wetting agent, dilute to 1 L with water, and mix.

Sodium salicylate-sodium nitroprusside solution, dissolve 150 g sodium salicylate ($NaC_7H_5O_3$) and 0.3 g sodium nitroprusside ($Na_2Fe(CN)_5 \cdot NO \cdot 2H_2O$) in 600 mL water,dilute to 1 L and mix. Vacuum filter through 0.45 μm porosity filter and transfer to light-resistant container.

Phosphate-tartrate buffer solution, pH 14.0, dissolve 50 g sodium potassium tartrate and 26.8 g sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$) in 600 mL water. Cool, dilute to 1 L with water and mix.

Sampler wash solution, 6% sulfuric acid, dissolve 60 mL H_2SO_4 in 800 mL water, cool, dilute to 1 L and mix.

Nitrogen standard solutions, dry ammonium sulfate [$(NH_4)_2SO_4$] primary standard for 2 hr at 105°C and prepare 6 standards by weighing to nearest 0.1 mg, 0, 60, 120, 180, 240, and 300 mg (each ± 10 mg) into digeston flask. Digest standards as described on page 63 beginning with step 2. Assume a theoretical value of 21.20% N and calculate the mg N/250 mL.

Distillation/titration quantification

Base (one of the following)

a) Sodium hydroxide, 45% w/w solution (for all catalysts except mercury)
Dissolve 2250 g low N NaOH and dilute to 5 L

b) Sodium hydroxide - Potassium sulfide solution (for mercury catalyst)
Dissolve 400 g low N NaOH in water and, while still warm, dissolve 30 g potassium sulfide (K_2S) in solution and dilute to 1 L

Trapping solution (one of the following)

a) Boric acid solution, 4%
dissolve 400 g boric acid (H_3BO_3) in distilled water containing
70 mL 0.1% alcoholic solution of methyl red and
100 mL 0.1% alcoholic solution of bromocresol green
dilute to 10 L with distilled water.

b) Hydrochloric acid standard solution, 0.5N
Prepare 0.5N standard acid solution by diluting 430.1 mL 36.5 to 38% HCl to 10 L with distilled water and standardize by method 3.1.1

Titrating solution (one of the following)

a) For boric acid trapping solution
Prepare 0.2 N standard hydrochloric acid solution by diluting 172 mL 36.5 to 30% HCl to 10 L with distilled water and standardize by method 3.1.1

b) For standard acid trapping solution
Prepare 0.1N sodium hydroxide (NaOH) solution by method 3.1.2

After standardizing acid and base, check one against the other by titrating one with the other and recalculating normality.

Indicator (one of the following)

a) Methyl red indicator
   dissolve 1 g methyl red (sodium salt) in 100 mL methanol

b) Methyl red - Bromocresol green
   see Boric Acid Solution

Safety Precautions:

- Handle acid safely: Use acid-resistant fumehood; always add acid to water unless otherwise directed in method; wear face shield and heavy rubber gloves to protect against splashes; if acids are spilled on skin, immediately wash with large amounts of water
- Sulfuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated acid or alkali. Use effective fume removal device to protect against acid fumes or alkali dusts or vapors. Always add concentrated sulfuric acid or sodium hydroxide pellets to water, not vice versa. Concentrated sodium hydroxide can quickly and easily cause blindness. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.
- Mercury in contact with ammonia, halogens and alkali can produce extremely toxic and cumulative vapors. Regard spills as extremely hazardous and clean up promptly. Powdered sulfur sprinkled over spilled mercury can assist in cleaning up spills. A high degree of personal cleanliness is necessary for persons who use mercury. Use skin and respiratory protection when dry mercuric salts are to be used.
- The sulfur oxide fumes produced during digestion are hazardous to inhale.
- Digests must be cool before dilution water is added to avoid a violent reaction during which the acid can shoot out of the flask. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

Procedure:

Digestion

1) Weigh ground sample into digestion tube, recording weight (W) to nearest 0.1 mg. Weight range should depend on protein content of sample as follows:

Protein, %	Sample, g
6 to 24	1.5±0.1
25 to 40	1.0±0.1
41 to 50	0.8±0.1
51 to 60	0.7±0.1
61 to 90	0.5±0.1
>90	Weigh sample equal to 50 mg N

Include reagent blank and high purity lysine as check of correctness of digestion parameters. Weigh a second subsample for laboratory dry matter determination.

2) Place in a fume hood. Add sufficient catalyst tablets to supply 9 g K_2SO_4 and 0.42 g HgO (or appropriate amount of alternative catalyst). Then add 15 mL sulfuric acid.
3) Place tubes in block digester preheated to 410°C. (Digester must be equipped with an exhaust system and/or placed in an acid fume hood.) Digest about 45 min.
4) Remove tubes and let cool about 10 min in a fume hood. Time will depend upon airflow around tubes. Direct rapid spray or stream of deionized water to the bottom of each tube to dissolve acid digest completely (total volume of 50 to 75 mL if using distillation/titration quantification).

Option A: Colorimetric quantification (automated ammonia determination)

5) Let cool, dilute to volume, and mix thoroughly. Transfer portion of each sample solution to analyzer beaker.
6) Place standards in tray in increasing order of concentration, followed by group of samples. Analyze lowest concentration standard in duplicate, discarding first peak. Follow each group of samples with standard references to correct for possible drift.
7) Plot mg N/250 mL vs average peak height of the two standards and determine mg N/250 mL for each sample.

Option B: Distillation/titration quantification

5) Place NaOH-K_2S (for mercury catalyst) or NaOH (for alternative catalyst) in alkali tank of steam distillation unit. Make sure that sufficient NaOH is dispensed from unit to neutralize all acid in tube (about 50 mL) before conducting distillation.
6) Place 250 mL tritration flask containing trapping solution and indicator on the receiving platform, with tube from the condenser extending below the surface of the trapping solution. The trapping solution will be either:
    a) about 25 mL 4% boric acid containing indicator
    b) appropriate volume (approximately 15 mL), accurately measured to nearest 0.1 mL (V_{HCl}) standard HCl solution and sufficient water to insure that the end of the condenser tube is submerged. Also add 3 to 4 drops methyl red indicator.
7) Attach digestion tube containing diluted, cooled digest to distillation unit.
8) Dispense appropriate volume of base solution.
9) Steam distill until 100-125 mL distillate collects.
10) Remove titrating flask from unit, rinsing condenser tip with water.
11) (For boric acid trapping solution)
    Titrate trapping solution with 0.2 N HCl to neutral gray endpoint. Record volume of acid (V_{HCl}) required to nearest 0.01 mL. Titrate reagent blank (V_B) similarly. Color change is green to gray to purple.

-or-

11) (For HCl trapping solution)
    Titrate excess HCl with standard sodium hydroxide solution to orange endpoint. (Color

change from red to orange to yellow). Record volume (V_{NaOH}) of sodium hydroxide to titrate acid to nearest 0.01 mL. Titrate reagent blank (B) similarly.

Comments:

- Include a reagent blank and at least one sample of high purity lysine hydrochloride in each day's run as check of correctness of digestion parameters. If digestion is not complete, make appropriate adjustments. A standard, such as NIST Standard Reference Material No. 194, ammonium phosphate ($NH_4H_2PO_4$), certified 12.15% N should also be included. Following is a list of some standards:

Standard	Theoretical Yield % nitrogen
Ammonium p-toluenesulfonate (Hach 22779-24)	7.402
Glycine p-toluenesulfonate (Hach 22780-24)	5.665
Nicotinic acid p-toluenesulfonate (Hach 22781-24)	4.743
Lysine monohydrochloride (Sigma L-5626 or Aldrich Gold label)	15.34
Various ammonium salts	
Diammonium hydrogen phosphate (100% assay)	21.21
Ammonium chloride (100% assay)	26.18
Ammonium sulfate (100% assay)	21.20
Ammonium dihydrogen phosphate (NIST SRM 194)	12.15
Citrus leaves (NIST SRM 1572)	2.86
Urea (NIST SRM 2141)	46.63

  The ammonium salts and glycine p-toluenesulfonate serve primarily as a check on distillation efficiency and accuracy in titration steps because they are digested very readily. Lysine and nicotinic acid are difficult to digest; therefore they serve as a check on digestion efficiency.
- Reagent proportions, heat input and digestion time are critical factors - do not change.
- Ratio of salt to acid (wt:vol) should be 1:1 at end of digestion for proper temperature control. Digestion may be incomplete at lower ratio; nitrogen may be lost at higher ratio. Each gram of fat consumes 10 mL sulfuric acid and each gram of carbohydrate consumes 4 mL sulfuric acid during digestion.
- Catalyst mixtures are commercially available in powdered or tablet form. Dispensers are available for convenient delivery of powdered catalyst mixtures.
- When using mercury catalyst, sodium thiosulfate can be added independently, rather than in the 45% NaOH, before distillation; however it must be added immediately before distillation to avoid production of H_2S gas. If added independently, add 15 mL of 8% $Na_2S_2O_3$ solution.
- Mercury containing Kjeldahl waste cannot be disposed directly to a sanitary sewer.

- Alternative catalysts are available, although not listed in AOAC Official Methods of

Analysis. Two examples are: copper catalyst tablets (each tablet contains 0.35 g K_2SO_4 and 0.1 g $CuSO_4$) and selenium catalyst tablets (each tablet contains 3.5 g K_2SO_4 and 0.035 g Se). When using these catalysts, increase digestion time to approximately 60 min.

Calculation: Percent Nitrogen (N)

For colorimetric determination:

$$\%N \text{ (DM basis)} = \frac{[(\text{mgN} / 250 \text{ mL from graph}) \times 100]}{W \times 1000 \times \text{Lab DM}/100}$$

Where W = sample weight in grams
1000 = conversion factor for grams to mg

For standard sodium hydroxide titrant:

$$\%N \text{ (DM basis)} = \frac{[(V_{HCl} \times N_{HCl}) - (B \times N_{NaOH}) - (V_{NaOH} \times N_{NaOH})] \times 1.4007}{W \times \text{Lab DM}/100}$$

Where
- V_{NaOH} = mL standard NaOH to titrate sample
- V_{HCl} = mL standard HCl pipetted into titrating flask for sample
- N_{NaOH} = Normality of NaOH
- N_{HCl} = Normality of HCl
- B = mL standard NaOH needed to titrate 1 mL standard HCl minus V_{BK}
- V_{BK} = mL standard NaOH needed to titrate reagent blank carried through method and distilled into 1 mL standard HCl
- 1.4007 = milliequivalent weight of nitrogen X 100
- W = weight of sample in grams

For standard HCl titrant:

$$\% \text{ N (DM basis)} = \frac{(V_A - V_B) \times N_{HCl} \times 1.4007}{W \times \text{Lab DM}/100}$$

Where V_A = Volume, in mL, of standard HCl required for sample
V_B = Volume, in mL, of standard HCl required for blank
N_{HCl} = Normality of acid standard
1.4007 = milliequivalent weight of N X 100
W = sample weight in grams

Calculation: Percent Crude Protein (CP)

$$\text{CP (DM basis)} = \% \text{ N (DM basis} \times F$$

Where F = 6.25 for all forages and feeds except wheat grains
F = 5.70 for wheat grains

Quality Control:

Include a reagent blank, one sample of high purity lysine hydrochloride, and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for crude protein ranges from about ±0.10 for samples with 10% CP to ±0.20 for samples with 20% CP, which results in warning limits (2s) ranging from ±0.20 to 0.40 and control limits (3s) ranging from ±0.30 to 0.60. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

3.3 Nitrogen Determination by Combustion MethodError! Bookmark not defined.

Reference:

Protein (Crude) in Animal Feed: Combustion Method. (990.03) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This method is applicable for the determination of nitrogen in all types of forages.

Basic Principle:

Nitrogen freed by combustion at high temperature in pure oxygen is measured by thermal conductivity detection and converted to equivalent protein by appropriate numerical factor.

Equipment:

Any instrument or device designed to measure nitrogen by combustion may be used which is equipped to provide following conditions:

a) Furnace to maintain minimum operating temperature of 950^{o}C for pyrolysis of sample in pure (99.9%) oxygen. Some systems may require higher temperature.

b) Isolation system to isolate liberated nitrogen gas from other combustion products for subsequent measurement by thermal conductivity detector. Device for converting NO_2 products to N_2 or measuring N as NO_2 may be required and included in the system.

c) Detection system to interpret detector response as % nitrogen (weight/weight). May include features such as calibration on standard material, blank determination and barometric pressure compensation. Any required calibration must be based on theoretical % nitrogen in pure primary standard organic material such as NIST SRM Uric Acid 913 or EDTA.

Safety Precautions:

- Follow manufacturer's recommendation for safe operation of instrument.
- Secure compressed gas cylinders and use proper gas regulators.

Procedure:

Operate instrument according to manufacturer's instructions; following are generalized instructions.

1) Turn furnaces on (or take off standby).
2) Turn gas regulators to desired flow rate.
3) Wait until furnaces have stabilized at desired temperature.
4) Enter sample number on console.
5) Enter other parameters as required by computer software.
6) Enter appropriate N content of pure primary standard.
7) Include two blanks and three dried or dessicated pure primary standards at the beginning of each run to calculate the calibration factor for determining N.

8) Weigh samples and transfer to autosampler tray. Weigh a second subsample to determine laboratory dry matter.
9) Run samples.

Comments:

- System must be capable of measuring nitrogen in feed materials containing 0.2 to 20% nitrogen.
- Suitable fineness of grind is that which gives relative standard deviation (RSD) ≤2.0% for 10 successive determinations of nitrogen in mixture of corn grain and soybeans (2/3and 1/3) that has been ground for analysis. RSD, % = (standard deviation divided by mean %N) times 100. Fineness of grind (about 0.5 mm) required to achieve this precision must be used for all mixed feeds and other nonhomogeneous materials.

Calculation: Percent Nitrogen (N)

$$\%\ N\ (DM\ basis) = \frac{\%\ N\ (from\ analyzer\ output)}{Lab\ DM/100}$$

Calculation: Percent Crude Protein (CP)

$$CP\ (DM\ basis) = \%\ N\ (DM\ basis) \times F$$

Where F = 6.25 for all forages and feeds except wheat grains
F = 5.70 for wheat grains

Quality Control:

Include a reagent blank, one sample of NIST SRM Uric Acid 913, and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

Accuracy of system is demonstrated by making 10 successive determinations of nitrogen in nicotinic acid and 10 successive determinations in lysine·monohydrochloride. Means of determinations must be within ±0.15 of the respective theoretical values, with standard deviations ≤0.15. Standard tryptophan may be substituted for lysine·monohydrochloride.

An acceptable average standard deviation among replicated analyses for crude protein ranges from about ±0.10 for samples with 10% CP to ±0.20 for samples with 20% CP, which results in warning limits (2s) ranging from ±0.20 to 0.40 and control limits (3s)ranging from ±0.30 to 0.60. Plot the results of the control sample(s) on a X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

3.4 Crude Protein by Near Infrared Reflectance Spectroscopy

Reference:

Fiber (Acid Detergent) and Protein (Crude) in Animal Feed and Forages: Near-infrared Reflectance Spectroscopic Method. (989.03) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Martin, G.C., J.S. Shenk, and F.E. Barton II. 1989. Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality. United States Department of Agriculture, Agricultural Research Service. Agricultural Handbook No. 643.

Scope:

This procedure is applicable for determining protein of ground, air-dry or partially dried (90 to 95% dry matter) forage. Samples must be ground through cyclone grinder with 1 mm screen and be 90 to 95% dry matter.

Basic Principle:

Random portions of a sample are loaded into a NIR sample holder and reflected light from the ground sample is measured in the infrared region (generally 1100 to 2500 nm). Instrument is part of a system that has been calibrated using representative samples from population to be tested. Equations selected based on calibration statistics, which have been validated, are used to calculate crude protein content of feed and forage samples.

Equipment:

Near infrared reflectance spectrophotometer, wavelength range at least 1100 to 2500 nm
Sample holders with infrared transmitting quartz window
Computer with software for collecting, storing and processing spectra

Reagents:

None.

Safety Precautions:

- Follow manufacturer's recommendation for safe operation of instrument.

Procedure:

1) Prepare samples by the same method as the calibration samples were prepared.
2) For best results run instrument (but not lamp) continuously. If instrument is cold, warm-up time should be 1 hr.
3) Clean sample holder with a camel hair brush or vacuum. Additional cleaning may be done with soft tissue or lint-free cloth. Glass should be free of finger prints and foreign material.

