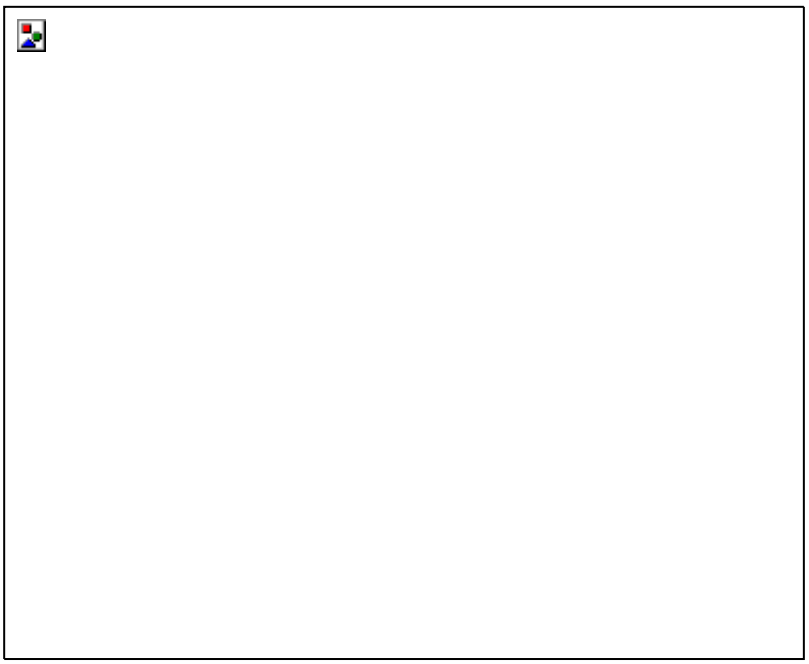
AFIA - Laboratory Methods Manual

A reference manual of standard methods for the analysis of fodder



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About the AFIA

The Australian Fodder Association (AFIA) is the National peak body for the Australian fodder industry and was established in March 1996. This formation came from the realisation that the fodder industry needed a coherent representative group.

The AFIA objectives are to:

- sustain a profitable industry for all participants,
- promote the objective testing of hay and silage
- represent the industry on a national basis and
- promote funding targeted at research and development in the fodder industry via RIRDC

AFIA members are a rare 'whole of supply chain' blend of fodder producers, consumers, traders, contractors, exporters, grower organizations, machinery manufacturers, input suppliers, state government departments and testing laboratories.

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FOREWORD

This manual is the result of extensive discussion and collaboration among fodder testing laboratories, animal nutritionists and representatives of the Australian fodder industry. Its objective is to foster uniform testing methods across Australian laboratories for the quality criteria of importance to the fodder industry.

The quality attributes of hay and silage vary greatly compared to other commodities such as grain. Consequently, the objective measurement of fodder quality is critical when seeking predictors of livestock performance.

Historically, laboratories have worked in isolation, developing and implementing their own procedures for testing the nutritive value of fodder, whether engaged in research, extension or commercial activities. This has meant that a client who divides a sample into three parts and sends a sub-sample to three different laboratories may well receive three different sets of results. These may include some common tests (eg. moisture and crude protein) but may also contain different fibre fractions and estimates of energy content using various tests and prediction equations. This can result in fodder producers and end-users becoming confused and continuing to rely on the old and unreliable subjective criteria such as colour, odour and texture.

Serious efforts to introduce a uniform objective system for fodder quality description and testing began in the early 1990's, with a working party set up by the Victorian Farmers Federation (VFF) Grains Group. However it soon became clear that for such a system to have any chance of success, it must be national rather than confined to any one State.

In 1994, the Rural Industries Research and Development Corporation (RIRDC) convened a national workshop to review its R&D for fodder crops. A key strategy emerging from this workshop was the need to 'develop a product description language, matching buyer specifications as a basis for developing quality standards'. Subsequently, research funding for this topic was provided by RIRDC to the Victorian Department of Primary Industries (DPI Vic) and to CSIRO Livestock Industries (WA).

A subsequent industry forum, funded from the DPI Vic project and supported by RIRDC and VFF, had two objectives: to seek agreement on a national uniform system for the measurement and description of fodder quality, and to determine the extent of industry support for the formation of a national fodder industry body, which up to that point had never existed in Australia. This forum resulted in the formation of the Australian Fodder Industry Association (AFIA), now a well-established and successful industry peak body.

The forum, which represented all sections of the industry, from production through to a variety of end-users, agreed that the basic objective measurements needed for fodder were dry matter, metabolisable energy and crude protein, with a suitable measurement to predict voluntary intake to be added later, pending successful research. It is significant to note that this forum was attended by representatives of all major fodder testing laboratories in Australia at that time, the first time this had ever occurred.

At various meetings following the forum, there was extensive discussion and debate on the details of laboratory methods for testing the agreed parameters. The group involved in the discussions, which included representatives of the laboratories and the fodder industry, has now been formalised as a sub-committee of AFIA, called the Quality Evaluation Committee (QEC). The agreed methods which now appear in this manual cover sampling, sample preparation, dry matter, crude protein, digestibility and estimated metabolisable energy.

A major output of the DPI Vic research project was the measurement of *in vivo* digestibility (using sheep) of 16 different hays which varied widely in quality. Large samples of these hays were

retained and agreement was reached between DPI Vic and RIRDC for the samples to become the property of AFIA. Sub-samples of the hays have been made available, together with this manual, to fodder testing laboratories affiliated with AFIA, with a specific objective of assisting laboratories to standardise their estimation of digestibility, which is a 'property' of fodder rather than a 'constituent'.

The next important step in obtaining uniformity in fodder testing has been the introduction of 'ring tests'. This involves sending carefully prepared sub-samples of six fodder samples to the participating laboratories on a regular basis, in order to determine the agreement between laboratories. This 'ring test' procedure has been and will continue to be a vital mechanism to aid quality control in laboratory testing of fodder.

Additional tests to those agreed as important to the industry in 1995 have now emerged, especially in relation to fodder exports and silage. These include acid and neutral detergent fibre (ADF and NDF), measures of total or water-soluble carbohydrates, ammonia nitrogen and pH of silage. Shear energy has been added to this latest edition of the Manual.

There are alternative methods possible for determining several forage quality values using more convenient equipment. The QEC decided that, where there were several alternative methods for analysing one fodder constituent, one would be referred to as the "Reference" method and given the symbol "R", whereas the others would be designated as "Approved" methods, given the symbol "A" followed by (a..n), when there is more than one approved method. A requirement for laboratories that use "Approved" methods is for them to demonstrate that they can produce the same results obtained using the "Reference" method.

Draft Methods are given the symbol "D" and may be added to this manual following consideration at a meeting of the QEC. People wishing to propose new methods need to send them to the AFIA EO. Once circulated to QEC members and discussed or amended at a QEC meeting proposals may become a draft method. At the subsequent August QEC meeting, a vote can be taken on any Draft Method to change it to Approved Method status, remove it from this manual, or retain it as a Draft Method.

Proposed changes to any existing methods should be provided to the AFIA EO for circulation to the QEC members and discussion at the next meeting. As this manual is a "living" document, AFIA have decided to provide it in electronic format.

Clearly, the whole issue of fodder testing methodology must be an ongoing process, as new research developments occur and new industry priorities emerge. Laboratories have to respond to these factors, but for the industry to benefit as a whole, a coordinated approach is essential. This requires the continuing dedication, cooperation, understanding and good-will of all parties, irrespective of commercial competition between laboratories and other industry participants.

It is hoped that this manual will be a valuable resource for fodder testing laboratories and others with an interest in this field. Many people and organisations have contributed to the effort of producing the manual, but particular thanks are due to G&M Murphy Consulting Services, John L Black Consulting, Peter Flinn of Kelspec Services, Alan Kaiser of NSW Department of Primary Industries and RIRDC for its ongoing support. This project was funded from industry revenue that is matched by funds provided by the Federal Government via RIRDC.

Acknowledgments have been included in the manual where methods have been adopted or modified from those listed in the "Forage Analyses Procedures" of the US National Forage Testing Association (1993). The note on the use of anthrone methods for carbohydrate analysis was provided by Mr A.B. Blakeney of Cereal Solutions.

The Title of the Manual was changed for Edition (3) to 'AFIA – Laboratory Methods Manual' from 'Fodder Analyst's Methods Manual'.

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SECTION 1

AFIA REFERENCE AND APPROVED METHODS

“Reference” methods are regarded as the AFIA benchmark methods. “Approved” methods involve more convenient equipment and can be used for AFIA approved fodder analysis provided laboratories can demonstrate that the “Approved” method produces results not significantly ($P<0.05$) different from the “Reference” method.