4) Load NIR sample cup placing one scoop of forage (previously dried to 90-95% dry matter or greater, ground with cyclone mill to pass 1 mm screen, thoroughly mixed) on each third of the glass surface to ensure that portions of different subsamples are scanned. Overfill the sample holder and scrape off any excess.
5) Press back into holder so that sample is firmly pressed against window.
6) If any abnormality appears in window, remove back and repeat process. Consistency in sample handling, preparation, and cell packing is crucial to success.
7) Scan sample, collect spectra, and process data.

Comments:

- NIR instrument should be maintained in a dust-free environment. Clean or change filter monthly. Clean ceramic standard and drawer assembly monthly.
- Never touch or clean grating.
- NIR instrument should be maintained in a stable-temperature (25 ±3^{o}C) room with controlled humidity (60 ±5%). Room should be free from vibration and have stable and dedicated electrical current.
- Sample must be dried and ground by the same methods as those samples used to develop the calibration equation.
- Spectrophotometer reads only a fraction of 1 mm depth of material touching glass of sample container. Therefore container must be loaded carefully with different subsample in each quadrant to make spectral reading more representative of entire sample.

Calculations:

Prediction is made by direct comparison to calibration. No additional calculations.

If calibrations were made using reference values on a dry matter basis, NIR results will be expressed on a dry matter basis.

Quality Control:

Include at least one set of duplicates in each run. These duplicates must be two subsamples each packed in a separate holder. Scanning the same sample twice is not a true replicated analysis using NIR.

An acceptable average standard deviation among replicated analyses for crude protein using reference methods ranges from about ±0.10 for samples with 10% CP to ±0.20 for samples with 20% CP, which results in warning limits (2s) ranging from ±0.20 to 0.40 and control limits (3s) ranging from ±0.30 to 0.60.

Quality control for NIR analysis involves monitoring the accuracy of both the instrument and the calibration equation. Instrument diagnostics should be run and recorded weekly, after instrument warm-up, to insure that maximum response, wavelength accuracy and repeatability are within acceptable manufacturer's tolerances. For example, for NIRSystems model 6250 and 6500 instruments, maximum response should be between 55000 and Method 2.2.2.4

58000 (adjust if below 51000), suggested wavelength error should be <0.5 times currently observed error (restandardize if observed error is too large) and root mean square corrected (RMSC) repeatability of multiple scans (32 revolution and 30 scans) should be less than 20 to 30 (correct problems if greater). Each manufacturer should provide acceptable performance specifications for their instrument, and often software is provided to monitor instrument accuracy. However, it is the responsibility of the operator to run the diagnostic software routinely and record the results a minimum of once a week.

In addition to meeting specifications, instrument operation should be monitored by scanning a check sample (that has been sealed in a sample holder) each day and storing spectral data weekly. Daily results for each analysis (DM, CP, ADF, NDF, and minerals) should be plotted on an X-control chart and the chart should be examined for trends. Standard deviation (s) for the check sample can be established after 10 scans and should be substantially lower than acceptable standard deviations of duplicate reference method analyses (.10, .20, .35 and .60 for DM, CP, ADF, and NDF, respectively) because the same sample is being scanned. Results outside of ±2s upper and lower warning limits are evidence of problems with the analytical system. Results outside of ±3s upper and lower control limits are evidence of loss of control and no NIR analyses should be done without detecting and correcting the problem. Two consecutive analyses falling on one side of the mean between warning limits and control limits also indicate a loss of control.

Monitoring the calibration equation consists of two tests that determine the existence of bias and unacceptable increases in standard error of prediction corrected for bias [SEP(C)]. Every 20 to 25th sample predicted by the NIR should be analyzed by the reference method used to develop the calibration equation (Note: Any bias or increased SEP(C) can be due to inaccuracies in NIR or differences in reference method analytical procedure). A continuous time chart of observed bias and SEP(C) should be plotted to observe trends. After nine (N) samples have been accumulated, analyze these samples by the reference method (A sample size of nine is a good compromise between the number of analyses required and the statistical accuracy desired for the confidence limits given below). Calculate the observed bias and SEP(C) using the equations given below. Determine the confidence limits for bias and SEP(C) based on the standard error of the calibration equation (SEC). If the SEP(C) of the nine samples is less than the SEP(C) limit and absolute value of the bias is less than the bias limit, the calibration equation is acceptable. If the SEP(C) of the nine samples is less than the SEP(C) limit but the absolute value of the bias exceeds the bias limit, the calibration equation may be corrected by adding the bias to the intercept of the calibration equation or to each value (bias adjustment) although recalibration is recommended. If both SEP(C) and bias exceed their limits, add samples to the calibration data set and recalibrate.

NIR Quality Control Calculations:

$D_i = X_i - Y_i$

$Bias = D_i / N$ Bias Confidence Limit = ±0.55 X (SEC)

Install Equation Editor and double-click here to view equation. SEP(C) Confidence Limit = 1.29 X (SEC);

where D_i = difference
X_i = reference method value
Y_i = NIR value for *i*th sample
N = 9 (number of samples)
Bias = average difference between reference and NIR values
SEP(C) = standard error for prediction corrected for bias
SEC = standard error of the calibration equation.

This monitoring procedure can be used with any NIR instrument and some manufacturers have incorporated this approach into their quality control monitoring software.

4. Acid Detergent Fiber

4.1 Determination of Acid Detergent Fiber by Refluxing Error! Bookmark not defined.

Reference:

Fiber (Acid Detergent) and Lignin in Animal Feed. (973.18) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This procedure is applicable for the determination of acid detergent fiber (ADF) in all types of forages.

Basic Principle:

An acidified quaternary detergent solution is used to dissolve cell solubles, hemicellulose and soluble minerals leaving a residue of cellulose, lignin, and heat damaged protein and a portion of cell wall protein and minerals (ash). ADF is determined gravimetrically as the residue remaining after extraction.

Equipment:

Refluxing apparatus
Berzelius beakers (600 mL)
Fritted glass (Gooch) crucibles (coarse porosity, 50 mL)
Analytical electronic balance, accurate to 0.1 mg
Suction filtering device with trap in line and valve to break vacuum
Forced-air drying oven set at 100°C

Reagents:

Acid detergent solution:

To prepare mix:

1 liter	1.00N Sulfuric acid ±0.005N. Normality must be verified by titration with a primary base standard (method 3.1.1) before adding CTAB. A solution approximately 1.0 N sulfuric acid can be made by adding 51.04 g (27.7 mL) of concentrated reagent grade sulfuric acid (95-98% purity) to 972.3 mL water (AOAC 935.70). Titrate by method 3.1.2 and add water (if normality too high) or sulfuric acid (if normality too low) to adjust normality to 1.00N ±0.005N.
20 g	Cetyl trimethylammonium bromide (CTAB), technical grade

Acetone, reagent grade

Safety Precautions:

- Always add sulfuric acid to water. Wear face shield and heavy rubber gloves. If acid is splashed on skin, wash immediately with copius amounts of water.

- CTAB powder will irritate mucous membranes, eyes and skin. Wear gloves and dust mask while handling.
- Acetone is highly flammable. Do not let vapors accumulate in work area. Use effective fume removal device. Also avoid inhaling or contact with skin. Make sure all traces of acetone have evaporated from the crucibles containing fiber residue before placing in the drying oven.

Procedure:

1) Samples should be microwave dried or oven dried at 55°C to ≥85% dry matter, then ground to pass a 1 mm screen.
2) Dry 50 mL fritted glass crucibles overnight at 100°C and hot weigh (W_1), recording weight to nearest 0.1 mg. (Hot weigh techniques described in method 2.2.2.2.)
3) Thoroughly mix and weigh sample (W_2) (approximately 0.9 to 1.1 g, record weight accurate to 0.1 mg) into Berzelius beaker. Weigh a second subsample for laboratory dry matter determination.
4) Add 100 mL acid-detergent solution at room temperature. Place beaker on heater under the cold water condenser.
5) Heat to boiling in 5-10 min; reduce heat to avoid foaming as boiling begins. Reflux 60 min from onset of boil, adjusting boiling to slow, even level.
6) After about 30 min, wash down sides of beaker with minimal amount of acid detergent solution. A wash bottle is convenient for dispensing solution.
7) Remove beaker, swirl, and filter through tared (step 2) fritted glass crucible, using minimal vacuum. Police and rinse the Berzelius beaker with boiling water while inverted over the crucible to insure quantitative transfer of all fiber particles into the crucible.
8) Soak twice with boiling (95-100°C) water by breaking up mat and filling crucible each time with vacuum off and allowing to soak a minimum of 15 to 30 sec (2 min recommended) after each wash. While filling the crucible with hot water or acetone, rinse the top edge and sides to remove residual acid detergent.
9) Rinse twice with 30-40 mL acetone by filling crucible each time with vacuum off, allowing a minimum of 15 to 30 sec (2 min recommended) before vacuuming dry.
10) Dry 3 hr or overnight in forced-air oven (100°C) and weigh hot, recording weight (W_3) to nearest 0.1 mg.

Comments:

- Sulfuric acid for acid detergent fiber solution must be standardized to be between 0.995 and 1.005 N. Variation in normality outside of this range can result in low or high ADF values.
- Timing of refluxing is critical and should not vary more than 5 min from the 60 min described by the method.
- Acid must be thoroughly washed from the sample because it will become concentrated when water is removed during drying. The combination of strong sulfuric acid and high temperature can char the sample and result in low ADF values. If black

discoloration occurs during drying, repeat the analysis.

- Difficult filtration may result from plugging of the fritted glass crucibles. Crucibles should be cleaned regularly with acid or alkaline cleaning solution. (Alkali cleaning will tend to deteriorate fritted disk faster.) The filtration rate of crucibles should be as uniform as possible for a given set of samples. To check the filtration rate of crucibles, fill them with 50 mL of distilled water and record the time required to drain completely without vacuum. This should be about 180 sec. If filtration takes more than 240 sec, crucibles need cleaning. If cleaning does not improve the filtration rate, the crucible should be discarded. If filtering takes less than 120 sec, check crucible for cracks or holes in the fritted disk. If filtering takes less than 100 sec, the crucible should be discarded.
- The proper vacuum is critical to good filtering. It should be sufficient to remove the solutions rapidly but not so great that fiber particles plug the fritted disk.
- Rinse water must be in excess of 95°C. This is particularly true of samples containing pectic substances, mucilages or glycoproteins.

Calculation: Percent Acid Detergent Fiber (ADF)

$$\% \text{ ADF (DM basis)} = \frac{W_3 - W_1}{W_2 \times \text{Lab DM}} \times 100$$

Where W_1 = tare weight of crucible in grams
W_2 = initial sample weight in grams
W_3 = dry weight of crucible and dry fiber in grams

Quality Control:

Include one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for acid detergent fiber ranges from about ±0.20 for samples with 20% ADF to ±0.35 for samples with 40% ADF, which results in warning limits (2s) ranging from ±0.40 to 0.70 and control limits (3s) ranging from ±0.60 to 1.05. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

4.2 Determination of Acid Detergent Fiber by Near Infrared Reflectance

Spectroscopy

Reference:

Fiber (Acid Detergent) and Protein (Crude) in Animal Feed and Forages: Near-infrared Reflectance Spectroscopic Method. (989.03) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Martin, G.C., J.S. Shenk, and F.E. Barton II. 1989. Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality. United States Department of Agriculture, Agricultural Research Service. Agricultural Handbook No. 643.

Scope:

This procedure is applicable for determining acid detergent fiber of ground, air-dry or partially dried (90 to 95% dry matter) forage. Samples must be ground through cyclone grinder with 1 mm screen and be 90 to 95% dry matter.

Basic Principle:

Random portions of a sample are loaded into an NIR sample holder and reflected light from the ground sample is measured in the infrared region (generally 1100 to 2500 nm). Instrument is part of a system that has been calibrated using representative samples from population to be tested. Equations selected based on calibration statistics, which have been validated, are used to calculate acid detergent fiber content of feed and forage samples.

Equipment:

Near infrared reflectance spectrophotometer, wavelength range at least 1100 to 2500 nm
Sample holders with infrared transmitting quartz window
Computer with software for collecting, storing and processing spectra

Reagents:

None.

Safety Precautions:

- Follow manufacturer's recommendation for safe operation of instrument.

Procedure:

1) Prepare samples by the same method as the calibration samples were prepared.
2) For best results run instrument (but not lamp) continuously. If instrument is cold, warm-up time should be 1 hr.
3) Clean sample holder with a camel hair brush or vacuum. Additional cleaning may be done with soft tissue or lint-free cloth. Glass should be free of finger prints and foreign material.

4) Load NIR sample cup placing one scoop of forage (previously dried to 90-95% dry matter or greater, ground with cyclone mill to pass 1 mm screen, thoroughly mixed) on each third of

the glass surface to ensure that portions of different subsamples are scanned. Overfill the sample holder and scrape off any excess.
5) Press back into holder so that sample is firmly pressed against window.
6) If any abnormality appears in window, remove back and repeat process. Consistency in sample handling, preparation, and cell packing is crucial to success.
7) Scan sample, collect spectra, and process data.

Comments:

- NIR instrument should be maintained in a dust-free environment. Clean or change filter monthly. Clean ceramic standard and drawer assembly monthly.
- Never touch or clean grating.
- NIR instrument should be maintained in a stable-temperature (25 ±3^{o}C) room with controlled humidity (60 ±5%). Room should be free from vibration and have stable and dedicated electrical current.
- Sample must be dried and ground by the same methods as those samples used to develop the calibration equation.
- Spectrophotometer reads only a fraction of 1 mm depth of material touching glass of sample container. Therefore container must be loaded carefully with different subsample in each quadrant to make spectral reading more representative of entire sample.

Calculations:

Prediction is made by direct comparison to calibration. No additional calculations.

If calibrations were made using reference values on a dry matter basis, NIR results will be expressed on a dry matter basis.

Quality Control:

Include at least one set of duplicates in each run. These duplicates must be two subsamples each packed in a separate holder. Scanning the same sample twice is not a true replicated analysis using NIR.

An acceptable average standard deviation among replicated analyses for acid detergent fiber using reference methods ranges from about ±0.20 for samples with 20% ADF to ±0.35 for samples with 40% ADF, which results in warning limits (2s) ranging from ±0.40 to 0.70 and control limits (3s) ranging from ±0.60 to 1.05.

Quality control for NIR analysis involves monitoring the accuracy of both the instrument and the calibration equation. Instrument diagnostics should be run and recorded weekly, after instrument warm-up, to insure that maximum response, wavelength accuracy and repeatability are within acceptable manufacturer's tolerances. For example, for NIRSystems model 6250 and 6500 instruments, maximum response should be between 55000 and

58000 (adjust if below 51000), suggested wavelength error should be <0.5 times currently observed error (restandardize if observed error is too large) and root mean square corrected (RMSC) repeatability of multiple scans (32 revolution and 30 scans) should be less than 20 to 30 (correct problems if greater). Each manufacturer should provide acceptable performance specifications for their instrument, and often software is provided to monitor instrument accuracy. However, it is the responsibility of the operator to run the diagnostic software routinely and record the results a minimum of once a week.

In addition to meeting specifications, instrument operation should be monitored by scanning a check sample (that has been sealed in a sample holder) each day and storing spectral data weekly. Daily results for each analysis (DM, CP, ADF, NDF, and minerals) should be plotted on an X-control chart and the chart should be examined for trends. Standard deviation (s) for the check sample can be established after 10 scans and should be substantially lower than acceptable standard deviations of duplicate reference method analyses (.10, .20, .35 and .60 for DM, CP, ADF, and NDF, respectively) because the same sample is being scanned. Results outside of ±2s upper and lower warning limits are evidence of problems with the analytical system. Results outside of ±3s upper and lower control limits are evidence of loss of control and no NIR analyses should be done without detecting and correcting the problem. Two consecutive analyses falling on one side of the mean between warning limits and control limits also indicate a loss of control.