“Reference” methods for a specific analysis are numbered and followed with the letter “R”, whereas “Approved” methods for the same analysis have the same number followed by “A(a..n)” for cases where there is more than one Approved method.

Method – 1.1R: How to Take Fodder Samples for Analysis

Scope: This procedure is used to obtain fodder samples for analysis; separate methods are used for hay and silage.

Principle: The accuracy of fodder analysis depends on the sample sent to the laboratory. It is critical that the sample represents the average composition of the "lot" of fodder sampled; otherwise the laboratory tests will not be useful.

A "lot" is defined as hay or silage taken from:
- the same species (pure or mixed) and variety;
- the same paddock or pit/bunker; and
- harvested within 48 hours.

Other factors influencing the definition of a "lot" include:
- rain damage;
- weed content;
- soil type;
- treatment after cutting; and
- storage effects.

A "lot" of baled hay or cubes should not exceed 200 tonnes.

Sampling Equipment: Representative hay samples can only be obtained with a probe or core sampling device. *Do not rely on a couple of handfuls or a "flake" from one bale.*

- Australian made corers are available from:
  KELSPEC SERVICES, PO Box 31, Dunkeld, Victoria 3294 (phone 03 5577 2624);
- Several types are also marketed in the USA.
- A home-made corer can be made from 32mm steel tubing. It should be at least 450mm long with a slightly scalloped and sharp cutting edge.
- Corers are driven using either a hand brace or by a continuously variable speed, electric drill (preferred option where practicable). A portable generator is useful and can be justified if many samples are to be taken. Cordless drills can be used if they meet power (torque) requirements.

Sample Size Reduction:

The original sample will normally require reducing in size prior to further processing. There are two ways this can be achieved: By use of a riffle box or by sample coning and quartering. The riffle box is preferred for certain sample types (e.g. fine chopped maize forage) in that it is faster and yields a marginally better sub-sample. However, it requires specialist equipment, and for some sample types, it can be prone to blocking.

Riffle Box: These devices are also known as sample splitters and are available commercially. The sample is split into two equivalent portions via a series of gates. One half is usually discarded, and the other retained for analysis, or for further sample reduction

Sample Coning and Quartering: This is the process used to reduce the size of a sample to a convenient amount for submission to the laboratory. It involves the following steps:

a) Spread the entire sample over a clean surface or tray and mix well to ensure even distribution of leaf and stem;

b) Fold the outer edges back into the middle to form a “Cone” or “Mound” of material;

c) Divide this “Cone” into four equal “Quarters”;

d) Select any two diagonally opposed “Quarters” and either transfer this sub-sample to a clean plastic bag or suitable container for sending to the laboratory or repeat the “Coning and Quartering” cycle until a final sample in the correct weight range is obtained. Discard the unselected “Quarters”.

Sampling Procedure: HAY

1. *Small square bales:*

    a) Sample between 10 and 20 small square bales, selected at random from the “lot”.

    b) Take one core from each bale selected, probing near the centre of the "butt" end and at right angles to the surface. **Ensure that the corer does not get hot.**

    c) Combine all cores into a single sample in a bucket and mix thoroughly. *Keep the whole sample intact - do not subdivide at this stage.*

2. *Large round or square bales:*

    a) Sample between 5 and 10 large bales, selected at random from the lot.

    b) Take one core from each side of all bales selected, probing at right angles to the surface at different heights.

    c) Combine all cores into a single sample in a bucket and mix thoroughly. *Keep the whole sample intact - do not subdivide at this stage.*

3. *Cubes or pellets:*

    a) Select a good handful of cubes or pellets from at least 6 locations or bags, which make up the complete lot.

    b) Combine the sub-samples in a bucket and mix thoroughly.

    c) Use the “Riffle Box” or “Coning and Quartering” process to reduce sample size to at least 250g, but not more than 500g.

Sampling Procedure: SILAGE

Silage is best sampled at least 3 weeks after it has been ensiled and as close to the time of feeding as is practicable.

1. *Pit or bunker silage:*

    a*)* *Unopened pit or bunker* - Obtain core samples for analysis using a long coring device that extends deeply into the pit or bunker. Sample from at least 3 to 5 locations to ensure a representative sample.

    b) *Opened pit or bunker* - Take random grab samples from at least 10 locations across a freshly cut face of the stack, although this will not provide as good a representative sample as multi-site coring.

    c) Combine all the material into a single sample in a bucket and mix thoroughly. Keep the whole sample intact and do not subdivide at this stage.

2. *Wrapped baled silage*

    a) Sample between 5 and 10 large bales at random using a coring device in the same manner as for large hay bales.

Note: This procedure is acceptable only if great care is taken to reseal the holes made in the plastic by the corer**.**

    b) Combine all cores into a single sample in a bucket and mix thoroughly. *Keep the whole sample intact - do not subdivide at this stage.*

Sample Handling: Immediately after sampling and mixing, use the “Coning and Quartering” process to reduce the sample size if necessary. The final fodder sample must be placed in a robust (preferably "press-seal") plastic bag and tightly sealed to exclude air. This is to ensure that the laboratory report of dry matter will approximate the dry matter content of the lot when it was sampled.

Sample Dispatch: Samples must be delivered to the laboratory as quickly as possible after being taken. Unless silage samples can reach the laboratory on the same day they are collected, they must be frozen immediately. This is especially important during hot weather.

Avoid mail delays over the weekend by posting samples early in the week.

Ensure that you follow closely the laboratory's instructions for labelling samples and filling out all the required details on the sample submission sheet. If you have any further queries or problems regarding sampling or sample handling, contact the appropriate AFIA recommended laboratory for further information.

Method – 1.2R: Preparation of Fodder Samples for Analysis

Scope: This procedure is used to prepare fodder samples for analysis.

Principle: Well-mixed (homogeneous) samples are essential to achieve accurate analytical results. This generally involves drying and/or grinding. Most fodder samples received at a laboratory fall into one of the following categories:

a) Dry enough to grind and analyse immediately (sample 90 to 95% or more dry matter);

b) Dry enough to be coarsely ground (to pass a 4mm sieve) but too wet to be finely ground; or

c) Those samples, which need to be partially dried before the sample can be ground coarsely (sample dry matter less than approximately 85%). This 'wet' material (less than 85% dry matter) is dried at 55 to 60^{o}C (maximum) in a forced air oven for a maximum of 24hr to reduce moisture content prior to grinding (Method 1.3R, Step 1)

Each sample type must be handled differently. Most forages can easily be ground to pass a 4mm screen 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 (Udy, Cyclotec or similar) to grind forage samples to pass a 1mm screen, 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 4mm screen. The coarse ground sample is then reduced in size by "Coning and Quartering". When necessary, the reduced sample is partially dried (Method 1.3R, Step 1) and the sample is ground again to the fineness desired for analysis.

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

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

Equipment:

Laboratory forage chopper

Cyclone pulverizing mill - 1mm screen (Udy or Cyclotec are required for samples using NIR)

Cutting mill - 4mm screen (Wiley or equivalent)

Sample containers

Freezer and refrigerator

Safety precautions:

- Wear hearing protection when operating a mill. The noise level is hazardous.
- Wear a dust mask to 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:

1) *Preparing samples greater than 85% dry matter for grinding*:

   a) Remove sample from shipping container and discard any roots from plants and brush off dirt particles.

   b) Note and report any material removed from the sample and any other sample manipulation.

   c) Chop samples of whole plants into approximately 2cm long pieces using either hand shears or the laboratory forage chopper. Include any ears attached to maize plants. (This process is not necessary for cored hay or silage samples).

   d) Grind entire sample to pass 4mm screen in the Wiley or other large mill.

   e) Reduce the milled sample by coning and quartering to the amount desired for laboratory sub-sample.

   f) Transfer to a suitable airtight container, seal and label as the coarse sample.

   g) Grind the coarse material in a cyclone mill (Udy or similar) fitted with a 1mm screen to produce finely ground material for analysis.

   h) Thoroughly mix the ground sample. Transfer to a suitable airtight container, seal and label as the final finely ground sample.

2) *Preparing 'wet' samples (less than 85% dry matter) for grinding:*

   a) Remove sample from shipping container and discard any roots from plants and brush off dirt particles.

   b) Note and report any material removed from the sample and any other sample manipulation**.**

   c) Chop samples of whole plants into approximately 2cm pieces using either hand shears or the laboratory forage chopper. Cut open stalk and maize cob pieces to assist drying. Include any ears attached to maize plants.

   d) Place the chopped sample onto a clean surface and mix thoroughly. If the entire sample cannot be dried, reduce the sample size by coning and quartering.

   e) Repeat the cycle until the volume is reduced to an appropriate size. Make certain that representative ratios of stem and leaf occur in each pile.

   f) Transfer reduced sample to a tared container for determination of partial dry matter (Method 1.3R, Step 1).

   g) Transfer the remainder to a plastic bag, seal and label. Store in a refrigerator as a reserve until sample preparation is complete.

   h) Dry the reduced sample using the forced-air oven (Method 1.3R, Step 1).

   i) Grind the entire partially dried sample to pass a 4mm screen in the Wiley or other large mill.

   j) Reduce the milled sample by coning and quartering to the amount desired for laboratory sub-sample.

   k) Transfer to a suitable airtight container, seal and label as the coarse sample.

l) Grind the coarse material in a cyclone mill (Udy or similar) fitted with a 1mm screen to produce finely ground material for analysis.

m) Thoroughly mix the ground sample. Transfer to a suitable airtight container, seal and label as the final finely ground sample.

Comments:

- Samples to be scanned by NIR must be dried and ground 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 logbook.
- Transfer samples quantitatively. Do not leave sample portions in mill.
- Store ground samples in airtight containers away from heat and light.

Reference:

National Forage Testing Association - Forage Analysis Procedures 1 Laboratory Sample Preparation

Method – 1.3R: Determination of Dry Matter

Scope: This procedure is used to determine the dry matter content (%DM) of all types of fodder except silages. Silage loses volatile components during the drying process. Appendix 1.3R(1) outlines the correction factors to be used when determining the %DM for silage samples.

Principle: Three steps are involved in the determination of %DM:

Step 1: Wet material (less than 85% dry matter) is dried at 55 to 60°C (maximum) in a forced air oven for a maximum of 24 hours to reduce moisture content prior to grinding. The loss of moisture is recorded (partial dry matter%). The procedure has minimal effect on chemical composition. Samples containing less than 15% moisture (ie greater than 85%DM) are ground as received.

Step 2: Ground material is dried at 105°C in a forced air oven for 3 hours. The loss of moisture is recorded (laboratory dry matter%).

Step 3: Calculation of Total Dry Matter %

Total dry matter % (%DM) = { (Partial dry matter%) X (Laboratory dry matter%) } /100.

Equipment:

Step 1 - Partial dry matter %

Forced-air drying oven set at 55 to 60°C (maximum)

Top loading electronic balance, accurate to 0.01g

Numbered containers able to hold 100 to 250g coarse sample at 3.5cm maximum sample depth

Data recording sheets / computer

Step 2 - Laboratory dry matter %

Analytical balance, accurate to 0.1mg

Numbered aluminium moisture dishes (approximately 50mm diameter and 40mm deep) with lids

Laboratory oven, forced air - 105°C

Desiccator

Tongs

Data recording sheets / computer

Spatula

Tissues

Balance brush

Cotton gloves

Procedure:

Step1. Partial dry matter %

a) Weigh empty container on a top loading balance and record container number and weight (W_1) to nearest 0.01g.

b) Tare balance against empty container.

c) Add (150 - 250g) coarse forage to the container to a maximum sample depth of 3.5cm. Record sample number and wet weight to nearest 0.01g (W_2).

d) Dry in a forced-air oven at 55 to 60^{o}C (maximum) for 16 to 24 hours.

e) Remove container from oven and cool / air-equilibrate sample for at least 1 hour before weighing the sample and container. Record the weight to the nearest 0.01g (W_3).

Comments:

- Ensure free movement of air through oven and around samples. Do not stack samples on top of each other or overfill oven.
- The oven temperature must not exceed 60^{o}C or heat-damaged protein will be formed. This will affect fibre values.

Calculation:

Partial dry matter% = { (W_3 - W_1) / W_2} X 100%

Where: W_1 = Empty weight of container in grams

W_2 = Initial weight of sample in grams

W_3 = Dry weight of sample and container in grams

Step 2 - Laboratory dry matter %

Note: Cotton gloves should be worn when handling moisture dishes in this procedure. This is to prevent contamination of the moisture dishes with sweat and grease from the hands.

a) Dry aluminium dishes, plus lids, at 105^{o}C for at least 2 hours before use.

b) Cover dish with lid and cool in a desiccator to room temperature.

c) Remove dishes one at a time from the desiccator and weigh dish (plus lid) to 0.1mg (W_4). Record the dish number and weight against sample number.

d) Immediately weigh (to 0.1mg) about 2.0g of well-mixed sample. Record the weight (W_5).

e) Shake the dish gently to distribute the sample evenly and ensure the maximum surface area for drying.

f) Place lids on each dish during transfer to the oven.

g) At the oven, remove the lids and place the dishes in upturned lids in the oven.

h) Leave the dishes in oven for 3 hours at 105^{o}C.

i) Remove the dishes from the oven, quickly replace lids and transfer to a desiccator.

j) Cool for at least 30 minutes but not more than 3 hours before weighing. Record the weight (W_6) to 0.1mg.

Calculation:

Laboratory dry matter % (Lab DM %) = { (W_6 - W_4) / (W_5 - W_4) } X 100%

Where: W_4 = Empty weight of container in grams

W_5 = Initial weight of sample in grams

W_6 = Dry weight of sample and container in grams

Step 3: Calculation of Percent Total Dry Matter

Total Dry matter % (%DM) = { (Partial dry matter%) X (Laboratory dry matter%) } /100.

= { (W_3 - W_1) / W_2} X { (W_6 - W_4) / (W_5 - W_4) } X 100%

References:

National Forage Testing Association - Forage Analysis Procedures 2.2.1 Partial Drying of Wet Samples.

National Forage Testing Association - Forage Analysis Procedures 2.2.2.5 Dry Matter by Oven Drying for 3 hr at 105^{o}C.

FEEDTEST, DPI Vic, Hamilton, Victoria - Method Manual,
Method 2.1 Determination of Dry Matter.

Appendix 1.3R(1): Method for Silage Samples

Scope: Silages contain a volatile dry matter (DM) fraction that is lost with oven drying and freeze drying. This fraction contains volatile fatty acids, alcohols and volatile nitrogen (N) compounds. The loss of volatiles varies with the extent and type of silage fermentation, and is generally higher with low DM silages where there is usually a more extensive fermentation. With higher DM silages (> 50 % DM) there is a more restricted fermentation so the volatile loss on oven drying is less.

As the volatile compounds in silage are completely digestible, it is important to take this into account when calculating digestibility, otherwise digestibility will be underestimated. Owing to volatile N losses, the total N content of silages will also be underestimated if analyses are conducted on oven dried samples. All other analyses on silages will be overestimated unless a correction is made for the volatile DM content.

Principle: A laboratory reference method that specifically determines water content is needed to allow the calculation of the true DM content of silages and other fermented feeds. The Karl Fischer method has been found to be suitable and is now semi-automated through the use of Karl Fischer titrators. The method has been described by Kaiser *et al.* (1995) and involves the extraction of silage moisture in methanol, and then determining water content of the extract using a Karl Fischer titrator.

The above procedure, while suitable for research laboratories, is less appropriate for commercial feed testing laboratories. In this situation, an alternative approach is to use a correction equation(s) to predict true DM from an oven DM measurement. As the correction required will be influenced by the oven drying regime adopted, it is important that the correction equation selected is applicable to the oven drying temperature used.

Procedure:

An earlier correction equation provided by Kaiser *et al.* (1995) calibrated true DM against oven DM determined at 80ºC. This was based on a relatively small number of silages. To increase the number and the range of silages, two correction equations have been developed at the Wagga Wagga Agricultural Institute, for silages oven dried at 80ºC for 24 hours (one-stage method) and at 60ºC for 24 hours (first stage of two-stage method, i.e. Partial DM).

(a) Correction for silages dried at 80ºC

This correction equation has been based on 102 silage samples from studies at the Wagga Wagga Agricultural Institute (Kaiser *et al.* 1995; Kaiser and Kerr 2003). The silages covered a wide range of crops and pastures with oven DM contents (80ºC) in the range 18.0-72.8 %.

Estimated True DM (%) = 3.846 + (0.96 × oven DM %) (r^2 = 0.99; s.e. = 1.28)

(b) Correction for silages dried at 60ºC

This equation is based on 60 silage samples (a subset from the above study by Kaiser and Kerr 2003). The silages covered a wide range of crops and pastures and had DM contents (60ºC) in the range 20.6-78.1 %. This equation applies to silages with oven DM contents of 40% or below. **DO NOT USE** for silages with oven DM content above 40%.