Monitoring the calibration equation consists of two tests that determine the existence of bias and unacceptable increases in standard error of prediction corrected for bias [SEP(C)]. Every 20 to 25th sample predicted by the NIR should be analyzed by the reference method used to develop the calibration equation (Note: Any bias or increased SEP(C) can be due to inaccuracies in NIR or differences in reference method analytical procedure). A continuous time chart of observed bias and SEP(C) should be plotted to observe trends. After nine (N) samples have been accumulated, analyze these samples by the reference method (A sample size of nine is a good compromise between the number of analyses required and the statistical accuracy desired for the confidence limits given below). Calculate the observed bias and SEP(C) using the equations given below. Determine the confidence limits for bias and SEP(C) based on the standard error of the calibration equation (SEC). If the SEP(C) of the nine samples is less than the SEP(C) limit and absolute value of the bias is less than the bias limit, the calibration equation is acceptable. If the SEP(C) of the nine samples is less than the SEP(C) limit but the absolute value of the bias exceeds the bias limit, the calibration equation may be corrected by adding the bias to the intercept of the calibration equation or to each value (bias adjustment) although recalibration is recommended. If both SEP(C) and bias exceed their limits, add samples to the calibration data set and recalibrate.

NIR Quality Control Calculations:

$D_i = X_i - Y_i$

$Bias = D_i / N$ Bias Confidence Limit = ±0.55 X (SEC)

Install Equation Editor and double-click here to view equation. SEP(C) Confidence Limit = 1.29 X (SEC);

where D_i = difference
X_i = reference method value
Y_i = NIR value for *i*th sample
N = 9 (number of samples)
Bias = average difference between reference and NIR values
SEP(C) = standard error for prediction corrected for bias
SEC = standard error of the calibration equation.

This monitoring procedure can be used with any NIR instrument and some manufacturers have incorporated this approach into their quality control monitoring software.

5. Neutral Detergent Fiber - Amylase Procedure

5.1 Determination of Amylase Neutral Detergent Fiber by Refluxing

Reference:

Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analysis (apparatus, reagents, procedures, and some applications). USDA Agricultural Research Service. Handbook number 379 as modified by D.R. Mertens (1992, Personal Communication).

Van Soest, P.J, J.B. Robertson, and B.A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J. Dairy Science 74:3583-3597.

Mertens, D.R. 1992. Critical conditions in determining detergent fiber. Proceedings of NFTA Forage Analysis Workshop. Denver, CO. p C1-C8.

Scope:

This method is applicable for the determination of neutral detergent fiber in all types of forages and feeds.

Basic Principle:

A neutral detergent solution is used to dissolve the easily digested pectins and plant cell contents (proteins, sugars and lipids), leaving a fibrous residue (aNDF) that is primarily cell wall components of plants (cellulose, hemicellulose and lignin). Detergent is used to solubilize the proteins and sodium sulfite also helps remove some nitrogenous matter; EDTA is used to chelate calcium and remove pectins at boiling temperatures; triethylene glycol helps to remove some nonfibrous matter from concentrate feeds; and heat-stable amylase is used to remove starch. Two additions of amylase (one during refluxing and one during filtration) have been observed to aid aNDF analyses and minimize filtering difficulties. Heat-stable amylases are used in hot solutions to inactivate potential contaminating enzymes that might degrade fibrous constituents.

Equipment:

Refluxing apparatus
Berzelius beakers (600 mL)
Fritted glass (Gooch) crucibles (coarse porosity, 50 mL)
Analytical electronic balance, accurate to 0.1 mg
Suction filtering device with trap in line and valve to break vacuum
Forced-air drying oven set at 100^{o}C

Reagents:

Neutral detergent solution - To make approximately 18 liters mix:

17.82 L	Distilled water
540 g	Sodium lauryl sulfate, USP
335 g	Ethylenediaminetetraacetic acid (EDTA), disodium salt (may substitute 72 g sodium hydroxide (NaOH) and 263 g free acid EDTA as a less expensive alternative.)
122.6 g	Sodium borate, decahydrate ($Na_2B_4O_7 \cdot 10H_2O$), reagent grade
82.1 g	Sodium phosphate, dibasic (Na_2HPO_4), anhydrous, reagent grade

Preparing aNDF Solution

Pour one-half of distilled water into mixing container. Place on stir plate in hood and begin stirring. Add remaining reagents except for triethylene glycol. (Caution: wear dust mask when weighing and transferring the sodium lauryl sulfate.) Slowly add remaining distilled water to container to limit foaming of the detergent. When approximately three-fourths of the distilled water has been added to the container, add the triethylene glycol. The triethylene glycol will reduce foaming of the detergent solution. Allow to stir overnight. Use heated stirrer if material fails to dissolve. Keep container at 20°C or higher to avoid precipitation of the solution. Verify pH of solution to be between 6.95 and 7.05. Adjust with HCl or NaOH as required if not within range.

180 mL	Triethylene glycol, reagent grade

Reagents, continued

Sodium sulfite, anhydrous (Na_2SO_3), reagent grade
Acetone, reagent grade
Heat-stable alpha-amylase solution (standardized by method 5.1.1)

Safety Precautions:

- Acetone is highly flammable. Do not let vapors accumulate in work area. Use effective fume removal device. Also avoid inhaling or contact with skin. Make sure all traces of acetone have evaporated from crucibles containing the fiber residue before placing them into the oven.
- Sodium lauryl sulfate is irritating to mucous membranes. Wear dust mask and gloves while handling.

Procedure:

1) Samples should be microwave or oven dried at 55°C to greater than 85% dry matter, then ground to pass a 1 mm screen.
2) Dry 50 mL fritted glass crucibles overnight at 100°C and hot weigh (W_1), recording weight to nearest 0.1 mg. (Refer to method 2.2.2.2 for description of hot weighing techniques.)
3) Thoroughly mix sample, then weigh 0.45 to 0.55 g, recording to nearest 0.1 mg, (W_2) into 600 mL Berzelius beaker. Weigh a second subsample for laboratory dry matter determination.
4) Preheat extraction heating (reflux) unit to a temperature that permits boiling of neutral detergent solution within 5 min.
5) Add 0.5 g of sodium sulfite using previously calibrated scoop.
6) Add 50 mL of neutral detergent solution and swirl beaker until the sample and sodium sulfite are completely suspended.
7) Place beaker on the heating unit under a cool water condenser. Samples should come to a boil in 4 to 5 min. Samples normally foam vigorously for 1 to 2 min. Do not reduce temperature of heating unit during this time.
8) After 5 min, remove beaker from the reflux unit and add 2 mL of the standardized amylase solution.
9) Swirl beaker to thoroughly mix the amylase in the neutral detergent solution and resuspend any particles that have crept up the sides of the beaker. Detach any sample attached to the sides or bottom of the beaker using a rubber policeman. Rinse off policeman with aNDF solution.
10) Return beaker to the reflux unit and allow to come to a boil. Reflux for 60 min. Five to 10 min after adding amylase, rinse down the sides of the beaker with neutral detergent solution.
11) Remove sample from heating unit and allow to settle for 30-60 sec before filtering.
12) Preheat the fritted glass crucible for filtering by adding 40 mL of boiling water. Remove water with vacuum.
13) Carefully decant the first 30-40 mL of solution from the Berzelius beaker into the crucible. Rinse lip of beaker to prevent solution with particles from running down outside of beaker. Keep beaker in "decant" position while emptying. Remove the solution with a minimum amount of vacuum.
14) Close vacuum and rinse the remaining residue from the beaker into the crucible using a fine stream of boiling water. Be certain that no particles remain in the beaker or on the lip to run down the outside as the beaker is turned upright. Apply minimum vacuum to filter.
15) Immediately fill crucible half full of hot water and add 2 mL of standardized amylase solution. Allow to react for approximately 45 to 60 sec, while policing particles from sides of Berzelius beaker.

16) Rinse Berzelius beaker with boiling water while inverted over the crucible until all residue is transferred.
17) Filter and wash twice by adding 30 to 40 mL boiling water to residue in fritted glass crucible and allowing to soak for 2 min each time.
18) Rinse sample twice with 30 mL of acetone, allowing at least 2 min soaking time between rinses.
19) Rinse policeman, vacuum sample dry, and remove sample from manifold.
20) Dry crucibles at 100°C for 8 hr or overnight and hot weigh recording weight (W_3) to nearest 0.1 mg.

Comments:

- Difficult filtration may result from plugging of the fritted glass crucibles. Crucibles should be cleaned regularly with acid or alkaline cleaning solution. The filtration rate of crucibles should be as uniform as possible for a given set of samples. To check the filtration rate of crucibles, fill them with 50 mL of distilled water and record the time required to drain completely without vacuum. This should be about 180 sec. If filtration takes more than 240 sec, crucibles need cleaning. If cleaning does not improve the filtration rate, the crucible should be discarded. If filtering takes less than 120 sec, check crucible for cracks or holes in the fritted disk. If filtering takes less than 100 sec, the crucible should be discarded.
- The proper vacuum is critical to good filtering. It should be sufficient to remove the solutions rapidly but not so great that fiber particles plug the fritted glass disc.
- Rinse water must be in excess of 95°C. This is particularly true for samples containing pectic substances, mucilages or glycoproteins.
- Some sample types are consistently difficult to filter (corn silage, citrus pulp, sunflower meal, meat by-products and feces). Experience has shown that any sample that takes longer than 10 min to filter will provide erroneous results and must be repeated using modifications described by Mertens or Van Soest.
- Many amylase extracts are crude mixtures that may contain fiber degrading enzymes. Because heat will inactivate these contaminating enzymes, it is recommended that a heat-stable amylase be used in hot solution to minimize fiber loss.

Calculation: Percent Amylase Neutral Detergent Fiber (aNDF)

$$\text{aNDF (DM basis)} = \frac{W_3 - W_1}{W_2 \times \text{Lab DM}/100} \times 100$$

Where W_1 = tare weight of crucible in grams
W_2 = initial sample weight in grams
W_3 = dry weight of crucible and dry fiber in grams

Quality Control:

Include one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for neutral detergent fiber ranges from about ±0.35 for samples with 40% NDF to ±0.60 for samples with 70% NDF, which results in warning limits (2s) ranging from ±0.70 to 1.20 and control limits (3s) ranging from ±1.05 to 1.80. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

5.2 Standardizing Alpha-Amylase Activity for Neutral Detergent Fiber Determination

Reference:

(D. R. Mertens, 1992, USDA Dairy Forage Research Center, Personal communication)

Scope:

This method is applicable for the determination of alpha-amylase activity of solutions to be used in neutral detergent fiber procedure (5.1).

Basic Principle:

To insure that the amylase activity is sufficient to remove the majority of starch in samples and reduce filtering difficulties, it is critical to determine the amount of any specific enzyme

source that is needed for the aNDF method. Activity of alpha-amylase is determined under conditions existing during aNDF procedure.

Equipment:

Refluxing apparatus
Berzelius beakers (600 mL)
Analytical electronic balance, accurate to 0.1 mg
Ice bath

Reagents:

Dried hominy corn (sold in most grocery stores as corn grits, raw, not instant) that has been ground to pass a 1-mm screen (Wiley mill)
Burke's iodine solution (2 g KI, 1 g I_2 and 100 mL H_2O) or iodine solution from Sigma (Cat. No. 700-2)
Amylase test solution or extraction

The ratio of powder or stock solution to water can vary greatly depending on the enzyme activity. After some experience with an amylase source is gained, it may be possible to select a dilution or extraction rate that is most appropriate for the specific amylase source. If a liquid enzyme source is used, dilute a small volume to 100 mL final volume with distilled water (e.g. approximately 1.25 mL Sigma Cat. No. A5426 or 5 mL of NOVO Termamyl diluted to 100 mL). Powdered enzymes must be extracted with water. It is recommended to start with 5 g of powder extracted for 20 min with 100 mL of distilled water. Concentration of amylase test solution (C) is expressed as g (powder) or mL (liquid) of enzyme/100 mL test solution.

Safety Precautions:

- Sodium lauryl sulfate is irritating to mucous membranes. Wear dust mask and gloves while handling.

Procedure:

A. Determine appropriate amount of amylase needed.

Use a range of enzyme concentrations to detect differences in the activity of an enzyme and determine the amount of the enzyme that is needed for aNDF. It is suggested that six doses of most enzyme dilutions is all that need to be evaluated (0, 1.0, 1.5, 2.0, 2.5, & 3.0 mL). However, if an unknown source is used, it may be wise to use a geometric progression of doses (0, 0.5, 1.0, 2.0, 4.0 & 8.0 mL) the first time to establish the range to be used in a second trial that will determine the final amount of an unknown amylase to use.

1. Weigh 0.5 g (± 0.005 g) of ground, dried hominy corn into each of six Berzelius beakers.
2. Preheat extraction heating (reflux) unit to a temperature that permits boiling of neutral detergent solution within 5 min. Prepare an icebath for cooling the beakers after they have been removed from the hotplates.
3. Add 50 mL of neutral detergent solution and swirl beaker. Do not add 0.5 g of sodium sulfite as in method 5.1.
4. Put beakers onto preheated refluxing apparatus at 1 minute intervals. Samples should come to a boil in 4 to 5 min. Timing is critical to the evaluation of the enzymes because reaction rates are temperature and time dependent. It is also important that enzymes be used in ascending order of concentration.
5. After the samples begin to boil (approximately 5 min after placing on hot plates), add one of the doses of enzyme solution to each of the respective beakers.
6. Reflux for 10 min. One hr refluxing is not needed to evaluate amylases.
7. At 1 minute intervals remove each beaker from the refluxing apparatus. Add the second dose of amylase, swirl to mix and rinse down the sides of the beaker using a minimum amount of room temperature neutral detergent solution. Let the amylase react with the beaker on the benchtop for 60 sec (until after the next sample is removed from the hotplate and enzyme is added to it).
7. Place the beaker in the ice bath to cool for 5 min.
8. Remove each beaker and place it on the benchtop until all beakers have been cooled.
9. Arrange beakers in order of increasing enzyme concentration, preferably on a white sheet of paper or other light background color surface.
10. Quickly add 0.5 mL of Burke's iodine solution to each beaker, then swirl each beaker in turn. Set timer to alarm in 90 sec.

11. After 90 sec evaluate the efficacy of raw corn starch (grits) hydrolysis using the following scale:
    Purple = not adequate enzyme
    Amber = not adequate enzyme
    Yellow = adequate enzyme.
    The volume of the amylase test solution or extraction (V_s) that indicates no starch (yellow color) is used to calculate the standardized amylase solution.

    It is best not to look at the beakers while waiting for the 90 sec to elapse, instead look away from the beakers and after the alarm sounds make a quick decision (before 120 sec has elapsed) about the lowest amount of enzyme that gives a yellow starch reaction with iodine. Caution: Excess enzyme is not beneficial and can be detrimental. Too much amylase is expensive, can cause retrograde starch synthesis and may increase the amount of contaminating enzymes in the amylase preparation that can cause problems.

12. Calculate the amount of enzyme needed in 2 mL of standardized amylase solution to remove interfering starch.

B. Verify the absence of undesirable fiber-degrading enzyme activity in any unknown amylase source

Use Novo Termamyl or the AOAC Dietary Fiber method amylase (Sigma Cat. No. A5426) which have minimal fiber-degrading activity under the conditions of aNDF analysis. However, if other sources are used, use beta-glucan (barley - Sigma No. G-6513), arabinogalactan (larchwood - Sigma No. A-2012 or Dietary Fiber control - Sigma No. A9788) and pectin (citrus-ICN No. 102587 or Sigma No. P9135) to determine unacceptable fiber-degrading activities.

1. Weigh each of the following into separate Berzelius beakers in duplicate: 0.1 g of beta-glucan, 0.5 g arabinogalactan, or 0.5 g pectin.
2. Follow the usual aNDF procedure using the standardized amylase solution determined previously, EXCEPT: Add the amylase to one of the duplicate beakers, but not the other. To aid filtering of these difficult materials, add 0.25 g of glass wool or glass microfiber filters (Whatman GF/D, 4.25 cm) to the crucibles prior to initial drying of the crucible (W_1) before obtaining the tared weight. Make sure wash water is boiling.
3. After calculating the percentage aNDF for each compound, divide the %aNDF with amylase by the %aNDF without amylase. If this ratio is less than 0.9 for any of the compounds, reject the source of enzyme and choose another source to use because the unknown amylase source has significant fiber degrading activity.

Comments:

- Each new source or lot of enzyme should be standardized.
- If a single lot is being used over a period of time it should be checked every month for activity.

Calculation: Amount of enzyme preparation needed for the standardized amylase solution.

Rather than vary the amount of enzyme solution to use with each source or lot, it is desirable to calculate concentration of a specific enzyme that will be added in the aNDF method as 2 mL of solution. Then an automatic pipet or syringe, precalibrated to 2 mL, can be used to dispense the enzyme regardless of its initial activity. Two mL of dilute enzyme is used to minimize the error associated with a drop of solution that may be retained on the syringe or pipet, because one drop of a concentrated enzyme solution contains significant activity.