Estimated True DM (%) = 4.686 + (0.89 × oven DM %) (r^2 = 0.98; s.e. = 1.61)

Comments:

- The correction in (a) above was developed in a research project at the Wagga Wagga Agricultural Institute, Department of Primary Industries NSW, with support from Dairy Australia, Meat & Livestock Australia, and the Rural Industries Research and Development Corporation.

- This method is recommended by the TopFodder Silage project in their “Successful Silage” manual.

- The correction in (b) above should be applied to the PARTIAL oven dry matter before it is multiplied by the laboratory dry matter to obtain TOTAL dry matter, according to this AFIA reference method.

- The results from a more recent study at Wagga Wagga (Kaiser *et al.* 2006) indicated that no change is currently justified to the correction equations in (a) and (b) above.

References:

Kaiser, A.G. and Kerr, K.L. (2003). *More Accurate Laboratory Tests for Assessing Silage Quality,* Final Report for DRDC Project DAN 100 and RIRDC Project DRD-4A, 50pp.

Kaiser, A.G., Mailer, R.J. and Vonarx, M.M. (1995). *J. Sci. Food Agric.* **69**: 51-59.

Kaiser, A.G., Bailes, K.L and Piltz, J.W. (2006). *An Evaluation of Drying Regimes used to Determine Forage DM,* Final Report for DRDC Project DAN 12109 and RIRDC Project DRD-5A, 22pp.

Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion)

Scope: This method enables the determination of all protein nitrogen (N) and most non-protein N in all types of forages and feeds. Silage requires special precautions as outlined in Appendix 1.4R(1) of this method.

Principle: The Kjeldahl method involves:

Step 1: Digestion of the sample in concentrated sulphuric acid with a catalyst. This results in the conversion of susceptible nitrogenous compounds to ammonium sulphate.

Step 2: The resultant solution is then neutralised with excess caustic soda. Ammonia is liberated by steam distillation and captured in boric acid. The borate formed is titrated with dilute sulphuric acid. Depending on equipment chosen, titration can be fully automated or carried out manually.

Application: The following Equipment, Reagent and Procedure Sections provide a generic description of the Kjeldahl process based on block digestion, automated steam distillation and manual titration.

Equipment:

Fume hood with neutralising tank for acidic vapours

Block digester, capable of attaining 420^{o}C and digesting 20 samples at a time (Tecator or similar)

Steam distillation apparatus (Tecator Kjeltec System 1026 or similar)

Analytical electronic balance, accurate to 0.1 mg

Data recording sheets / Computer

Digestion tubes

Digestion tube stand

Forceps

Scoop

Spatula

Variable dispenser - 25ml volume

Conical flasks, Glass 250ml

Digital burette

Protective gloves

Reagents:

1. Digestion acid - concentrated, commercial grade sulphuric acid (98 - 99% purity - SG 1.84).

2. Catalyst - Kjeltabs, 1527-0003 (containing 3.5g K_2SO_4 and 0.035g Se) or Kjeltabs TCT (containing 3.0g K_2SO_4, 0.105g $CuSO_4$ and 0.105g TiO).

3. Sodium Hydroxide solution, typically 35 - 45% w/v for use in steam distillation apparatus (refer operating instructions for required concentration). To prepare 6 litres of stock concentrate 45% NaOH solution, proceed as follows:

    a) Weigh 2.7kg of commercial grade NaOH into a plastic container.

    b) Transfer 6 litres of deionised water to a suitably sized stainless steel bucket.

    c) Slowly add the NaOH to the water.

    d) Stir to dissolve.

    e) Allow to cool and transfer to a suitable plastic reservoir.

4. Boric Acid/Indicator Solution, 4% w/v. To prepare 10 litres of solution proceed as follows:

    a) Weigh 400g of commercial grade boric acid and transfer to an appropriately sized electric blender bowl. Add enough deionised water to ¾ fill the bowl. Cover and blend on 'high' for about 1 minute.

    b) Transfer the slurry to a stainless steel bucket (capacity > 10l) ¾ filled with deionised water. Stir to dissolve to a clear solution.

    c) Add 100ml of bromocresol green solution (0.1g bromocresol green in 100ml of ethanol).

    d) Add 70ml of methyl red solution (0.1g methyl red in 100ml of ethanol).

    e) Add deionised water to make to the 10 litre mark and continue to stir until all crystalline material has dissolved.

    f) Transfer to a suitable container.

5. Sulphuric Acid - approximately 0.1000N, standardised to 0.0001N.

Safety Precautions:

- Sulphuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. If splashed on skin or in eyes, flush with copious amounts of water and seek medical attention.

- Handle concentrated acid and alkali safely. Wear heavy rubber gloves and face shield to protect against concentrated acid or alkali. Use effective fume removal device to protect against acid fumes, alkali dusts and vapours.

- Always add concentrated sulphuric acid or sodium hydroxide pellets to water, not *vice versa*.

- The sulphur dioxide fumes produced during digestion are hazardous to inhale.

- Digests must be cool before dilution water is added to avoid a violent reaction. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

Procedure:

Note: *Analyses are usually performed only on samples previously dried at* 55 *to 60°C (maximum) and ground to pass a 1mm screen. Exceptions do occur, refer Appendix 1.4R(1), this Method.*

Step1: Digestion

1. Weigh (to 0.1mg) about 1g of well mixed sample into a tared scoop. Transfer to a digestion tube without loss. Record sample number, digestion tube number and weight of sample.

2. Weigh a second sub-sample for laboratory dry matter determination.

3. Place digestion tubes in a fume hood. Add two (2) Kjeltabs to each tube then add concentrated sulphuric acid. Usual volume is 15 - 18ml but actual volume will depend on digestion system selected. Mix carefully by swirling the tube by hand.

4. Place tubes in block digester preheated to 420°C. (Digester must be equipped with an exhaust system and/or placed in an acid fume hood).

5. Digest for 45 - 60 minutes.

Note: Actual temperature and time of digestion vary with equipment. For example, some block digestion systems reduce temperature to 390°C after the initial 10 minutes and then digest for a further 50 minutes.

6. When time has elapsed, remove tubes from the digestor block and let cool for at least 20 minutes in a fume hood. Time will depend upon airflow around tubes. Do not cool to point of solidification or loss of Nitrogen will occur. Rapid solidification is an indication of insufficient acid or excessive digestion times.

7. When cool, direct a rapid spray or stream of deionised water to the bottom of each tube to dissolve the acid digest completely. Add about 50ml of water.

Step 2: Distillation and Titration

1. Place NaOH in alkali tank of steam distillation unit. Make sure that sufficient NaOH (about 50 ml) is dispensed from the unit to neutralise all of the acid in the tube before distillation starts.

2. Place a 250ml conical titration flask containing 25ml 4% boric acid/indicator solution on the receiving platform. Ensure the tube from the condenser reaches below the surface of the boric acid.

3. Attach digestion tube containing diluted, cooled digest to steam distillation unit.

4. Dispense appropriate volume of NaOH solution.

5. Steam distil until 100 - 125ml distillate collects or the automatic process is completed.

6. Remove the receiver flask, rinse the condenser tip with deionised water and place on the bench ready for titration. Put the next sample into distillation unit and proceed from Step 8.

7. While the next sample is distilling, titrate the distillate from the previous sample to a neutral grey with 0.1N H_2SO_4. Record the volume of titrant (H_2SO_4) used (V_S) to the nearest 0.01ml. Titrate the reagent blank (V_B) similarly. The colour change is to a neutral grey.

8. When all the samples have been distilled and distillate has been titrated, empty the digestion tubes and flasks down the sink with copious amounts of water. Wash tubes and flasks as appropriate.

9. Shut down the distillation unit according to manufacturer's instructions.

Calculations: For standard H_2SO_4 titrant, results are calculated using the following equations:

- **% Nitrogen (%N)** = $$\frac{(V_S - V_B) \times N\ (H_2SO_4) \times 14.007 \times 100}{W \times 1000}$$

Where: V_S = Volume, in ml, of standard H_2SO_4 required to titrate sample

V_B = Volume, in ml, of standard H_2SO_4 required to titrate blank

N (H_2SO_4) = Normality of the acid titrant

14.007 = equivalent weight of Nitrogen

W = sample weight in grams

- **% Crude Protein (%CP) = %N X 6.25** and

- **% Nitrogen Dry Matter basis (%N DM) = %N X [100 / Lab DM%]**

Where: Lab DM% = Percent Laboratory Dry Matter of the sample analysed

Comments:

- Reagent proportions, heat input and digestion time are critical factors - Do not change. *Choose the settings for temperature and length of digestion, which give a 98% recovery of N from an EDTA standard*.