The calculation that follows is for dilution of an enzyme stock solution or extraction to allow 2 mL of standardized amylase solution to be added during the aNDF procedure.

$$E = 100\ mL \times (V_s \times C) / 2\ mL$$

Where:	E	=	Enzyme powder (g) or liquid (mL) needed to make 100 mL of standardized amylase solution
	V_s	=	Minimum volume of test solution or extract resulting in yellow color with iodine (no starch)
	C	=	Concentration of solution (g powder/100 mL or mL liquid/100 mL)
	2 mL	=	Volume to be added in aNDF procedure
	100 mL	=	Volume of standardized amylase solution to be prepared

5.3 Determination of Neutral Detergent Fiber by Near Infrared Reflectance Spectroscopy

Reference:

Martin, G.C., J.S. Shenk, and F.E. Barton II. 1989. Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality. United States Department of Agriculture, Agricultural Research Service. Agricultural Handbook No. 643.

Scope:

This procedure is applicable for determining acid detergent fiber of ground, air-dry or partially dried (90 to 95% dry matter) forage. Samples must be ground through cyclone grinder with 1 mm screen and be 90 to 95% dry matter.

Basic Principle:

Random portions of a sample are loaded into an NIR sample holder and reflected light from the ground sample is measured in the infrared region (generally 1100 to 2500 nm). Instrument is part of a system that has been calibrated using representative samples from population to be tested. Equations selected based on calibration statistics, which have been validated, are used to calculate neutral detergent fiber (NDF) or amylase neutral detergent fiber (aNDF) of feed and forage samples.

Equipment:

Near infrared reflectance spectrophotometer, wavelength range at least 1100 to 2500 nm
Sample holders with infrared transmitting quartz window
Computer with software for collecting, storing and processing spectra

Reagents:

None.

Safety Precautions:

- Follow manufacturer's recommendation for safe operation of instrument.

Procedure:

1) Prepare samples by the same method as the calibration samples were prepared.
2) For best results run instrument (but not lamp) continuously. If instrument is cold, warm-up time should be 1 hr.
3) Clean sample holder with a camel hair brush or vacuum. Additional cleaning may be done with soft tissue or lint-free cloth. Glass should be free of finger prints and foreign material.

4) Load NIR sample cup placing one scoop of forage (previously dried to 90-95% dry matter or greater, ground with cyclone mill to pass 1 mm screen, thoroughly mixed) on each third of the glass surface to ensure that portions of different subsamples are scanned. Overfill the sample holder and scrape off any excess.
5) Press back into holder so that sample is firmly pressed against window.
6) If any abnormality appears in window, remove back and repeat process. Consistency in sample handling, preparation, and cell packing is crucial to success.
7) Scan sample, collect spectra, and process data.

Comments:

- NIR instrument should be maintained in a dust-free environment. Clean or change filter monthly. Clean ceramic standard and drawer assembly monthly.
- Never touch or clean grating.
- NIR instrument should be maintained in a stable-temperature (25 ±3^{o}C) room with controlled humidity (60 ±5%). Room should be free from vibration and have stable and dedicated electrical current.
- Sample must be dried and ground by the same methods as those samples used to develop the calibration equation.
- Spectrophotometer reads only a fraction of 1 mm depth of material touching glass of sample container. Therefore container must be loaded carefully with different subsample in each quadrant to make spectral reading more representative of entire sample.

Calculations:

Prediction is made by direct comparison to calibration. No additional calculations.

If calibrations were made using reference values on a dry matter basis, NIR results will be expressed on a dry matter basis.

Quality Control:

Include at least one set of duplicates in each run. These duplicates must be two subsamples each packed in a separate holder. Scanning the same sample twice is not a true replicated analysis using NIR.

An acceptable average standard deviation among replicated analyses for neutral detergent fiber using reference methods ranges from about ±0.35 for samples with 40% NDF to ±0.60 for samples with 70% NDF, which results in warning limits (2s) ranging from ±0.70 to 1.20 and control limits (3s) ranging from ±1.05 to 1.80.

Quality control for NIR analysis involves monitoring the accuracy of both the instrument and the calibration equation. Instrument diagnostics should be run and recorded weekly, after instrument warm-up, to insure that maximum response, wavelength accuracy and repeatability are within acceptable manufacturer's tolerances. For example, for NIRSystems model 6250 and 6500 instruments, maximum response should be between 55000 and

58000 (adjust if below 51000), suggested wavelength error should be <0.5 times currently observed error (restandardize if observed error is too large) and root mean square corrected (RMSC) repeatability of multiple scans (32 revolution and 30 scans) should be less than 20 to 30 (correct problems if greater). Each manufacturer should provide acceptable performance specifications for their instrument, and often software is provided to monitor instrument accuracy. However, it is the responsibility of the operator to run the diagnostic software routinely and record the results a minimum of once a week.

In addition to meeting specifications, instrument operation should be monitored by scanning a check sample (that has been sealed in a sample holder) each day and storing spectral data weekly. Daily results for each analysis (DM, CP, ADF, NDF, and minerals) should be plotted on an X-control chart and the chart should be examined for trends. Standard deviation (s) for the check sample can be established after 10 scans and should be substantially lower than acceptable standard deviations of duplicate reference method analyses (.10, .20, .35 and .60 for DM, CP, ADF, and NDF, respectively) because the same sample is being scanned. Results outside of ±2s upper and lower warning limits are evidence of problems with the analytical system. Results outside of ±3s upper and lower control limits are evidence of loss of control and no NIR analyses should be done without detecting and correcting the problem. Two consecutive analyses falling on one side of the mean between warning limits and control limits also indicate a loss of control.

Monitoring the calibration equation consists of two tests that determine the existence of bias and unacceptable increases in standard error of prediction corrected for bias [SEP(C)]. Every 20 to 25th sample predicted by the NIR should be analyzed by the reference method used to develop the calibration equation (Note: Any bias or increased SEP(C) can be due to inaccuracies in NIR or differences in reference method analytical procedure). A continuous time chart of observed bias and SEP(C) should be plotted to observe trends. After nine (N) samples have been accumulated, analyze these samples by the reference method (A sample size of nine is a good compromise between the number of analyses required and the statistical accuracy desired for the confidence limits given below). Calculate the observed bias and SEP(C) using the equations given below. Determine the confidence limits for bias and SEP(C) based on the standard error of the calibration equation (SEC). If the SEP(C) of the nine samples is less than the SEP(C) limit and absolute value of the bias is less than the bias limit, the calibration equation is acceptable. If the SEP(C) of the nine samples is less than the SEP(C) limit but the absolute value of the bias exceeds the bias limit, the calibration equation may be corrected by adding the bias to the intercept of the calibration equation or to each value (bias adjustment) although recalibration is recommended. If both SEP(C) and bias exceed their limits, add samples to the calibration data set and recalibrate.

NIR Quality Control Calculations:

$D_i = X_i - Y_i$

Bias = D_i / N Bias Confidence Limit = ±0.55 X (SEC)

Install Equation Editor and double-click here to view equation. SEP(C) Confidence Limit = 1.29 X (SEC);

where D_i = difference
X_i = reference method value
Y_i = NIR value for *i*th sample
N = 9 (number of samples)
Bias = average difference between reference and NIR values
SEP(C) = standard error for prediction corrected for bias
SEC = standard error of the calibration equation.

This monitoring procedure can be used with any NIR instrument and some manufacturers have incorporated this approach into their quality control monitoring software.

6. Acid Detergent Insoluble Nitrogen and Acid Detergent Fiber Crude Protein

References:

Goering, H.K. and P.J. Van Soest. 1970. Forage fiber analysis (apparatus, reagents, procedures, and some applications). USDA Agricultural Research Service. Agriculture Handbook No. 379.

Goering, H.K., C.H. Gordon, R.W. Hemken, D.R. Waldo, P.J. Van Soest, and L.W. Smith. 1972. Analytical estimates of nitrogen digestibility in heat damaged forages. Journal of Dairy Science. 55(9): 1275-1280.

Scope:

This method is applicable for the determination of acid detergent insoluble nitrogen in all forages.

Basic Principle:

Acid detergent insoluble nitrogen (ADIN) is the nitrogen remaining in the acid detergent fiber residue and, while some occurs naturally in all plant material, is generally considered to be an estimate of heat damage occurring during storage or processing. Nitrogen in excessively heated samples is usually unavailable to animals.

ADIN is determined as the nitrogen in ADF residue. The two options used to determine ADIN differ in the amount of the ADF residue that is analyzed for nitrogen. If the total ADF residue is collected on filter paper and analyzed for nitrogen, ADIN (DM basis) is determined by measuring the nitrogen (corrected for a filter paper blank) in the total ADF residue and dividing by the original dry sample weight. It is difficult, if not impossible to collect all ADF residue from fritted glass (Gooch) crucibles, therefore only a subsample of the total ADF residue is analyzed for nitrogen. When only a part of the ADF residue is analyzed, by sampling the ADF residue from a fritted glass crucible (or from filter paper), the nitrogen content of the ADF residue must be determined by dividing the nitrogen in the ADF sample by the ADF sample weight. Then ADIN (DM basis) is calculated by multiplying the nitrogen content of ADF by the ADF content in the dry matter. When sampling ADF residues from fritted glass crucibles, be careful not to scrape glass particles into the partial ADF residue that is analyzed for nitrogen.

Acid detergent fiber crude protein (ADFCP) is ADIN expressed as crude protein on a dry matter basis.

Equipment:

See method 3.1, 3.2, or 3.3 for equipment for appropriate nitrogen determination method
See method 4.1 for equipment for acid detergent fiber procedure
Filter paper, acid hardened, Whatman 54 or equivalent

Reagents:

See method 3.1, 3.2, or 3.3 for reagents for appropriate nitrogen determination method
See method 4.1 for reagents for acid detergent fiber procedure.

Safety Precautions:

See method 3.1, 3.2, or 3.3 for safety precautions for appropriate nitrogen determination method
See method 4.1 for safety precautions for acid detergent fiber procedure.

Procedure:

Option A: Determination of ADIN using total ADF residue (filter paper)

1) Dry 6 papers overnight at 100°C to determine average filter paper DM. Weigh filter papers to be used to collect ADF residues to nearest 0.1 mg (W_1).
2) Thoroughly mix and weigh sample (W_2) (approximately 0.9-1.1 g, record weight accurate to 0.1 mg) into Berzelius beaker. Weigh a second subsample for laboratory dry matter determination.
3) Add 100 mL acid-detergent solution at room temperature. Place beaker on heater under the cold water condenser.
4) Heat to boiling in 5-10 min; reduce heat to avoid foaming as boiling begins. Reflux 60 min from onset of boil, adjusting boiling to slow, even level.
5) After about 30 min, wash down sides of beaker with minimal amount of acid detergent solution. A wash bottle is convenient for dispensing solution.
6) Remove beaker, swirl, and filter onto filter papers using Buchner funnels with a retainer ring to hold the filter papers in place during filtering. Use minimal vacuum. Include 2 blanks (filter paper washed with ADF solution) to estimate nitrogen in paper and reagents.
7) Police and rinse the Berzelius beaker with boiling water while inverted over the funnel to insure quantitative transfer of all fiber particles into the filter paper.
8) Soak twice with boiling (90-100°C) water by breaking up mat and filling funnel each time with vacuum off and allowing to soak a minimum of 15 to 30 sec (2 min recommended). While filling the Buchner funnel with hot water or acetone, rinse the top edge and sides to remove residual acid detergent.
9) Rinse twice with 30 to 40 mL acetone by filling funnel each time with vacuum off, allowing a minimum of 15 to 30 sec (2 min recommended) before vacuuming dry.
10) Fold filter paper to retain sample, dry 3 hr in forced-air oven (100°C) and weigh hot, recording weight (W_3) to nearest 0.1 mg.
11) Insert filter paper and sample into Kjeldahl flasks, add 5 mL additional acid to digest the filter paper and determine nitrogen by method 3.1 or 3.2.

Calculation: Percent Acid Detergent Insoluble Nitrogen (ADIN), DM basis
using total ADF residue (filter paper)

When determining nitrogen using colorimetric method:

$$\%\text{ADIN (DM basis)} = \frac{[(\text{mgN in ADF+filter paper /250 mL}) - (\text{mgN in blank filter paper/250 mL})] \times 100}{W_2 \times 1000 \times \text{Lab DM}/100}$$

Where W_2 = sample weight in grams
1000 = conversion factor for grams to mg

When determining nitrogen using standard sodium hydroxide titrant:

$$\%\text{ADIN (DM basis)} = \frac{[(V_{HCl} \times N_{HCl}) - (B \times N_{NaOH}) - (V_{NaOH} \times N_{NaOH})] \times 1.4007}{W_2 \times \text{Lab DM}/100}$$

WhereV_{NaOH}=mL standard NaOH to titrate ADF + filter paper

V_{HCl}	=	mL standard HCl pipetted into titrating flask
N_{NaOH}	=	Normality of NaOH
N_{HCl}	=	Normality of HCl
B	=	mL standard NaOH needed to titrate 1 mL standard HCl minus V_{BK}
V_{BK}	=	mL standard NaOH needed to titrate filter paper blank carried through method and distilled into 1 mL standard HCl
1.4007	=	milliequivalent weight of nitrogen X 100
W_2	=	weight of sample in grams

When determining nitrogen using boric acid trapping solution/standard HCl titrant:

$$\% \text{ ADIN (DM basis)} = \frac{(V_A - V_B) \times N_{HCl} \times 1.4007}{W_2 \times \text{Lab DM}/100}$$

Where V_A = Volume, in mL, of standard HCl required for ADF residue + filter paper
V_B = Volume, in mL, of standard HCl required for filter paper blank
N_{HCl} = Normality of standard HCl
1.4007 = milliequivalent weight of N X 100
W_2 = sample weight in grams

Calculation: Acid Detergent Insoluble Nitrogen (as percent of total nitrogen). Also called ADIN to N ratio.

$$\% \text{ ADIN (of Total N)} = \frac{\% \text{ ADIN (DM basis)}}{\% \text{ N (DM basis)}} \times 100$$

Where %ADIN = ADIN (% of Dry matter) calculated above
%N = % nitrogen of original sample

Calculation: Percent Acid Detergent Fiber Crude Protein (ADFCP), DM basis

$$\% \text{ ADFCP (DM basis)} = \%\text{ADIN (DM basis)} \times 6.25$$

Where %ADIN = ADIN (% of Dry matter) calculated above

Option B: Determination of ADIN using partial ADF residue (from fritted glass crucibles)

1) Samples should be microwave dried or oven dried at 55°C to ≥85% dry matter, then ground to pass a 1 mm screen.
2) Dry 50 mL fritted glass crucibles overnight at 100°C and hot weigh (W_1), recording weight to nearest 0.1 mg. (Hot weigh techniques described in method 2.2.2.2.)
3) Thoroughly mix and weigh sample (W_2) (approximately 0.9 to 1.1 g, record weight accurate to 0.1 mg) into Berzelius beaker. Weigh a second subsample for laboratory dry matter determination.
4) Add 100 mL acid-detergent solution at room temperature. Place beaker on heater under the cold water condenser.
5) Heat to boiling in 5-10 min; reduce heat to avoid foaming as boiling begins. Reflux 60 min from onset of boil, adjusting boiling to slow, even level.
6) After about 30 min, wash down sides of beaker with minimal amount of acid detergent solution. A wash bottle is convenient for dispensing solution.
7) Remove beaker, swirl, and filter through tared (step 2) fritted glass crucible, using minimal vacuum. Police and rinse the Berzelius beaker with boiling water while inverted over the crucible to insure quantitative transfer of all fiber particles into the crucible.
8) Soak twice with boiling (95-100°C) water by breaking up mat and filling crucible each time with vacuum off and allowing to soak a minimum of 15 to 30 sec (2 min recommended) after each wash. While filling the crucible with hot water or acetone, rinse the top edge and sides to remove residual acid detergent.
9) Rinse twice with 30-40 mL acetone by filling crucible each time with vacuum off, allowing a minimum of 15 to 30 sec (2 min recommended) before vacuuming dry.
10) Dry 3 hr or overnight in forced-air oven (100°C) and weigh hot, recording weight (W_3) to nearest 0.1 mg.
11) Sample a portion of the ADF residue from fritted glass crucible using a teflon or plastic policeman into Kjeldahl flasks. Do not scrape so hard as to dislodge glass from the fritted disk.
12) Weigh partial ADF residue (W_8), recording weight to nearest 0.1 mg.
13) Determine nitrogen content of the ADF residue subsample using the Kjeldahl (method 3.1 or 3.2) or combustion methods (method 3.3).

Calculation: Percent Acid Detergent Insoluble Nitrogen (ADIN), DM basis
using partial ADF residues (from fritted glass crucibles or filter paper)

$$\text{ADIN (DM basis)} = \%N_s \times \%\text{ADF (DM basis)}/100$$

Where $\%N_S$ = percent nitrogen of ADF residue (using W_8)

W_8 = weight of ADF residue used for nitrogen analysis in grams
%ADF (DM basis) = percent ADF calculated using W_1 to W_3 (Method 4.1)

Calculation: Acid Detergent Insoluble Nitrogen (as percent of total nitrogen). Also called ADIN to N ratio.

$$\%\ ADIN\ (of\ Total\ N) = \frac{\%\ ADIN\ (DM\ basis)}{\%\ N\ (DM\ basis)} \times 100$$

Where %ADIN = ADIN (% of Dry matter) calculated above
%N = % nitrogen of original sample

Calculation: Percent Acid Detergent Fiber Crude Protein (ADFCP), DM basis

$$\%\ ADFCP\ (DM\ basis) = \%ADIN\ (DM\ basis) \times 6.25$$

Where %ADIN = ADIN (% of Dry matter) calculated above

Quality Control:

Include a filter paper blank and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for acid detergent insoluble nitrogen (expressed as % of total N) ranges from about ±0.22 to ±0.40, which results in warning limits (2s) ranging from ±0.44 to 0.80 and control limits (3s) ranging from ±0.66 to 1.20. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

7. Total Ash in Forages

Reference:

Ash of Animal Feed. (942.05) Official methods of Analysis. 1990. Association of Official Analytical Chemists, 15th Edition.

Scope:

This procedure is applicable for the determination of ash in all types of dried, ground forages and feeds. It is not applicable for ash determination in liquid feeds or feeds high in sugar content.

Basic Principle:

A dried, ground sample is ignited in a furnace at 600°C to oxidize all organic matter. Ash is determined by weighing the resulting inorganic residue.

Equipment:

Crucibles, porcelain, low wide form, 30 mL, with covers numbered with furnace-proof ink
Muffle furnace with pyrometric controller
Analytical balance, sensitive to 0.1 mg
Desiccator, with vented lid
Drying oven

Reagents:

None.

Safety Precautions:

- Use standard precautions when working around electrical equipment or glassware.
- Make sure that electrical equipment is properly grounded and installed and maintained by qualified electricians.

Procedure:

1) Remove crucibles with cover which have been dried for at least 2 hr at 100°C from oven, to desiccator. Cool, and record weight of crucibles with cover to the nearest 0.1 mg (W_1).
2) Weigh 1.5 to 2.0 g of sample into the crucible, recording weight of crucible with cover and sample to the nearest 0.1 mg (W_2).
3) Ash in furnace at 600°C for 2 hr after the furnace reaches temperature.
4) Allow crucibles to cool in furnace to less than 200°C and place crucibles with cover in desiccator with vented top. Cool and weigh crucible with cover and ash to the nearest 0.1 mg (W_3).

Comments:

- Time and temperature described must be adhered to closely.
- Samples should be placed in ashing furnace so that air can circulate freely. Crucibles should not touch each other.
- Slide the desiccator lid open. Do not place the lid on the countertop with the grease side down. The grease will pick up dirt, preventing formation of a seal.
- Seals should be kept clean and well greased and the lid should always slide easily on or off. If a lid "grabs," it is time to remove the old grease and apply fresh lubricant.
- If a lid can be directly lifted off the desiccator, either the desiccator was not properly sealed or, more likely, it needs fresh lubricant.
- Rubber stoppers in the lid should always be pliable.
- Crucibles should not be packed excessively tight in a desiccator. Air movement is necessary to cool crucibles. Crucibles should not touch each other.
- The desiccator lid should be left open for minimal amount of time.
- Desiccant should be checked and dried periodically. Replace desiccant twice annually or more often depending on use. Use of desiccant with color indicator for moisture is recommended.
- Open a loaded desiccator very slowly after samples have cooled. A vacuum forms during cooling and abrupt opening results in turbulence which can blow samples out of crucibles.
- If determining ash after fiber analysis, set furnace at 500^{O}C and ash until carbon-free and grey ash color (3 to 5 h). Lower ashing temperatures require longer ashing times.
- Higher temperatures will melt glass and ruin filter crucibles. A practical maximum service termperature for pyrex glass is 510^{O}C and the annealing temperature is 560^{O}C.

Calculation: Percent Ash, DM basis

$$\% \text{ ASH (DM basis)} = \frac{(W_3 - W_1) \times 100}{(W_2 - W_1) \times \text{Lab DM}/100}$$

Where W_1 = tare weight of crucible in grams
W_2 = weight of crucible and sample in grams
W_3 = weight of crucible and ash in grams

Quality Control:

Include one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least

one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for ash is about ±0.10, which results in a warning limit (2s) of about ±0.20 and a control limit (3s) of about ±0.30. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2s (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3s (99 percent confidence limits), indicate loss of control and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

8. Crude Fat (Ether Extract) in Forages

Reference:

Fat (Crude) or Ether Extract in Animal Feed. (920.29) Official Methods of Analysis. 1990. Association of Official Analytical Chemists. 15th Edition.

Scope:

This method is applicable for the determination of crude fat in dried forages and mixed feeds. It is not applicable for oilseeds, baked and/or expanded products (pet foods), liquid feeds, sugar products, and feeds containing dairy products. For determining fat in oilseeds, consult Official Methods and Recommended Practices of the American Oil Chemists Society.

Basic Principles:

A dried, ground sample is extracted with diethyl ether which dissolves fats, oils, pigments and other fat soluble substances. The ether is then evaporated from the fat solution. The resulting residue is weighed and referred to as ether extract or crude fat.

Both the ether and the samples must be free of moisture to avoid coextraction of water-soluble components in the sample such as carbohydrates, urea, lactic acid, glycerol, etc. If water-soluble components are present in large amounts in the sample, they are washed out of the sample prior to drying.

Low temperatures are used to evaporate the ether and remove residual moisture to prevent oxidation of the fat.

Petroleum ether does not dissolve all of the plant lipid material, and therefore it cannot be substituted for diethyl ether.

Equipment:

Goldfisch fat extraction apparatus, 6-flask unit, equipped with glass thimble holders and ether
Extraction thimbles, 22 x 80 mm, Alundum (porous clay), coarse
Fat beakers, pyrex, with ground lip, engraved with a number, 50 x 85 mm
Drying oven, 102^{o}C gravity convection
Analytical balance, sensitive to 0.1 mg
Desiccator and tongs
Filter paper, Whatman #1, 11 cm, or equivalent
Steambath in a hood (optional)
Gloves, white nylon, lintless

Reagents:

Anhydrous Diethyl Ether, purified for fat extraction Mallinkrodt #0844 or equivalent. To prevent ether from absorbing water, purchase it in small containers and keep containers tightly closed.

Safety Precautions:

- Ether has an extremely low flash point. Have no open flames nearby. Avoid inhaling ether vapors. Store ether in metal containers. Handle open containers (reagent cans and fat beakers) in a hood. Conduct the extractions in a well ventilated area.
- Peroxides can accumulate in open containers of ether. These are explosive and shock sensitive. Check each container opened for more than 30 days for peroxides. Ether-containing peroxides must be disposed with special techniques.
- Electrical equipment is to be grounded. Extractors should be spark-proof.
- Make sure all ether is evaporated from the beakers before placing them in the oven to avoid a fire or explosion.

Procedure:

Sample drying

1) Weigh 1.5 to 2 g of ground sample into a thimble recording the weight to nearest 0.1 mg (W_1). Weigh a second subsample for dry matter determination.

- Or -

1A) If the sample contains large amounts of carbohydrates, urea, glycerol, lactic acid or water-soluble components, weigh 2 g sample to nearest 0.1 mg (W_1) into a small filter cone. Extract with five 20 mL portions of deionized water allowing each portion to drain, then insert the paper and sample into thimble.

2) Dry for 5 hr at 100^{o}C.
3) Dry beakers to be used for fat determination for at least 1 hr at 100^{o}C. Cool the appropriate number of fat beakers in a desiccator. Weigh and record the weight to the nearest 0.1 mg (W_2).
4) When the drying period is over, remove the samples from the oven to a desiccator. (This is a convenient stopping point. The samples should be stored in a desiccator if not immediately extracted.)

Extraction

5) Line the fat beakers up in front of the extractor and match the thimbles with their corresponding fat beakers.
6) Slip the thimble into a thimble holder and clip the holder into position on the extractor.
7) Add about 40 mL of diethyl ether (one glass reclaiming tube full) to each fat beaker.
8) Wearing white gloves, slip the beaker into the ring clamp and tightly clamp the beaker onto the extractor. If the clamp is too loose, insert another gasket inside the ring.
9) Raise the heaters into position. Leave about a 1/4 inch gap between the beaker and the heating element.
10) Turn on the heater switch, the main power switch, and the condenser water.

11) After the ether has begun to boil, check for ether leakage. This can be detected by sniffing around the ring clamp. If there is leakage, check the tightness of the clamp and if necessary replace the gasket(s).
12) Extract for minimum of 4 hr on a Hi setting (condensation rate of 5 to 6 drops per second), or for 16 hr on a Low setting (condensation rate of 2 to 3 drops per sec).
13) After extraction, lower the heaters, shut off the power and water, and allow the ether to drain out of the thimbles (about 30 min). This is a good stopping point.

Ether Distillation and Weighing of Fat Residue

14) Remove the thimble from the holder, and rinse the holder with a small portion of diethyl ether from the washbottle. Clip an ether reclaiming tube in place and reattach the fat beaker.
15) Reposition the heaters and turn on the electricity and water. Proceed to distill the ether using a Hi setting. Watch Closely.
16) Distill until a thin layer of ether remains in the bottom of the beaker, and then lower the heater. Do not allow beakers to boil dry. Overheating will oxidize the fat. When the last beaker has finished, shut off the power and water.
17) Wipe the exterior of the beaker clean with a Kimwipe as it is being removed from the extractor.
18) Empty the reclaiming tubes into the "USED" diethyl ether container.
19) Place the tray of beakers in an operating hood to finish evaporating the ether. If there is no hurry, air moving through the hood will be sufficient without heat. A steambath may be used to speed up the evaporation. Beakers should remain in the hood until all traces of ether are gone. Carefully sniff each beaker to determine if any ether remains.
20) Place the beakers in a 102^{o}C gravity convection oven. Warning: If a beaker containing ether is placed in the oven an explosion may occur.
21) Dry for 1/2 hr. No longer. Excessive drying may oxidize the fat and give high results.
22) Cool in a desiccator and weigh and record weight to the nearest 0.1 mg (W_2).
23) The fat beakers are best cleaned by warming on a steambath or on a hot plate on a low setting. Add some used ether to dissolve the fat. The use of a rubber policeman is helpful. After soaking the beakers in Alconox detergent, wash them using hot water and vigorous brushing. The thimbles are best cleaned by blowing out with air.

Comments:

- If doing a proximate analysis, the residue left in the thimble may be used to determine crude fiber.

Calculations: Percent Crude Fat (Ether Extract), DM basis

$$\% \text{ Crude Fat (DM basis)} = \frac{(W_3 - W_2) \times 100}{W_1 \times \text{Lab DM}/100}$$

Where W_1 = initial sample weight in grams
W_2 = tare weight of beaker in grams
W_3 = weight of beaker and fat residue in grams

Quality Control:

Include a reagent blank and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made.

An acceptable average standard deviation among replicated analyses for crude fat about ±0.10, which results in a warning limit (2s) of ±0.20 and a control limit (3s) of ±0.30. Plot the results of the control sample(s) on an X-control chart and examine the chart for trends. Results outside of upper or lower warning limits, ±2 standard deviations (95 percent confidence limits), are evidence of possible problems with the analytical system. Results outside of upper or lower control limits, ±3 standard deviations (99 percent confidence limits), indicate loss of control, and results of the run should be discarded. Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

Appendix A - Calculated Values

A1. Relative Feed Value Index

References:

Rohweder, Dwayne A. 1984. Estimating forage hay quality. In National Alfalfa Hay Quality Testing Workshop. pp 31-37 Chicago, IL. March 22-23.

Mertens, David. 1985. Effect of fiber on feed quality for dairy cows. In Proc. Minnesota Nutrition Conference. pp 204-224.

Linn, J.G. Neal P. Martin, W.T. Howard. and D.A. Rohweder. 1987. Relative feed value as a measure of forage quality. Minnesota Forage UPDATE. vol XII, No. 4. pp 2,4. Minnesota Forage and Grassland Council.

Rohweder, D.A., R.F. Barnes, and Neal Jorgensen. 1978. Proposed hay grading standards based on laboratory analysis for evaluating quality. J. Anim. Sci. 47:747-759.

Scope:

Digestible Dry Matter (DDM), Dry Matter Intake (DMI), and Relative Feed Value Index (RFV) calculations are applicable to legume, legume-grass and cool season grass fresh forages, hays and haylages.

Basic Principle:

Relative feed value index is an index which ranks cool season legumes, grasses and mixtures by potential digestible dry matter intake. It is an index used to allocate forages to the proper livestock class with a given level of expected performance. Relative feed value is calculated from digestible dry matter and dry matter intake.

Digestible dry matter is an estimate of the total digestibility of the feed and is calculated from acid detergent fiber.

Dry matter intake is an estimate of the amount of feed an animal will consume in percent of body weight and is calculated from percent neutral detergent fiber.

Calculation: Relative Feed Value (RFV)

$$DDM = \text{Digestible Dry Matter} = 88.9 - (.779 \times \%ADF)$$

$$DMI = \text{Dry Matter Intake} = \frac{120}{\%NDF}$$

$$RFV = \frac{DDM \times DMI}{1.29}$$

Where %ADF = acid detergent fiber, dry matter basis
%NDF = neutral detergent fiber, dry matter basis

A2. Adjusted Crude Protein

Reference:

Goering, H.K., C.H. Gordon, R.W. Hemken, D.R. Waldo, P.J. Van Soest and L.W. Smith. 1972. Analytical estimates of nitrogen digestibility in heat damaged forages. J. Dairy Sci. 55:1275-1280.

Klopfenstein, T.J. 1991. Efficiency of escape protein utilization. Proc. Distillers Feed Conf. 46:77-81.

Merchen, N.R., and L.D. Satter. 1983. Changes in nitrogenous compounds and sites of digestion of alfalfa harvested at different moisture contents. J. Dairy Sci. 66:789-801.

Merchen, N. R. 1990. Effects of heat damage on protein digestion by ruminants: Alternative interpretations. Proc. Distiller Feed Conf. 45:57-65.

Mertens, D.R. 1979. Adjusting heat-damaged protein to a CP basis. J. Animal Sci. 42:259.

Satter, L.D. 1991. USDA Dairy Forage Research Center, personal communication

Thomas, J,W. Y. Yu, T. Middleton and C. Stallings. 1980. Estimations of protein damage. Proc. Inter. Symp. Protein Requirements for Cattle. (F.N. Owens, ed.) Oklahoma State Univ., pp 81-98.

Van Soest, P.J. 1984. Nitrogen fractions in NDF and ADF. Proc. Distillers Feed Conf. 39:73-81.

Van Soest, P.J. 1965. Use of detergents in analysis of fibrous feeds. III. Study of effects of heating and drying on yield of fiber and lignin in forages. J.A.O.A.C. 48:785-790.

Weiss, W.P., H.R. Conrad and W.L. Shockey. 1986. Digestibility of nitrogen in heat-damaged alfalfa. J. Dairy Sic. 69:2658-2670.

Scope:

The calculation for adjusted crude protein is applicable to forages.

Basic Principle:

In non-heat-damaged forages, CP is highly correlated with the digestible protein (DP) content of forages [%DP = .94 (%CP) - 3.5; r = .95]. The regression coefficient of this equation (.94) estimates the true digestibility of CP in forages and the intercept (-3.5) estimates endogenous fecal loss of CP. Crude protein reflects the total nitrogen (N) content of feeds and provides a good estimate of DP in feeds that are not heat-damaged. However, there are instances when CP overestimates protein availability.