- Ratio of salt to acid (wt:vol) should be 1:1 at end of digestion with proper temperature control. Digestion may be incomplete at lower ratio and nitrogen may be lost at higher ratio.

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.

- Analyse all samples in duplicate with, wherever possible, the duplicates being analysed in a different analytical batch. An acceptable average standard deviation (s) 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:

  a) 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.

  b) Two consecutive analyses falling on one side of the mean between the warning limits and the control limits also indicate loss of control.

References:

National Forage Testing Association - Forage Analysis Procedures 3.2 Nitrogen Determination by Kjeldahl (Block Digestion).

FEEDTEST, DPI Vic, Hamilton, Victoria - Method Manual, Method 2.6 Determination of Crude Protein - Kjeldahl Method.

Appendix 1.4R(1): Determining the Crude Protein of Silage Samples

Crude protein values for silages are inevitably underestimated when analyses are conducted on oven dried samples. This is due to the loss of volatile nitrogen (N) compounds during the drying process, even when drying takes place at 60ºC.

This problem can be overcome by conducting a macro-Kjeldahl digestion on a fresh (wet) silage sample, a procedure adopted by most research laboratories. The method adopted is generally similar to that already described, with the analysis being conducted on a 2g sample of fresh finely chopped silage. Fine chopping of silages and other fresh forages can most easily be achieved by chopping the material in a rotating bowl chopper (e.g. Hobart Model 8185 food processor). This equipment is commonly used in the food processing industry and comprises a bowl which rotates in the horizontal plane, delivering the forage to knives which chop in the vertical plane.

Where crude protein analyses can only be conducted on dried silage samples, an attempt can be made to correct for the loss of volatile N compounds during oven drying. A "correction" equation was developed at the Wagga Wagga Agricultural Institute (Kaiser and Kerr 2003) to predict the "fresh" N content of a silage (if the analysis had been conducted on a fresh silage sample), from an analytical result obtained from an oven dried sample (80ºC). The "correction" can be applied as follows:-

Step 1:
Adjust the N result obtained on the oven dried sample to a true DM basis if this has not already been done.

N_{OD} as % estimated true DM = N_{OD} as % oven DM × (oven DM % / estimated true DM %)

where N_{OD} is the N result from an analysis conducted on an oven dried sample

Step 2:
Use the following equation to estimate the "fresh" or "corrected" N content of the silage. This regression is based on 88 silage samples varying widely in DM and N content (0.53-3.65 % N estimated true DM basis, determined on fresh samples) and covering a wide range of crops and pastures. It predicts the N content for an analysis conducted on a fresh silage sample from an analysis conducted on a dried sample of the silage.

N_C (% estimated true DM) = 0.0653 + (1.051 × N_{OD} as % estimated true DM)

where N_C is the corrected N content

Step 3:
Calculate crude protein content from the N result in the normal way.

Corrected CP (% estimated true DM) = 6.25 × N_C as % estimated true DM

Notes:

- The volatility of the N component will vary from silage to silage. Hence the equation in step 2 provides only an estimate of the corrected result.

- The above equations are based on an oven-drying temperature of 80ºC. As there was no significant difference between (a) oven-drying at 80ºC and (b) oven-drying at 60ºC overnight followed (after grinding) by drying at 105ºC for 3 hours (ie. Method 1.3R) in a recent DM study (Kaiser *et al.* 2006), it is recommended that the above equations can be used for both drying methods.

Reference:

Kaiser, A.G. and Kerr, K.L. (2003). *More Accurate Laboratory Tests for Assessing Silage Quality,* Final Report for DRDC Project DAN 100 and RIRDC Project DRD-4A, 50pp.

Kaiser, A.G., Bailes, K.L and Piltz, J.W. (2006). *An Evaluation of Drying Regimes used to Determine Forage DM,* Final Report for DRDC Project DAN 12109 and RIRDC Project DRD-5A, 22pp.

Method – 1.5R: Determination of Crude Protein by the Dumas Method (Combustion)

Scope: This method enables the determination of all protein nitrogen (N) and all non-protein N in all types of forages and feeds. The method will provide higher estimates of nitrogen and crude protein than the Kjeldahl method for samples with high concentrations of nitrate. Silage requires special precautions as outlined in Appendix 1.4R(1).

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

Equipment:

Any instrument or device designed to measure nitrogen by combustion may be used providing it can meet the following conditions:

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

2. System to isolate liberated nitrogen gas from other combustion products for subsequent measurement by thermal conductivity detector.

3. Device for converting NO_2 products to N_2 or measuring N as NO_2 may be required and included in the system.

4. Detection system to interpret detector response as % nitrogen (weight/weight). Features such as calibration on standard material, blank determination and barometric pressure compensation may be included. Any required calibration must be based on theoretical % nitrogen in pure primary standard organic material such as National Institute of Standards and Technology Standard Reference Material 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 the instrument according to manufacturer's instructions; the following are generic instructions:

1. Turn furnaces on (or take off standby).

2. Turn gas regulators to desired flow rate.

3. Wait until furnaces have stabilised 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 desiccated pure primary standards at the beginning of each run to calculate the calibration factor for determining N. The weights of the primary standards should be selected to ensure that the amount of N generated covers the expected range in N contents of the samples.

8. Weigh samples and transfer to autosampler tray.

9. Weigh a second sub-sample to determine laboratory dry matter.

10. Run samples.

Comments:

- System must be capable of measuring nitrogen in feed materials containing 0.2 to 10% nitrogen.

- Suitable fineness of grind is that which gives relative standard deviation (RSD) ±2.0% for 10 successive determinations of nitrogen in a 2:1 mixture of maize grain and soybeans that has been ground for analysis. RSD% = (standard deviation divided by mean %N) X 100.

- Fineness of grind (about 1.0mm or less) required to achieve this precision must be used for all mixed feeds and other nonhomogeneous materials.

Calculation: Percent Nitrogen (N)

% N (DM basis) = {% N (from analyser output) } / Lab DM/100

Calculation: Percent Crude Protein (CP)

% CP (DM basis) = % N (DM basis) X 6.25 (for all forages).

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 (s) among replicated analyses for crude protein ranges from about ±0.10 for samples with 10% CP to ±0.20 for samples with 20% CP. This 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 limit and the control limit also indicate loss of control.

References:

National Forage Testing Association - Forage Analysis Procedures 3.3 Nitrogen Determination by Combustion Method.

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

Method – 1.6R: Determination of the Ammonia-N Content of Silages

Scope: This method determines the ammonia-N content of silages to evaluate silage fermentation quality.

Principle: An acid extract is prepared from a chopped silage sample stored in a sealed container in a cold room for a period of at least 24 hours. Ammonia-N is absorbed into the solution as ammonium ion. There are various methods available for determining the ammonia-N concentration in an aliquot of the filtrate from the silage extract. The following procedure is based on an automated distillation and titration method (Method 1.4R), where the ammonium ion is converted back to ammonia and the titrant volume is recorded for calculation of the result, which is expressed as a % of the total N. An alternative method (1.15A) determines ammonia-N using an ion specific electrode.

Apparatus:

Volumetric flask (5L)

Fume hood with neutralising tank for acidic vapours

Digestion tubes

Digestion tube stand

Steam distillation apparatus Kjeltec Analyser or equivalent)

Analytical electronic balance, accurate to 0.1 mg

Forceps

Spatula

Variable dispenser - 25ml volume

Conical flasks, Glass 250ml

Burette

Protective gloves

Reagents:

Sodium Hydroxide (NaOH) —technical grade, (35–40%)

Sulphuric acid (H_2SO_4)—0.3 M

Boric acid (H_3BO_3)

Bromocresol green Indicator

Methyl red Indicator

Sodium carbonate (Na_2CO_3)

Hydrochloric acid—32% HCl (or 36%), or alternatively use 0.1N sulphuric acid as per Method 1.4R

Methanol

Water - Type II

Working solutions:

1. NaOH - 40% (Alkali):

    (a) Decant 1 ± 0.01 L water in plastic container

    (b) Gradually add 4 ± 0.01 L of 50% sodium hydroxide while stirring. This solution gets hot. Container may be placed in cold water to help cool.

    (c) Transfer into Kjeltec Alkali reservoir.
    or,

    (a) prepare 40% solution by adding 400 ± 1g NaOH pellets or flake, CAREFULLY into 900mL of water, and making up to a litre.

2. 1% Bromocresol green solution:

    (a) Add 0.25 ± 0.005g Bromocresol Green in 250 mL methanol, and stir till dissolved.