Studies indicate that heat-damage lowers protein digestibility in some low moisture silages (haylages). The degree of caramelization and browning of the forage is evidence of heat damage. Feeds that are dark brown or black have been extensively damaged by heating. The nonenzymatic browning (Maillard) reaction requires water, but not oxygen, and produces heat. It involves the condensation of carbohydrate degradation products with protein to form dark-colored, insoluble polymers. Sugar residues appear to condense with amino groups at a 1:1 ratio.

While developing the detergent system of fiber analyses in forages, Van Soest observed that materials dried at high temperatures resulted in high fiber values and the fiber contained nitrogen which was difficult to remove with detergent or pepsin. From this observation, he developed the concept that detergent could be used to partition protein into fractions that vary in their digestibility and availability in the rumen. Acid detergent insoluble nitrogen (ADIN, sometime

called ADFN) in forages represents primarily heat-damaged or bound protein that is indigestible or poorly digested by animals.

Mertens has shown that ADIN also exists in forages that have not been heated and observed that this non-heat-damaged ADIN is related to the lignin and to a fraction of protein in forages. His work suggests that 5-12% of the nitrogen in non-heat-damaged forages is isolated as ADIN (lower values for grasses than legumes). This naturally occurring ADIN probably is the truly indigestible protein in feeds. The ADIN in heat-damaged feeds contains both the ADIN naturally occurring in the feed and the ADIN associated with heat-damaged Maillard products. The heterogeneity of ADIN may explain the difficulty in measuring it (especially with NIR) and its lack of constant biological availability.

Neutral detergent insoluble nitrogen (NDIN), which is measured without the use of sodium sulfite as is used in the amylase neutral detergent fiber (aNDF) method, consists of ADIN plus insoluble fibrous proteins and cell wall bound protein. Van Soest suggests that the difference between NDIN and ADIN represents the protein that is slowly degraded in the rumen but is digested in the intestines. Cornell scientists use the difference between NDIN and ADIN in a computer model to estimate the amount of protein that escapes fermentation in the rumen (so called escape, bypass or undegraded protein).

It should be recognized that heating can have both positive and negative effects on protein utilization by the animal. Heating generally results in lowered digestibility of protein. Digestibility of ADIN varies from 0 to 60% depending on the feed ingredient, and the time and intensity of heating. At low heat inputs, this negative effect of heating can be compensated by reducing the solubility of proteins, which makes them less degradable in the rumen This reduces the potential loss of protein in the rumen (as ammonia) and actually increases protein utilization efficiency when feeds are slightly heated. Thus, for most feeds, no adjustment of CP is needed when ADIN (% of N) is less than 14%, CP is partially adjusted when ADIN is between 14 and 20%, and CP is completely adjusted for ADIN when it is above 20%.

Calculation: ADFCP/CP Ratio

$$\text{RATIO} = \frac{\text{ADFCP}}{\text{CP}} \times 100$$

Where ADFCP = % acid detergent fiber crude protein
CP = % crude protein

Calculation: Adjusted Crude Protein (ACP)

1) If ADFCP/CP ratio is less than 14 (all ADIN is considered digestible):

$$ACP = CP$$

Where CP = % crude protein

2) If ADFCP/CP is equal to or greater than 14 and less than or equal to 20 (only ADIN above 7% is indigestible):

$$ACP = CP - \left[\frac{RATIO - 7}{100}\right] X\ CP$$

Where Ratio = ADFCP/CP ratio calculated above
CP = % crude protein

3) If ADFCP/CP ratio is greater than 20 (all ADIN is considered indigestible):

$$ACP = CP - ADFCP$$

Where CP = % crude protein
ADFCP = % acid detergent fiber crude protein

A3. Estimates of Energy Availability

Scope:

The following equations are applicable for the estimation of energy in forages for ruminants. Prediction of energy availability from laboratory analyses usually requires specific equations for each type of feed. The accuracy of energy predictions is a function of the accuracy of laboratory analyses and the accuracy of the animal experimentation used to develop the prediction equation. Digestibility and energy value can be measured under a variety of conditions that influence the values that are obtained. Compared to cattle, sheep will obtain different digestibilities for the same feed. In addition, the level of feed intake of the animal has a significant effect on the digestibility of the feed and the utilization of its energy. For dairy cows, each level of intake above maintenance (the amount of feed needed to maintain a nonproducing animal's weight) reduces digestibility by about 4%. The dairy NRC assumes that lactating cows eat at 3X maintenance and reduces digestibility to 92% of that measured at maintenance.

Another major variable affecting the measurement of digestibility is the amount of selection allowed by the animal. Given a choice, most animals will eat the high protein, low fiber part of the feed (leaves) and leave the high fiber part (stems). Methods used to measure digestibility vary. Some scientists restrict the amount offered to the animal thereby encouraging the animal to consume it all. In this case a core sample of the feed represents what the animal consumed. However, most scientists measure ad libitum intake and digestibility in the same trial by offering the animals 5 to 15% more than they consume. Because they selectively consume the feed, a core sample may not represent the feed actually consumed and regression equations from these trials will be biased. Unfortunately it is difficult to uncover the exact techniques used to develop many of the equations for predicting energy value.

Basic Principle:

Available energy and digestibility cannot be measured in the laboratory and is estimated from chemical composition. Most energy values are predicted from fiber analyses because fiber is negatively related to the animal's ability to digest and use nutrients in the feed. Various groups have developed equations for predicting energy value and several are provided in the following tables for your consideration. Comparisons of the predictions of the various equations are given in tables 6 and 7. National Research Council (NRC) values are given for comparison, but it should be recognized that the source and accuracy of NRC values are also unknown. Total digestible nutrients (TDN) is the sum of digestible protein, digestible carbohydrates and digestible fat (fat is multiplied by 2.25 to adjust for its higher energy content). In general TDN is highly correlated with digestible dry matter (DDM) and digestible energy (DE). Estimated net energy (ENE) is a term formerly used to estimate net energy for production (weight gain or milk). Net energy of lactation (NE_L) is the current term used by NRC for assessing the energy requirements and feed values for lactating cows. All equations express ADF, NDF, TDN and DDM as percentages (1 to 100) and ENE and NE_L are expressed as Mcal/lb.

Table 1. Prediction Equations from Midwest

Legume and Grass Forages
%DDM = 88.9 - (0.779 x ADF)[a]

Corn Silage
%TDN = 87.84 - (.70 x ADF)[b]

Shelled Corn
%TDN = 92.22 - (1.535 x ADF)[c]
NE_L (Mcal/lb) = 0.905 - (0.0026 x ADF)[c]

Ear Corn
%TDN = 99.72 - (1.927 x ADF)[c]
NE_L (Mcal/lb) = 1.036 - (0.0203 x ADF)[c]

TDN conversion to NE_L
NE_L (Mcal/lb) = (TDN x .01114) - 0.054[d]

[a] Source: Rohweder, Barnes and Jorgensen, J. Anim. Sci. 68:403
[b] Source: Schmidt *et al.*, Agron. J. 68:403
[c] Source: Pennsylvania State
[d] Source: NRC, Dairy Update, 1989

Table 2. Prediction equations from Pennsylvania State[a]

Legumes
%TDN = 4.898 + (89.796 x NE_L)
ENE (Mcal/100 lb) = NE_L x 82.6
NE_L (Mcal/lb) = 1.044 - (0.0119 x ADF)

Mixed Forages
%TDN = 4.898 + (89.796 x NE_L)
ENE (Mcal/100 lb) = NE_L x 82.6
NE_L (Mcal/lb) = 1.0876 - (0.0127 x ADF)

Grasses
%TDN = 4.898 + (89.796 x NE_L)
ENE (Mcal/100 lb) = NE_L x 82.6
NE_L (Mcal/lb) = 1.0876 - (0.0127 x ADF)

Corn Silage
%TDN = 31.4 + (53.1 x NE_L)
ENE (Mcal/100 lb) = NE_L x 82.6
NE_L (Mcal/lb) = 1.044 - (0.0124 x ADF)

Sorghum, Small Grain Forages
%TDN = 4.898 + (89.796 x NE_L)
ENE (Mcal/100 lb) = NE_L x 82.6
NE_L (Mcal/lb) = 0.7936 - (0.00344 x ADF)

Complete Rations
%TDN = 93.53 - (1.03 x ADF)
ENE (Mcal/100 lb)= 82.04 - (0.76 x ADF)
NE_L (Mcal/lb) = (TDN x 0.0234) - 0.5448

Grain Mixtures (CF = crude fiber)
%CF = ADF x .80
%TDN = 81.41 - (0.60 x CF)
ENE (Mcal/100 lb) = 90.02 - (1.0532 x CF)
NE_L (Mcal/lb) = (TDN x 0.0234) - 0.5448

Ear Corn
%TDN = 99.72 - (1.927 x ADF)
ENE (Mcal/100 lb) = TDN x 1.025
NE_L (Mcal/lb) = 1.036 - (0.0203 x ADF)

Shelled Corn
%TDN = 92.22 - (1.535 x ADF)
ENE (Mcal/100 lb) = TDN x 1.053
NE_L (Mcal/lb) = 0.950 - (0.0026 x ADF)

Small Grains
%TDN = 4.898 + (89.796 x NE_L)
ENE (Mcal/100 lb) = 96.0548 - (0.8929 x ADF)
NE_L (Mcal/lb) = 0.9265 - (0.00793 x ADF)

[a] Source: Proceedings 41st Semiannual Meeting, 1981. Am. Feed Manufacturers Association. Lexington, Ky. p16-17.

Table 3. Equations from Western Region[a]

Alfalfa

%TDN = 82.38 - (0.7515 x ADF)

NE_L (Mcal/lb) = 0.8611 - (0.00835 x ADF)

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[a]Bath, Donald L. and Vern L. Marble. 1989. Testing Alfalfa for Its Feeding Value. Univ of CA. Cooperative Extension. Leaflet 21457. (WREP 109).

Table 5. Prediction equations from D.R. Mertens (personal communication)

Legumes
$\%TDN_m = 86.2 - (0.513 \times NDF)$
NE_L (Mcal/lb) = 1.054 - (0.0098 x NDF)

$\%TDN_m = 84.2 - (0.598 \times ADF)$
NE_L (Mcal/lb) = 1.011 - (0.0113 x ADF)

Grasses
$\%TDN_m = 105.2 - (0.667 \times NDF)$
NE_L (Mcal/lb) = 1.297 - (0.119 x NDF)

$\%TDN_m = 97.6 - (0.974 \times ADF)$
NE_L (Mcal/lb) = 1.120 - (0.0159 x ADF)

Table 4. Prediction Equations from New York State

Grasses
%TDN = 34.9 + (53.1 x NE_L)
ENE (Mcal/lb) = NE_L x 0.826
NE_L (Mcal/lb) = 1.085 - (0.0150 x ADF)

Legumes
%TDN = 29.8 + (53.1 x NE_L)
ENE (Mcal/lb) = NE_L x 0.826
NE_L (Mcal/lb) = 1.044 - (0.0123 x ADF)

Mixed Forages
%TDN = 32.4 + (53.1 x NE_L)
ENE (Mcal/lb) = NE_L x 0.826
NE_L (Mcal/lb) = 1.044 - (0.0131 x ADF)

Complete Feed
%TDN = 95.88 - 0.911 x ADF
ENE (Mcal/lb) = 1.0123 - (0.01432 x ADF)
NE_L (Mcal/lb) = 0.866 - (0.007 x ADF)

Grain mix
%TDN = 81.41 - (0.48 x ADF)
ENE (Mcal/lb) = 0.9002 - (0.0084 x ADF)
NE_L (Mcal/lb) = [(TDN x 0.0245) - 0.12] x 0.454

Ear Corn
%TDN = 99.72 - (1.927 x ADF)
ENE (Mcal/lb) = TDN x 1.025
NE_L (Mcal/lb) = 0.94 - (0.008 x ADF)

Shell Corn
%TDN = 92.22 - (1.535 x ADF)
ENE (Mcal/lb) = TDN x 0.01053
NE_L (Mcal/lb) = 0.94 - (0.008 x ADF)

Corn Silage
%TDN = 31.4 + (53.1 x NE_L)
ENE (Mcal/lb) = NE_L x 0.826
NE_L (Mcal/lb) = 0.94 - (0.008 x ADF)

Table 6. Comparison of TDN prediction equation for alfalfa and legumes.

ADF	NRC Alfalfa %TDN	Table 3 Western Alfalfa %TDN	Table 1 Midwest Gr.&Leg %TDN	Table 2 Penn St Legume %TDN	Table 4 NY Legume %TDN	Table 5 Mertens Legume $\%TDN_m$	Mertens Legume $\%TDN_{3X}$
27	68	62.1	67.9	69.8	67.6	68.1	62.6
29	63	60.6	66.3	67.7	66.3	66.9	61.5
31	60	59.1	64.8	65.5	65.0	65.7	60.4
33		57.6	63.2	63.4	63.7	64.5	59.3
35	58	56.1	61.6	61.2	62.4	63.3	58.2
37	55	54.6	60.1	59.1	61.1	62.1	57.1
39		53.1	58.5	57.0	59.8	60.9	56.0
41		51.6	57.0	54.8	58.5	59.7	54.9
43		50.1	55.4	52.7	57.2	58.5	53.8

Table 7. Comparison of TDN prediction equations for grasses.

ADF	NRC Grass %TDN[a]	Table 1 Midwest Gr.&Leg %TDN	Table 2 Penn St Grass %TDN	Table 4 NY Grass %TDN	Table 5 Mertens Grass $\%TDN_m$	Mertens Grass $\%TDN_{3X}$
27		67.9	71.8	71.0	71.3	65.6
29	74	66.3	69.5	69.4	69.4	63.8
31	71	64.8	67.2	67.8	67.4	62.0
33	69	63.2	64.9	66.2	65.5	60.2
35	67	61.6	62.6	64.6	63.5	58.4
37	64	60.1	60.4	63.0	61.6	56.6
39	62	58.5	58.1	61.5	59.6	54.8
41	60	57.0	55.8	59.9	57.7	53.1
43	57	55.4	53.5	58.3	55.7	51.3

[a] NRC Grass is an average of bromegrass, orchardgrass and ryegrass. NRC timothy was 7 to 8 %-units lower in TDN at each level of ADF.

Appendix B - Predicting Minerals by NIR

Reference:

McNunn, Lora. 1992. Predicting Minerals in Mixed Feeds. *In* ISI Training Manual. Perstorp Analytical.

Inorganic molecules do not absorb light energy in the near infrared regions. Mineral calibrations are based on an indirect relationship with the organic molecules due to the association of minerals with organic molecules. In other words, the NIR reading reflects something in the organic region that is correlated to the mineral composition of the sample. Since a correlation exists, a calibration can be created to estimate the mineral content. However, because the reading is not a direct prediction, errors will be much higher than for organic constituents.

Further, the correlation is only valid for minerals that are associated with the organic matrix of plant or grain tissues. When manufacturing a mixed feed, inorganic mineral sources, such as limestone, are added to the complete mix. Since these sources are not related to the organic composition of the feed ingredients, they cannot be detected by NIR instruments. Mineral calibrations created for mixed feeds would only predict the mineral composition of the organic portion of the mixed feed, ignoring any added inorganic sources. This type of calibration is eroneous and misleading because the NIR prediction will be much lower than the expected value, based on the known amount of added mineral.

Similarly, calcium added to soymeal as a "flow agent" cannot be detected by NIR.

NIR can only be used to estimate macro mineral content of organic tissues (excluding inorganic additions, dirt, etc.) and that any result outside of a normal range must be verified by reference methods.

Appendix C - Quality Assurance

Reference:

Dux, James P. Handbook of Quality Assurance for the Analytical Chemistry Laboratory. 2nd Edition. Van Nostrand Reinhold. New York, NY.

Quality control (QC) refers to those laboratory operations used to ensure that the data generated by the laboratory are of known accuracy to some stated, quantitative degree of probability. This means that every laboratory should be able to attach to every result a range within which the true value of the analyte can be stated to lie with a certain degree of confidence. The range is called a confidence limit with a specified probability. This does not mean that the confidence limit must be reported with the result, but that the laboratory should be able to make this statement, if required.

The kinds of operations referred to in this definition are those that are generally applied as good scientific practice: instrument calibration, personnel training, use of pure reagents, use of

standards and reference materials, etc.

Quality assurance (QA) refers to the ability of the laboratory to demonstrate or prove to someone not working in the laboratory that the quality of the data is what the laboratory says it is. Quality assurance is those laboratory operations undertaken to achieve the following objectives of documentation:

1. *That quality control procedures are being implemented in the laboratory.* For example, quality control requires that all pH meters be calibrated before use; quality assurance requires that the fact shall be recorded that the meter was calibrated and the results obtained.