3. 1% Methyl red solution:

    (a) Add 0.25 ± 0.005g Methyl Red in 250 mL methanol, and stir till dissolved

4. Receiver Solution:

    (a) Dissolve 50.0 ±0.1g of boric acid and made up to 5 L with water.

    (b) Add 50 ± 1 mL 1% Bromocresol green solution and add 35 ± 1 mL 1% Methyl red solution. Mix thoroughly.

    (c) Gradually add NaOH until the solution is between a red and green colour (grey/green) and add to container beneath Kjeltec Analyser.

5. HCl Standard acid solution - 0.1M HCl:

    (a) Add 20.0 ± 0.1 mL of 32% (10M HCl) to approx 1200 mL of water and make to 2 L volume with water.

    (b) Depending on the sample size and ammonia N content, standard acid solutions with concentrations ranging from 0.02 to 0.1 M may be used for titration of the receiver flask solution.

    (c) The standard acid solution can be prepared from ampoules of various concentrations or by diluting concentrated hydrochloric acid (36%, 12 M) with water.

6. Mixed Indicator solution:

    (a) Use equal volumes of solutions 1% Bromocresol green and 1% Methyl red solutions.

Procedure:

Silage extract

1. Weigh 50 g of chopped silage (for chopping procedure see Silage pH Method 1.12R) into an open neck glass bottle with a screw cap.

2. Cover with 250-450 ml 0.3M sulphuric acid and replace the cap. Stir gently. The higher volume will be required for drier silages.

3. Record the volume of acid used as it will be required when calculating the result. Do not add excessive acid as this will dilute the concentration of ammonia-N in the filtrate.

4. Store the sample in a refrigerator for at least 24 hours (preferably 3 days) before filtering.

5. Stir gently and filter approximately 30-50 ml into small plastic bottles using fast filter paper, and store frozen until ready for analysis.

Acid Standardisation

1. The concentration of the standard acid must be checked by titration against a predetermined solution of sodium carbonate.

2. Dry approx. 10 g of anhydrous sodium carbonate (Na_2CO_3) at 200° C for 2 hours and store in desiccator.

3. For 0.1 M HCl, weigh 0.1 g recorded 0.0001, into five conical beakers and dissolve with approx 30 mL of water

4. Add 3–4 drops mixed indicator. Titrate to neutral grey, with standard acid, and record volume (Vol 1).

5. Boil gently for 3 mins, should return to green colour. Cool rapidly under running tap water. Continue titrating to neutral grey and note titrant volume (Vol. 2).

   Note: 0.1 g will require approx 18 mL of 0.1 M HCl.

$$\text{Molarity, M} = \frac{18.868 \times \text{weight}}{\text{Vol.1} + \text{Vol.2}}$$

Kjeltec Analysis (Distillation and titration)

1. Thaw out previously prepared solutions at room temperature.

2. Invert gently to obtain a homogenous solution.

3. Pipette out 10.0 ± 0.1 mL of the solution into a distillation tube.

4. Analyse the solutions according to Method 1.4R (Step 2: Distillation and Titration) in this manual. Note that Method 1.4R uses sulphuric acid rather than hydrochloric acid.

Calculations:

Step 1:
Calculate the ammonia-N in the original fresh silage sample:-

$$\text{Af} = \frac{(\text{mL - blank}) \times \text{M} \times 1.401 \text{ x V1}}{\text{weight of sample (g) x V2}}$$

where
- Af = ammonia-N (g/kg fresh silage)
- mL = volume of titrant
- blank = sample blank digested with samples
- M = molarity of the titrant acid
- V1 = Volume of acid used to extract sample (mL)
- V2 = Aliquot volume used in KJ analysis (mL) (usually 10-50mL)

Step 2:
Convert the result to a true DM basis:-

$$Ad = \frac{(\mathrm{Af} \times 100)}{\mathrm{TDM}}$$

where TDM = true DM content of the silage (g/kg)

Step 3:
Express the result as a % of total N (g/kg true DM) previously determined by Kjeldahl digestion

$$\% \text{ Silage NH3-N of total N} = \frac{Ad \times 100}{\% \text{ N}}$$

Where % N = Total % nitrogen corrected for true DM content of the silage.

Comments:

- Silage is a fermented feed and the extent of breakdown of the protein or nitrogen (N) fraction is a good indicator of silage fermentation quality. During the early stages of the ensiling process, while anaerobic conditions are being established, and before there has been a significant drop in pH, breakdown of the protein in forage (proteolysis) commences due to the activity of plant enzymes. Further protein degradation can occur due to the activity of undesirable bacteria, particularly if there is a delay in achieving anaerobic conditions and/or there is a slow fall in pH. The ultimate breakdown product from this process is ammonia-N. High levels of ammonia-N, expressed as a % of total N, are an indicator of extensive protein breakdown and poor silage preservation. Ammonia-N is an excellent indicator of silage fermentation quality, and for an interpretation of these results refer to Kaiser and Piltz (2003).

- Silage is a perishable product and should be stored in a sealed bag (anaerobically) at low temperature. If the sample is not to be analysed immediately it should be frozen.

- The use and interpretation of ammonia-N data as a measure of silage fermentation quality is provided by Kaiser and Piltz (2003)

- Refer to the method for determining total N in silage. Where total N is determined on an oven dried silage sample, failure to take account of the loss of volatile N will influence the calculated result (% or g/kg total N). Also where total N is determined by the Dumas method rather than the Kjeldahl method, some adjustment may be required for any differences between the two methods in the total N result.

References:

AOAC Official Method 2001.11: Crude protein in animal feed, forage, grain and oilseed.

Kaiser, A.G. and Piltz, J.W. (2003). 12. Feed testing: assessing silage quality. In: *"Successful Silage"*, eds A.G. Kaiser, J.W. Piltz, H.M. Burns and N.W. Griffiths (Dairy Research and Development Corporation and NSW Agriculture: Australia), 24pp.

Method – 1.7R: Determination of Digestibility using the Pepsin - Cellulase Method

Scope: This procedure deals with the determination of digestibility of plant material to be fed to ruminant livestock. It is applicable to material with digestibility in the range covered by the *in vivo* standard feed samples available. The pepsin cellulase method has historically used the 16 hay *in vivo* standard feeds owned by AFIA, but is now intended for use with the 7 *in vivo* standard feeds developed at the Wagga Wagga Agricultural Institute (WWAI).

Principle: This procedure is in three steps, carried out over four days and attempts to simulate ruminant digestion. The sample is digested at 40°C with acidified pepsin, heated to 80°C and digested with thermostable alpha amylase and then digested at 40°C with a buffered cellulase solution following pH adjustment to 4.6. The disappearance of dry matter or organic matter is determined. This value is adjusted to predict *in vivo* digestibility using a linear regression based on samples of known *in vivo* digestibility and analysed in the same way.

Equipment:

Analytical electronic balance, accurate to 0.1 mg

Data recording sheets / Computer

Pyrex test tubes, 25 mm x 150 mm (50 mL)

Plastic caps, fitted with Bunsen valves

Scoop

Spatula

Tissues

Dispensers (3) to deliver 10, 15 and 0.8 mL of solutions

Magnetic stirrer and stirrer bars

Test tube racks

Covered water baths, suitable for 200 tubes

Immersion thermostat, set for 40°C

Immersion thermostat, set for 80°C

Sintered glass crucibles, porosity 1, 30 mL vol

Adaptors for crucibles

Vacuum pump

Suction filtration manifold

Filtering flasks

Oven, 105°C

Muffle furnace

Reagents:

1. Hydrochloric Acid, 32% (w/v), AR grade
2. Pepsin powder (derived from porcine source) 469 units/mg or greater.
3. Sodium Acetate, anhydrous AR grade
4. Sodium Carbonate, AR grade
5. Acetic Acid, glacial AR grade
6. Cellulase, 'Onozuka FA'
7. Heat stable α-amylase solution (NOVO Termamyl; Sigma Cat No A5426 or similar) , 18,000 units/mL or greater.
8. Acidified Pepsin Solution (0.3% w/v in 0.125 M HCl) - to prepare:
   a) Add 11.00 ± 0.05 mL HCl to 500 mL dH_20 and dilute to 1.0 L. Mix thoroughly and dissolve 3.00 ± 0.01 g pepsin in the solution.
   b) Prepare this solution just before use. Adjust these quantities as required, depending on the number of samples in the batch (15 mL required per tube).
9. Sodium Carbonate Solution (1 M):
   a) Dissolve 106.0 ± 0.2 g sodium carbonate in distilled water and dilute to 1.0 L. Mix well.
10. Buffered Cellulase Solution:
    a) Dissolve 20.4 g sodium acetate and 8.7 mL acetic acid in dH_20 and dilute to 1.0 L.
    b) Dissolve 12.5 g cellulase in the solution and mix thoroughly.
    c) Prepare this solution just before use. Adjust these quantities as required, depending on the number of samples in the batch (10 mL required per tube).
11. α-amylase, 2% (v/v): -
    α-amylase stock reagent should be standardised so that 2 additions of 2 mL of α-amylase solution will remove 0.5 g of starch as per Appendix 1.8A(b)(1).
    a) Dilute an appropriate aliquot of heat stable α-amylase (NOVO Termamyl; Sigma Cat No A5426 or similar, 18,000 units/mL or greater) in dH_20.
    b) Dilute 2.0mL α-amylase per 100mL dH_20, to give a final activity of 360U/mL or greater.
    c) Allow 4.0 ± 0.1 mL of the dilute enzyme preparation per tube.
    d) Prepare diluted solution fresh daily.
12. *In vivo* fodder standards (WWAI)

Procedure:

Note: *Analyses are performed only on samples previously dried at* 55 *to 60°C (maximum) and ground to pass a 1 mm screen.*

Day 1

1. Weigh 0.25 g (± 0.005 g) well-mixed laboratory dry sample onto previously tared scoop, record the weight to at least 0.01 mg (0.0001 g) accuracy (W_S) along with the sample number and number of the test tube.
2. Carefully transfer weighed sample into numbered test tube.
3. Weigh a second sub-sample for laboratory dry matter determination and ash determination.

4. Weigh out unknown samples and *in vivo* standard feeds in duplicate and include at least 1 blank tube in each rack of samples.

5. When all samples have been weighed out, add 15 mL acidified pepsin solution to each tube and mix using vortex mixer or equivalent to ensure sample is well mixed. Place cap on the tube.

6. Place tubes (in racks) in a water bath (or incubator) previously heated to 40ºC. Ensure that the bath is maintained at this temperature with sufficient water for 24 hr. Agitate tubes regularly (or use shaking water bath) and ensure feed material is washed down from sides of tubes.

Day 2

1. After 24 hr, remove tubes from the water bath and add 4 mL of diluted α-amylase solution to each tube. Mix using vortex or equivalent and place in second water bath (or incubator, previously heated to 80ºC) for 45 min.

2. After 45 min, remove tubes from 80ºC water bath and cool to room temperature. Add 0.8 mL sodium carbonate solution to each tube. Mix using vortex or equivalent.

3. Add 10 mL buffered cellulase solution to each tube and mix using vortex or equivalent.

4. Cap tubes and return them to the 40ºC water bath for a further 24 hr.

5. Place clean sintered glass crucibles (porosity 1) in 105ºC oven overnight. Glass fibre cases can also be used in place of sintered glass crucibles.

Day 3

1. Remove crucibles from 105ºC oven and place in desiccator for 1 hr. When cool, weigh and record crucible number and weight (W_C).

2. Remove tubes from 40ºC water bath after 24 hr and filter under vacuum into the weighed sintered glass crucibles. Ensure all residue is transferred from the tubes using dH_20 from wash bottle to rinse the tubes into the crucibles. No further washing is required. Do not over fill the crucible.

3. Place crucibles in 105ºC oven to dry overnight.

Day 4

1. Remove crucibles from oven and place in desiccator for 1 hr. Weigh and record weight (W_R).

2. Place crucibles in COLD muffle furnace and heat to 550-600ºC, maintaining this temperature for 2 hr.

3. Turn furnace off and allow it to cool BELOW 200ºC before removing crucibles. Transfer crucibles to a desiccator and cool for 1 hr. Weigh and record weight (W_A).

Calculations:

1. Calculate pepsin cellulase dry matter digestibility (PCDMD %DM):

Firstly, calculate sample dry matter weight (DM_S):

DM_S = W_S X Lab $DM_{(g/g)}$

Where: W_S = Sample laboratory weight (g)

Lab $DM_{(g/g)}$ = Laboratory dry matter (g DM / g sample)

Then calculate PCDMD (% DM):

$$\textbf{PCDMD (\%DM)} = \frac{[DM_S - ((W_{R(sample)} - W_C) - (W_{R(blank)} - W_C))] \text{ X } 100}{DM_S}$$

Where: W_S = Sample weight (g)

$W_{R(sample)}$ = Weight of sample residue plus crucible (g)

$W_{R(blank)}$ = Weight of blank residue plus crucible (g)

W_C = Weight of crucible (g)

2. Calculate pepsin cellulase organic matter digestibility (PCOMD %OM):

Firstly, calculate sample organic matter weight (OM_S):

$$\textbf{OM}_S = DM_S \text{ X } OM_{(g/g)}$$

Where: DM_S = Sample dry matter weight (g)

$OM_{(sample\ g/g)}$ = Sample organic matter (g OM / g DM)

Then calculate PCOMD (% OM):

$$\textbf{PCOMD (\%OM)} = \frac{[OM_S - ((W_{R(sample)} - W_{A(sample)}) - (W_{R(blank)} - W_{A(blank)}))] \text{ X } 100}{OM_S}$$

Where: OM_S = Sample organic matter weight (g)

$W_{R(samle)}$ = Weight of sample residue plus crucible (g)

$W_{A(sample)}$ = Weight of sample ash residue plus crucible (g)

$W_{A(blank)}$ = Weight of blank ash residue plus crucible (g)

W_C = Weight of crucible (g)

3. Calculate pepsin cellulase digestible organic matter in the dry matter (PCDOMD %DM):

$$\textbf{PCDOMD (\%DM)} = \frac{[OM_S - ((W_{R(sample)} - W_{A(sample)}) - (W_{R(blank)} - W_{A(blank)}))] \text{ X } 100}{DM_S}$$

4. Calculate predicted *in vivo* DOMD (ivDOMD %DM):

The PCDOMD values calculated for the standards and their known *in vivo* DOMD values are used to calculate a linear regression equation;

$PCDOMD_{(standard)}$ = *in vivo* $DOMD_{(standard)}$ X slope + intercept

This equation is then used to predict the *in vivo* DOMD (ivDOMD) of the samples:

$$\textbf{ivDOMD (\%DM)} = \frac{(PCDOMD_{(sample)} - \text{intercept})}{}$$

slope

Correction for silage DMD

For silages, a correction is required for true dry matter (TDM) from oven dry matter (ODM) as per Method 1.3R, Appendix 1.3R(1):

For silages dried at 80ºC: Estimated TDM (%) = 3.846 + (0.96 × ODM %)
For silages dried at 60ºC: Estimated TDM (%) = 4.686 + (0.89 × ODM %)

Corrected *in vivo* predicted Dry Matter Digestibility ($ivDMD_C$) is calculated as follows:

$$\textbf{ivDMD}_C\textbf{ (\%TDM) =} \quad 100 - \left[\frac{(100 - ivDMD\%) \text{ X } ODM\%}{TDM\%} \right]$$

Correction for silage DOMD

To correct the DOMD (and OMD if required) of silages for volatile losses during drying, it can be assumed that the volatile compounds are 100% organic matter and are 100% digestible during the PC assay. Therefore, *in vivo* predicted DOMD needs to be corrected for TDM:

Corrected *in vivo* predicted Digestible Organic Matter in the Dry matter ($ivDOMD_C$) is calculated as follows:

$$\textbf{ivDOMD}_C\textbf{ (\%TDM) =} \quad 100 - \left[\frac{(100 - ivDOMD\%) \text{ X } ODM\%}{TDM\%} \right]$$

Calculation of Metabolisable Energy (ME)

The *in vivo* predicted DOMD values (iv DOMD or $ivDOMD_C$) are used to estimate metabolisable energy of the sample (MJ ME/kg DM) according to AFIA Method – 2.2R: Calculation of Metabolisable Energy.

Secondary Standards:

- It is recognised by the AFIA QEC that the 7 WWAI *in vivo* standard feeds are not available in an endless supply and further standards for the PC assay will be required in the future. Laboratories can select a range of other forage samples, representative of the types to be analysed, which fit closely on the regression line for the 7 WWAI in vivo standard feeds and cover the same digestibility range. These samples could then be used as secondary standards in future PC assays. However, these secondary standards must be made available to other laboratories in the QEC in order to validate their use through AFIA ring-testing.

References:

Clarke, T., Flinn, P.C. and McGowan, A.A. 1982. Low-cost pepsin-cellulase assays for prediction of digestibility of herbage. Grass and Forage Science 37:147-150.