2. *Assure the accountability of the data is maintained.* "Accountability" means that the data reported do, in fact, reflect the sample as it was received in the laboratory, i.e. that mix-up was avoided and the sample was properly preserved prior to analysis.

3. *Facilitate traceability of an analytical result.* Every result reported should be traceable to the date of analysis, the analyst who ran the sample, the method used, the instrument(s) used and its (their) condition, and the status of the analytical system at the time of the analysis.

4. *Ensure that reasonable precautions are taken to protect the raw data against loss, damage, theft, or tampering.*

The establishment, maintenance and enforcement of QA/QC programs in the laboratory is the responsibility of laboratory management.

The checklist below can serve as a guide for a quick review of the status of quality assurance practices in the laboratory.

- Information on standards
  - source
  - purity or activity
  - preparation
  - adequate labeling
  - storage method
  - date received
  - date prepared
  - lot number
  - identity of preparer
  - certification information
  - review and acceptance
  - acceptance criteria

- Laboratory sample handling/preparation
  - protocol for sample log-in
  - protocol for sample preservation
  - protocol for sample pretreatment (drying, grinding, etc)
  - protocol for sample storage
  - protocol for sample disposal

- Analytical methods
  - selection of analytical method
    - source (AOAC, AACC, AOCS, etc)
  - evaluation of analytical methods
    - validation
      - reference materials or samples
      - spiked samples
      - replicate analysis
      - negative control checks (blanks)
    - comparison to established method
    - ruggedness testing
  - method documentation
    - reference
    - scope - applicability of method
    - principle of method
    - equipment/apparatus needed
    - identification of safety precautions
    - procedure
    - comments
    - calculations
  - review and approval

- Quality control
  - calculation checks: must include spike and recovery and replicate analysis scheme; must be uniform for all samples of a given type and method.
  - systematic series of negative controls: blanks must be specified, i.e. reagent blanks, method blanks
  - performance evaluation samples (check samples): recommended for method, matrix, analyte customarily performed; these are not substitutes for regular controls but are used in addition to regular spikes and/or reference materials.
  - control charts
    - type
      - X charts
      - Spiked-sample control charts
      - R-charts
    - charts should be established for the analyte, matrix, and concentration of interest; results should be readily available.
    - limits/acceptance criteria should be established
  - blind samples

- Reporting of results
  - units must be specified
  - records must be kept for a specified time
  - timeliness: dates and times of analysis must be recorded and available
  - significant figures must be correct
  - form for reporting data must be uniform
  - report must be reviewed for correctness

- Instruments, equipment, materials
  - specifications met when purchased to perform task
  - preventative maintenance and control
  - calibration
    - type
    - frequency

- Personnel
  - resumes should be on record.
  - on-the-job training should be listed.

- Standard operating procedures
  - analytical methods (see above)
  - calibration of equipment and glassware
  - preparation of reagents
  - quality control procedures
  - data reporting procedures
  - personnel safety, training and evaluation
  - documentation of sample receipt, tracking, storage, and final disposition
  - scheme for data document storage, archiving, and retrieval
  - equipment and instrument operation and maintenance
  - waste disposal
- Corrective action

Appendix D - Quality Control

Statistics

Precision: Precision refers to the reproducibility of replicate results about a mean which is not necessarily the true value. Replicate analysis is the primary means of evaluating data variability or precision. Two commonly used measures of variability which adjust for the magnitude of analyte concentration are coefficient of variation and relative percent difference.

The coefficient of variation is used most often when the size of the standard deviation (s) changes with the magnitude of the mean. Coefficient of variation (CV), also called relative standard deviation (RSD), is defined:

$$CV = RSD = (s/\bar{y}) \times 100\%$$

where: $\bar{y}$ = mean of replicate analyses

s = sample standard deviation, defined as:

Install Equation Editor and double-click here to view equation.

where: y_i = measured valued of the ith replicate

$\bar{y}$ = mean of replicate analyses

n = number of replicates

Sample standard deviation and coefficient of variation are used when there are at least three replicate measurements.

The second measure of variability which adjusts for the magnitude of the analyte is relative percent difference (RPD) or relative range (RR). This measure is used when duplicate measurements are made and is defined:

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where: Y_1 = larger of the two observed values

Y_2 = smaller of the two observed values

Bias: Bias is the nearness of a result to the true value and is often described as systematic error. Bias estimates are frequently based on the recovery of the analyte of interest from certified reference materials (such as NIST reference materials) or from matrix or surrogate spikes when reference materials are not available. The percent recovery is calculated as:

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where: %R = percent recovery (of known materials)
M = measured concentration
K = known concentration

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where: %R = percent recovery (of matrix or surrogate spikes)
S = measured concentration in spiked sample
U = measured concentration in unspiked sample
C_{sa} = actual concentration of spike added

Control Chart: Precision and bias are monitored by plotting control charts to determine if the measurement system is in control. Standard deviation (s) is calculated from repeated analysis of in-house quality control samples or from the recoveries of known or spiked materials. The ±2s value is used as an "alert" or warning marker on the control. The ±3s value serves as the outer bound of control. Once control charts have been established, they are easily used to determine if the analysis is "in control" or "out of control." If the system is determined to be "out of control," all analytical work must be stopped until an "in control" situation is established.

Control Charting Three types of control charts are used in laboratories: the X-chart, the spiked-sample chart, and the range or R-chart.

X-Chart: A standard reference material or control sample is selected and analyzed with each batch of unknowns or, if a large number of unknowns is run in a batch, one control sample for each 10 or 20 unknowns. After 15 to 20 analyses, the mean and standard deviation of the data are calculated and a control chart constructed. An example of the chart is shown below:

The center line represents the mean, the two outer lines represent the upper (UCL) and lower (LCL) control limits, or 99 percent confidence limits, and the two lines closest to the mean line are the 95 percent confidence limits, or upper (UWL) and lower (LWL) warning limits. One analysis outside the 95 percent confidence limits is not cause for alarm; however, two consecutive analyses falling on one side of the mean line between the 95 and 99 percent limits would certainly be cause for an investigation. Control charts are very useful in visualizing trends. The chart below is an example of a chart showing drift.

The X-chart would be appropriate for monitoring nitrogen (crude protein), acid detergent fiber, neutral detergent fiber and acid detergent insoluble nitrogen determinations in forages.

Spiked-sample control chart: Spiked sample control charts are frequently used in cases where check samples of appropriate analyte concentrations are not easily prepared or obtained. The spiked-sample control chart is superficially similar to the X-chart, but instead of using a check standard, one of the unknown samples is analyzed and then spiked with a known amount of the analyte of interest. The percent recovery is calculated and plotted on the control chart. The control chart lines on the spiked-sample chart correspond to the mean recovery and the 95 and 99 percent limits calculated from the standard deviation of the recovery data. The resultant chart can be used and interpreted in the same way as the X-chart. Since this type of chart would seldom be used for the type of analyses discussed in this manual, it will not be discussed further.

R-chart: It is common practice in analytical laboratories to run duplicate analyses at frequent intervals as a means of monitoring the precision of analyses and detecting out-of-control situations. This is often done for analyses for which there are no suitable control samples or reference materials available, such as moisture determinations in forages. Usually the mean of the duplicates is reported and the difference between the duplicates, or range, is examined for acceptability. Frequently, there is no quantitative criterion for acceptability. The use of duplicate range in a control chart is one system of deciding acceptability of the individual ranges.

Youden and Steiner (*Statistical Manual of the Association of the Official Analytical Chemists*, Washington, DC: Association of the Official Analytical Chemists, 1975) have published a table for the variation of duplicate differences or ranges. Using the factors they published, a control chart can be set up for the differences between duplicate analyses, or ranges of the duplicates. Based on this table it can be shown that 50 percent of the ranges are below the 0.845R value, 95 percent are below 2.45R and 99 percent are below 3.27R, where R is the average range for a set of duplicate analyses.

After 15 or 20 duplicates are run, the average range is calculated, and the mean and control limits are drawn, using the 95 and 99 percent confidence limits and the 50 percent line is also drawn on the chart. The resulting chart is used to monitor the precision of the analysis.

A typical control chart for a moisture determination is shown below. The solid lines on the chart represent the 50 percent line and the two dashed lines are the 95 and 99 percent confidence limits.

In interpreting duplicate control charts the 50 percent line plays the same role as the mean or average line in X-charts. If more than five or six points in succession fall on one side or the other of the line, it is a strong indication that something has changed and should be investigated. If the points are on the high side, the precision has deteriorated, while if they are on the low side, it has improved.

Duplicate control charts are extremely useful for monitoring the precision of analyses for which there are no acceptable reference check materials. Since they are based on the difference between two results, errors due to bias are effectively canceled, and no conclusions can be drawn regarding the bias of the analysis.

Appendix E - Taking a Good Forage Sample for Analysis

References:

Subcommittee on Laboratory Certification of the U.S. Alfalfa Hay Quality Committee. 1984. Hay Testing Certification Manual. Publication No. 2.

Shenk, J.S., M.O. Westerhaus, and M.R. Hoover. 1980. The application of hay grades derived from near infrared reflectance analysis to hay marketing. American Society of Agronomy Abstracts p. 128.

Sampling is a major factor affecting the accuracy of forage quality analyses. Chemical analysis is valid only to the extent that the sample analyzed represents the lot of hay or haylage to be fed. The true analysis value of the lot is never known. Thus, the analysis of any sample is an estimate with a defined range within which the true value has a certain likelihood of occurring.

Sampling equipment, sampling method, and sample handling, once the sample has been obtained, will affect estimates of quality. Variation of forage quality within storage packages is always present and will affect the accuracy of any sampling technique. The sample to be analyzed must be representative of the designated lot of hay or silage. One should obtain a small, but representative, amount of hay or silage from many different locations or bales in a lot to adequately represent all the variations present.

Take samples by "lots" of hay or silage. A "lot" is defined as hay or silage which has been made from the same cutting, field, and stage of maturity. A sample should not represent more than 200 tons dry matter. For lots larger than 200 tons, two or more samples should be taken and the average of the results used to represent the lot.

Hay. Hay is harvested and preserved in a number of different packages. It may be in the form of pellets, cubes, small two-wire or string bales, small three-wire bales, large square bales (1 ton), large round bales, or stacked as loose hay.

The most commonly used sampling method for baled or stacked hay employs a hollow tube (probe) to extract core samples from the hay. Use a probe that travels at least 12 to 18 inches into the hay package for most hay packages. The internal diameter of the probe should be at least 3/8 of an inch. Probes with sharpened tips must be kept sharp to cut through hay. A dull tip may reduce the amount of stem material in the sample due to the tip sliding past rather than cutting through the stems.

Baled hay packages are not uniform products because the initial windrows were not uniform and the baling process affects the distribution of leaves and stems (bale structure) within the bale. Based on the structure of the hay package to be sampled, the hay should be probed in such a way as to adequately sample the various concentrations of stems and leaves. At least 20 cores (one core per bale) should be taken and composited to develop one sample per lot. Bales within a lot of hay should be sampled at random. Random means that there should be no prechosen reason for selecting a specific bale to

sample (i.e., location, color, leafiness, etc.). Techniques to guard against nonrandom sampling are to sample every fourth or fifth bale going around the stack, truck, or down the row in the field or take at least five random samples from each of the four sides of a stack.

Sample rectangular bales, regardless of size, using a probe centered in the end of a bale and drill horizontally into the bale.

Sample round bales by drilling horizontally into the curved side of the bale. Deteriorated hay from the exterior of the bale should not be sampled if it will not be fed to animals or they can be selective in their feeding. However, if hay to be sold includes the deteriorated exterior, it should be included in the sampling. Bales stored outside should be sampled within 2 to 4 weeks of feeding so that continued deterioration does not significantly lower bale quality from the sample taken for analysis.

For loose hay use a probe at least 30 inches long with 3/4 inch or larger internal diameter and drill at an angle from the side of the stack to the probe's full depth in 20 random locations throughout the stack. In a mow, hold the probe vertically and drill at the spot where the hay is compressed by the weight of the operator. Discard any weather damaged surface layer that would not be included in the part being fed or sold. Hay stored outside should be sampled within 2 to 4 weeks of feeding so that continued deterioration does not significantly lower bale quality from the sample taken for analysis.

Hay cubes or pellets should be sampled by collecting several hay cubes or handfuls of pellets from 15 to 20 locations in each "lot" so that a minimum of 40 cubes or 2 lb of pellets are selected. Each lot should be limited to 200 tons or less.

Silage. Collect a 1- to 2-lb sample from the silo unloader while it is operating or a comparable amount from several sites in a bunker or silo tube. Do not collect a silage sample until at least two weeks after ensiling. Do not collect a silage sample from the top 2 to 3 feet in a top-loading upright silo. Avoid sampling from moldy or spoiled areas in silo, bunker or tube. Also, avoid sampling silage that has been exposed to the air for several hr. Sample bunker silos by sampling 12 to 15 sites from the face of the silage in the silo.

Sampling chopped forage as it is being put into the silo will give an indication of forage quality but will not account for changes occurring during the ensiling process. Fiber changes are usually less than 1 unit and occur primarily because digestible material is lost through respiration or juices leaching out. Protein content and solubility can change significantly during the ensiling process depending on the fermentation process.

Total Mixed Rations (TMR). Total mixed rations are difficult to sample because they are seldom homogeneous or well mixed. When it is unlikely that a sampling method can produce a representative sample, it is recommended that the components of total mixed rations be sampled and analyzed individually. When confident that a representative

sample can be obtained, a TMR sample may be analyzed by wet chemistry. NIR calibration on TMR samples has not been successful.

Sampling silages, haylages and total mixed rations may produce a large amount of sample. The sample should not be divided because stems and leaves will separate and settle in the sample. The sample should be taken early in the week, placed in a polyethylene, airtight (e.g. freezer) bag, sealed tightly and immediately mailed or delivered to the laboratory. Perishable samples should be mailed immediately after collection and should be mailed early in the week so they arrive at the laboratory without spending the weekend in shipment. Samples can be frozen before shipment if freezing will not affect analytical results (prussic acid analysis). For samples handled in either manner, the laboratory report of dry matter "as received" will approximate the dry matter content of the lot of hay or silage when sampled.

The sample should be properly labeled for the lot and locations where the cores were taken when it was sampled. The sample should also be labeled for source (area where grown), forage type (species), cutting, stage of maturity, and special conditions (frost, drought, etc.). Further information such as cutting date and interval between cuttings may also help a laboratory make a decision about atypical samples.

Appendix F - Critical Conditions in Determining Detergent Fibers

References:

Mertens, D.R. 1992. Critical conditions in determining detergent fibers. Proc. NFTA Forage Analysis Workshop, Sept. 16-17, Denver,CO. pp. C1-C8.

Abstract

Fiber is a uniquely nutritional entity that we attempt to measure using chemical solubility methods. Because there is no direct relationship between solubility and nutritional availability, the method used to isolate fiber, in effect, defines it. This indicates that fiber methods must be followed exactly to obtain values that are valid and reproducible. Unfortunately laboratories sometimes modify fiber methods for convenience or speed without understanding the effects these changes have upon results. Some of the conditions and steps in the detergent fiber methods are critical in obtaining accurate results. Among these are: subsampling, drying, grinding (sample particle size), sample amount, standardization of reagents, removal of starch and nitrogen contamination, timing and temperature of refluxing, transferring residues, washing fibrous residues, type of filtration vessel and weighing method. Some types of samples are difficult to filter in the NDF procedure, and modifications are proposed that aid the filtering of starch-, pectin- and fat-containing samples.

Introduction

Fiber is a uniquely nutritional term that can be defined as the indigestible or slowly-digesting components of feeds that occupy space in the gastrointestinal tract of animals. Chemically these components are a variable mixture of cellulose, hemicelluloses, some pectins and lignin, with indigestible proteins and lipids. This nutritional definition of fiber indicates that it can truly be measured only by the animal. However, nutritionists need a practical and routine way of measuring fiber and must compromise between the theoretical concept of fiber and the utility of using chemical solubility to isolate and measure fractions that closely resemble the nutritionally defined fraction called fiber. Because there is no guarantee of direct correspondence between chemical solubility and nutritional availability, in reality, fiber is defined by the method used to isolate it. The actual definition of fiber becomes method dependent, which explains why there are so many different fiber analyses (crude fiber, acid detergent fiber, neutral detergent fiber, amylase-neutral detergent fiber, dietary fiber, etc.).

The abundance of fiber methods is further complicated by the modifications of each method that are commonly used. Sometimes these modifications are developed to meet the specific needs of a particular application or research project. Other times modifications are made for convenience or to increase the speed of fiber analysis. Because fiber is defined by the method used to isolate it, it should be clear that modifications have the potential for defining a new fiber value that is not comparable with the parent method. The sensitivity of fiber values to method suggests that fiber methods must be followed exactly to be reproducible. To be acceptable, any modification of fiber methods must be evaluated thoroughly with several feed samples representing each type of feed ingredient. The objective of this paper is to discuss some of the critical steps and conditions in detergent fiber analyses and indicate the potential problems

inherent in modifying these methods.

Factors Causing Variation in Detergent Fiber Analyses

Subsampling and Segregation. In general, the fiber content of large particles is greater than that of small particles for most feed ingredients. Thus, any process that segregates a sample by size (such as shaking during shipment, grinding or grab sampling) can lead to a subsample that differs from the true average of the sample. One of the most insidious segregation processes is grinding. The tough, large particles that are retained in the grinder and finally pulverized to the extent they pass through the screen are high in fiber. If they are brushed or vacuumed from the mill and not included in the sample, there is an error. If they are ground until they pass, but the sample is not mixed thoroughly after grinding, there is an error because the last material (high fiber) out of the mill is on the top of the sample due to grinder segregation of the sample.

Sample Drying. Proteins and carbohydrates can form insoluble compounds (Maillard or browning products) when exposed to high temperatures in the presence of moisture. These Maillard products are measured as artifact fiber and lignin in the detergent system. Thus, feed samples should never be exposed to temperatures above 60°C (140°F) during drying and a maximum of 50°C (125°F) is preferred.

Sample Grinding. Fiber methods function by extracting and solubilizing non-fibrous compounds from feed particles. It is expected that extraction efficiency should increase as the size of particles decreases because reagents and washing solvents have less matrix to penetrate. Furthermore, fibrous residues are filtered on coarse porosity membranes suggesting that fine fiber particles may be washed out of the residue or plug the filter membrane. These factors explain why finer grinding of feed samples results in lower fiber values. However, a compromise is necessary between fine grinding to increase extraction efficiency and coarse grinding to prevent lost of fiber particles and plugging of the filtration vessel. The recommended grinding procedure uses a Wiley mill with a 1 mm screen. Cyclone mills generate particle size distributions that are smaller than Wiley mills when similar size screens are used because cyclone mills force the particles through the screen at an angle. Using the same size screen, Udy cyclone mills will produce an average particle size that is one half that of Wiley mills resulting in slightly lower fiber values and greater filtering difficulties during detergent analyses.

Standardizing reagents. To provide accurate ADF and NDF values, both reagents should be standardized. Measurement of ADF depends on the use of 1 N sulfuric acid. Normality of the acid used for acid detergent (AD) solution should be verified by titrating it against a primary base standard. We use TRIS or THAM (Tris- hydroxymethyl-aminomethane) as a primary base standard. Make a .9-1.0 N solution of THAM (weigh and calculate normality to 4 decimal places) and use it in the buret. Titrate (to the nearest .01 ml) 10 ml of acid in a beaker with stirring using a pH indicator that changes near pH 7.0. If AD solution is not between .99 and 1.01 N, adjust normality by adding water or concentrated sulfuric acid.

Neutral detergent (ND) solution should be standardized to a pH of 6.9 to 7.1 (I prefer a narrower range of 6.95 to 7.05). If pH differs by more than .2 from 7.0, check reagents to make

sure the wrong chemicals were not used and consider discarding the neutral detergent solution. If pH is between 6.8 and 7.2, adjust pH by adding either HCl or NaOH to obtain a pH of 7.00. The original ND solution developed by Van Soest contained 2-ethoxyethanol which has been found to be toxic. Triethylene glycol should be substituted for it on an equal volume basis. This chemical should not be eliminated from ND solution because it aids in the removal of non-fibrous residues from some feeds. Amylase solution should be standardized so that 2 ml of enzyme solution added at boiling and during the first filtration step removes all traces of corn starch from the fritted disk of Gooch crucibles. Make sure that 0.5 gm of sodium sulfite is added to each sample before refluxing. It is important for the removal of protein from NDF and is especially critical in the removal of nitrogenous contamination from cooked or heated feeds, animal byproduct feeds and fecal or digesta samples.

Sample Amount. The ratio of sample to detergent solution can have a small, but significant, effect on fiber analyses. The standard procedure for ADF uses 1.0 g of sample with 100 ml of AD solution, while the NDF procedure uses 0.5 g of sample in 50 ml of ND solution. The selection of sample amount to analyze is a compromise among extraction efficiency, reagent cost and weighing errors. Larger sample amounts increase reagent costs when maintaining the same sample:solution ratio. Smaller sample amounts magnify any weighing errors, e.g., if the residue weighs .0100 g with a weighing error of .0002 g, the error is 2%; however, if the residue weighs only .0020 g, the same error is 10%. Since ADF has smaller residues and the residue is often used for lignin analysis, a larger sample amount is recommended for ADF.

Pre-soaking of Samples Before Refluxing. Some laboratories weigh and add AD or ND solutions to the samples the night before analysis. This is NOT recommended. AD is a strong acid and it begins to degrade some fiber components when exposed to the sample for prolonged times. Sodium sulfite makes the ND solution unstable (that is why it is not added to the solution) and soaking in ND increases extraction efficiency. ALWAYS add detergent reagents at the time of refluxing.

Varying Refluxing Times and Temperatures. Extraction of detergent fibers is both time and temperature dependent. As the time and temperature increase, the amount of fibrous residue recovered decreases. It is critical to each method, especially ADF, that the time of refluxing be 60 minutes from the time of boiling. Refluxing should be at a temperature that causes a rolling agitation of feed particles. We calibrate our heating elements so they will bring 100 ml of water at room temperature to a boil in 3 to 4 minutes. This setting is marked on the heater and used during fiber refluxing.

Incomplete Transfer of Residues to the Crucible. The greatest source of poor technique is the loss or incomplete transfer of all fibrous residue from the Berzelius beaker to the Gooch crucible. Sometimes residues are stuck to the sides or bottom of the beaker. These residues must be policed free before they can be transferred. At other times the last drop from the beaker is allowed to flow down the outside of the vessel when it is turned upright after pouring contents into the crucible. We recommend that the beaker be kept inverted over the crucible and be rinsed with a fine stream of hot water to transfer all particles. If the beaker must be turned upright during transfer, be sure to wipe the last drop from the lip of the beaker onto the

lip of the crucible. Often this last drop contains significant fiber because particles have settled in the beaker during transfer. Transfer should be so complete that beakers do not need to be washed between uses. Beakers should be checked routinely for cleanliness to insure that previous transfers were complete.

Washing Residues with Hot Water and Acetone. The most common error made by fiber analysts is incomplete washing of fiber residues to remove detergent and soluble feed components. All too often residues are rinsed, rather than soaked during the washing steps. Feed particles are filled with voids that can trap solutions and components. These voids cannot be washed free of contaminants by simply rinsing the outside of the particle. The laws of mass action must be used to equilibrate the liquids within the void with clean wash water on the outside of the particle. This is a time dependent process. Thus, fibrous residues must be soaked in 30-40 ml of clean hot water (95-100°C) at least 2 minutes each time to remove the detergent and soluble compounds trapped in the voids of particles. The larger the volume of water and the longer the time of soaking, the more complete will be the extraction of soluble contaminants of fiber. We normally extract with 40 ml of water for 5 minutes.

The same principles are true for acetone washes which are used to remove residual lipids (fats) from the sample. Simply washing the outside of particles with acetone will not extract all the lipid. Both the time and amount of clean acetone are important. A minimum of 20 ml of acetone for 2 minutes is needed and we normally use 30 ml extracted for 5 minutes.

It is especially important that all traces of acid be washed from ADF residues and filtration vessels. With crucibles it is desirable to rinse the underside of the crucible, and with filter paper it is wise to rinse the edges of the paper. If residual acid remains, it will migrate to the edges of particles and become concentrated during drying. The concentrated acid will char the fiber or filter paper at 100°C. Charring signifies oxidation and loss of organic matter resulting in low weights. In crucibles only the fiber is lost and low fiber values are obtained. When filter paper is used, the loss of weight can be from both fiber and paper. Because the acid migrates to the edge of the paper, mostly paper weight is lost resulting in high fiber values**.**

Filtration. Several factors are important in making filtration of fiber residues effective and efficient. Normally minimum filtration vacuum should be used to prevent plugging the filter membrane with fiber residues and losing fine particle. The vacuum source should be constant and have reserve capacity. Some laboratories successfully use water aspirators, but we have not found them acceptable in our lab. We use a two-stage diaphragm pump with two 18 liter glass reservoirs as waste traps and vacuum reserves. It is also important that the manifold and vacuum lines be constructed to minimize the trapping of foam. Foam will greatly reduce the effective vacuum at the crucible.

We have designed a manifold that minimizes vacuum leaks and foam in the system, yet is durable and economical to construct. The manifold is designed for Gooch crucibles, but can easily be modified for use with Buchner funnels or paper funnels. The basic design fits crucibles tightly and allows back flushing of problem crucibles by removing and reinserting them into the holder.

The choice of filtration vessel is a compromise between filtration ease and fiber recovery. Coarse membranes will allow some fine fiber particles to be lost, but fine membranes often plug, making filtration difficult. The retention size of some common filtration vessels indicates the potential variation that can occur:

Vessel or Membrane	Retention Size (micrometers)
Extra coarse fritted disk, Gooch crucibles	170-220
FiberTec P0 special crucible	160-250
FiberTec P1 special crucible	90-150
California Buchner funnel with 200 mesh screen	70-85
FiberTec P2 standard crucible	40-90
Coarse fritted disk, Gooch crucible (50 ml)*	40-60
FiberTec P3 special crucible	14-40
Whatman 41/54/541 filter paper	20-25
Medium fritted disk, Gooch crucible	10-15
Whatman 40 filter paper	8
Fine fritted disk, Gooch crucible	4.0-5.5
Whatman GF/D glass microfibre filters	2.7
Very fine fritted disk, Gooch crucibles	2.0-2.5

*Recommended crucible for ADF and NDF analyses.

Filtration difficulties also can be caused by gradual plugging of the fritted disks of crucibles with ash after repeated use. We ash our crucibles as a part of the NDF procedure, but if ashing is not used to determine ash-free fiber values, it should be the first step in cleaning the crucibles after each use. Ash for 5 h at 500-525°C. Do not use a higher temperature or the glass will melt or glaze the surface of the fritted disk. Remove residual ash from crucibles with a brush and place them upside down in a sonicating bath containing MICRO cleaning solution and sonicate for 5 minutes (sonication is optional; crucibles also can be cleaned with brush using a detergent solution). Rinse crucibles by pulling water through the fritted disk in the reverse of normal use by connecting a No. 9 rubber stopper with a tube through the middle to a vacuum line containing a trap and applying vacuum. By putting the stopper in the top of the crucible, an air tight fit is achieved for sucking water through the crucible in reverse.

Occasionally the crucibles are cleaned with 6N HCl and/or an alkaline cleaning solution containing 5 g of disodium EDTA, 50 g of trisodium phosphate and 200 g of potassium hydroxide per liter of water. The crucibles should be allowed to soak in either solution for 30 minutes and the alkaline solution should be used with heat. The alkaline treatment can weaken the glass so we use it only on crucibles that do not filter normally. We check the filtration rate of crucibles by measuring the time it takes for 50 ml of water to pass through each crucible without vacuum. It should take approximately 180 seconds. If it takes less than 120 seconds, check the crucible to insure it is not cracked and leaking. If it takes longer than 240 seconds, clean the

crucible with acid and measure again. If it is still takes 240 seconds, clean with alkali. If cleaned crucibles take longer than 240 seconds, discard them because they will cause filtration problems.

Drying and Weighing Fibrous Residues. Acetone residues should be removed as completely as possible with vacuum. We recommend that extracted samples be placed near or on the drying oven to evaporate residual acetone before placing them in the oven. We also recommend that all samples be placed in the oven at one time at the end of the day. This prevents moisture from wet samples placed in the oven contaminating samples that have been dried in the oven. Samples should remain in the oven (100-105°C) until they achieve a constant dry weight. This normally takes 8 h or overnight drying.

Weighing technique is critical for obtaining dry weights of samples and fibrous residues. We recommend the hot weighing technique because it is more accurate and precise for hygroscopic residues such as dry fiber. The hot weighing technique is faster, requires less handling of the sample and is less prone to the errors associated with desiccator use. If too many samples are placed in the desiccator at one time, if the lid is held open during transfer from the oven or weighing, or if the desiccant is the wrong type or is not changed often, dry weights obtained using a desiccator are incorrect regardless of the oven temperature or drying time. We normally dry residues overnight and weigh them directly out of the oven using a Teflon pad on the balance pan to prevent heat transfer to the balance.

Calculation and Dry Matter Errors. Although it is rare, laboratories have been known to have errors in the equations used to calculate results. The most common source of discrepancies in fiber results among labs is due to differences in dry matter estimates and the variation associated with adjusting fiber values to a dry matter basis. Because dry matter determinations among laboratories are often not in agreement, we routinely compare results among labs on an as-is basis to remove variation due to dry matter adjustments.

Determining Fiber in Difficult-to-Filter Samples

Any sample that takes more than 10 minutes of filtration time under vacuum should be discarded because the results will be inaccurate. Instead, rerun the sample using a modification of the NDF method. Several modifications can be used on any or all samples that are difficult to filter:

1. Reduce the sample amount to 0.3 g. This will increase the errors associated with weighing and subsampling, but it often is the best approach to use with difficult samples.
2. Use filter aids. Glass wool (about 0.25 g) or glass microfibre filter mats (Whatman GF/D, 4.25 cm) will keep gelatinous materials and ash or fine residues from plugging the fritted disk of the crucible. The filter mats are expensive, but they sometimes provide the only means of obtaining NDF analyses.
3. Back-flush the crucible by removing then reinserting it into the crucible holder to force air back through the fritted disk.

High Starch Samples. If filtration is difficult, inject additional standardized amylase solution. Many times this will unplug the fritted disk and allow filtration. Modifications of the original NDF method that use an amylase treatment have solved this source of filtering difficulty in most cases.

High Pectin, Mucilage or Glycoprotein Samples. These samples must be kept hot to filter. Decrease soaking time to the minimum and keep water at boiling temperature. Preheat the crucible by filling it with hot water before beginning to transfer the residue from the beaker. Do not let residues settle in the beaker before transferring to the crucible; instead transfer as quickly as possible. Adding glass wool or glass filter mats to the crucible keep the gelatinous residue from plugging the filter. Adding acetone before the last water wash has been completely filtered (less than 5 ml of water remaining in the crucible) can salvage some samples, but recognize that acetone will precipitate any residual detergent in the residue.

High Fat Samples. Pre-extract samples with acetone to remove some of the lipids before fiber analysis. To do this, weigh the sample into the previously tared crucible that will be used for fiber analysis. Add 30-40 ml of acetone to the crucible and let the sample soak for 5 minutes, stirring occasionally. Remove the acetone with vacuum and repeat the acetone extraction three additional times. After the last extraction, vacuum the sample dry and quantitatively transfer the residue to a Berzelius beaker for fiber analysis. Do fiber analysis in the usual manner and filter the sample into the crucible used initially to extract the lipid. To remove any ND soluble matter that may be retained in the crucible and improve filtration, place it in a heated, covered glass dish with ND solution during the time the sample is being refluxed.

High Ash, Fecal or Digesta Samples. Fecal samples can be especially difficult to filter. It appears that fine material in these samples plugs the pores of the filtering vessel and slows or prevents evacuation. Using microfibre filter mats is usually essential to the determination of NDF in these samples. Filtration also can be enhanced by allowing the sample to settle in the beaker for 60 seconds after it has been removed from the refluxing apparatus and carefully decanting the liquid from the beaker with minimal transfer of particles to the crucible. It helps to

slowly transfer the liquid under vacuum in a way that does not cover the entire surface of the filter mat. If the sample begins to plug during the washing step, carefully scrape the surface of the mat to provide a new surface for filtration. Patience and minimum vacuum during the transfer step are important in obtaining accurate results with these samples.