De Boever, J.L., Cottyn, B.G., Buysse, F.X. and Vanacker, J.M. 1988. The use of a cellulase technique to predict digestibility, metabolisable and net energy of forages. Animal Feed Science and Technology 19:247-260.

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Mertens DR (1992). Critical conditions in determining detergent fibers. *In*. "Proceedings of the National Forage Testing Authority Forage Analysis Workshop", Sep 16-17, Denver, CO, pp. C1-C8. *Available in*: Appendix F, "Forage Analyses Procedures", National Forage Testing Association, Omaha, NE, pp. 132-139.

Version 3
Date 22/05/2011

Method – 1.8R: Determination of Amylase Neutral Detergent Fibre by Refluxing

National Forage Testing Association - Forage Analysis Procedure 5.1

Scope: This method is applicable for the determination of amylase neutral detergent fibre (aNDF) in all types of forages and feeds.

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 solubilise the proteins and sodium sulphite also helps remove some nitrogenous matter; EDTA is used to chelate calcium and remove pectins at boiling temperatures; triethylene glycol helps to remove some non-fibrous 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 minimise 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 (600ml)

Fritted glass (Gooch) crucibles (coarse porosity, 50ml)

Analytical electronic balance, accurate to 0.1mg

Suction filtering device with trap in line and valve to break vacuum

Forced-air drying oven set at 100^{o}C

Reagents:

1. **Neutral detergent solution -** To make approximately 18 litres requires:
    - 17.82L Distilled water
    - 540g Sodium lauryl sulphate, USP

Caution: wear dust mask and gloves when using sodium lauryl sulphate.

- 335g Ethylenediaminetetraacetic acid (EDTA), disodium salt (may substitute 72g sodium hydroxide (NaOH) and 263g free acid EDTA as a less expensive alternative)
- 122.6g Sodium borate, decahydrate ($Na_2B_4O_7.10H_2O$), reagent grade
- 82.1g Sodium phosphate, dibasic (Na_2HPO_4), anhydrous, reagent grade and
- 180 ml Triethylene glycol, reagent grade.

Preparing an aNDF Solution:

a) Pour one-half of distilled water into mixing container. Place on stir plate in hood and begin stirring.

b) Add remaining reagents except for triethylene glycol.

Caution: wear dust mask and gloves when transferring the sodium lauryl sulphate.

c) Slowly add remaining distilled water to container to limit foaming of the detergent.

d) 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.

e) 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.

f) Verify pH of solution to be between 6.95 and 7.05. Adjust with HCl or NaOH as required if not within range.

Reagents (cont'd):

2. Sodium sulphite, anhydrous (Na_2SO_3).
3. Acetone, reagent grade.
4. Heat-stable alpha-amylase solution (standardised using procedure in Appendix 1.9R(1)) reagent grade.

Safety Precautions:

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

Procedure:

1. Samples should be dried at 55 to 60°C (maximum) to greater than 85% dry matter, then ground to pass a 1mm screen.
2. Dry 50ml fritted glass crucibles overnight at 100°C and hot weigh (W_1), recording weight to nearest 0.1mg.
3. Thoroughly mix sample, then weigh 0.45 to 0.55g, recording to nearest 0.1mg, (W_2) into 600ml Berzelius beaker.
4. Weigh a second subsample for laboratory dry matter determination.
5. Preheat extraction heating (reflux) unit to a temperature that permits boiling of neutral detergent solution within 5 minutes.
6. Add 0.5 g of sodium sulphite using previously calibrated scoop.
7. Add 50ml of neutral detergent solution and swirl beaker until the sample and sodium sulphite are completely suspended.
8. Place beaker on the heating unit under a cool water condenser. Samples should come to the boil in 4 to 5 minutes.

Note: Samples normally foam vigorously for 1 to 2 minutes. Do not reduce temperature of heating unit during this time.

9. After 5 minutes, remove beaker from the reflux unit and add 2ml of the standardised amylase solution.

10. 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.

11. Return beaker to the reflux unit and allow to come to a boil. Reflux for 60 minutes.

12. Five to 10 minutes after adding amylase, rinse down the sides of the beaker with neutral detergent solution. Continue with refluxing until 60 minutes have elapsed.

13. Remove sample from heating unit and allow to settle for 30 to 60 seconds before filtering.

14. Preheat the fritted glass crucible for filtering by adding 40ml of boiling water. Remove water with vacuum.

15. Carefully decant the first 30 to 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.

16. Remove the solution with a minimum amount of vacuum.

17. 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.

18. Apply minimum vacuum to filter.

19. Immediately fill crucible half full of hot water and add 2ml of standardised amylase solution.

20. Allow to react for approximately 45 to 60 seconds, while policing particles from sides of Berzelius beaker.

21. Rinse Berzelius beaker with boiling water while inverted over the crucible until all residue is transferred.

22. Filter and wash twice by adding 30 to 40ml boiling water to residue in fritted glass crucible and allowing to soak for 2 minutes each time.

23. Rinse sample twice with 30ml of acetone, allowing at least 2 minutes soaking time between rinses.

24. Rinse policeman, vacuum sample dry, and remove sample from manifold.

25. Dry crucibles at 100^{o}C for 8 hours or overnight and hot weigh recording weight (W_3) to nearest 0.1mg.

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 50ml of distilled water and record the time required to drain completely without vacuum. This should be about 180 seconds. If filtration takes more than 240 seconds, crucibles need cleaning. If cleaning does not improve the filtration rate, the crucible should be discarded.

- If filtering takes less than 120 seconds, check crucible for cracks or holes in the fritted disk. If filtering takes less than 100 seconds, 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 fibre particles plug the fritted glass disc.

- **Rinse water must be in excess of 95ºC**. 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 faeces). Experience has shown that any sample that takes longer than 10 minutes 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 fibre degrading enzymes. Because heat will inactivate these contaminating enzymes, it is recommended that a heat-stable amylase be used in hot solution to minimise fibre loss.

Calculation: Percent Amylase Neutral Detergent Fibre

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

Where: W_1 = tare weight of crucible in grams

W_2 = initial sample weight in grams

W_3 = dry weight of crucible and dry fibre 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 fibre 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 limit and the control limit also indicate loss of control.

References:

Goering, H.K. and Van Soest P.J. 1970. Forage fibre 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, Robertson J.B. and Lewis B.A. 1991. Methods for dietary fibre, neutral detergent fibre and non-starch polysaccharides in relation to animal nutrition. J. Dairy Science 74:3583-3597.

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Appendix 1.8R(1): NFTA Method 5.2: Standardising Alpha-Amylase Activity for Neutral Detergent Fibre Determination

Scope: This method is applicable for the determination of alpha-amylase activity solutions to be used in the neutral detergent fibre procedure (Method 1.9R and NFTA Method 5.1).

Principle: To ensure 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 0.1mg

Ice bath

Reagents:

1. Dried hominy corn (sold in most grocery stores as corn grits, raw, not instant) that has been ground to pass a 1mm screen (Wiley mill)

2. Burke's iodine solution (2g KI, 1g I_2 and 100ml H_2O).

3. 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 100ml final volume with distilled water (eg. 1.25ml AOAC Dietary Fibre method amylase, Sigma Cat. No. A5426 or 5 ml of NOVO Termamyl diluted to 100ml).

4. Powdered enzymes must be extracted with water. It is recommended to start with 5g of powder extracted for 20 min with 100ml of distilled water.

5. Concentration of Amylase Test Solution (C) is expressed as g (powder) or ml (liquid) of enzyme/100ml test solution.

Safety Precautions:

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

Procedure A: Determine appropriate amount of amylase needed.

1. Use a range of Amylase Test Solution volumes to detect differences in the activity of an enzyme and determine the amount of the enzyme that is needed for aNDF.

2. It is suggested that six volumes of Amylase Test Solution is all that need to be evaluated (0, 1.0, 1.5, 2.0, 2.5, & 3.0ml). However, if an unknown source is used, it may be wise to use a geometric progression (0, 0.5, 1.0, 2.0, 4.0 & 8.0ml) 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.

3. Weigh 0.5g (± 0.005g) of ground, dried hominy corn into each of six Berzelius beakers.

4. Preheat extraction heating (reflux) unit to a temperature that permits boiling of neutral detergent solution within 5 minutes.

5. Prepare an ice bath for cooling the beakers after they have been removed from the hotplates.

6. Add 50ml of neutral detergent solution and swirl beaker.

Note: Do not add 0.5 g of sodium sulphite as in Method 1.8R (NFTA method 5.1).

7. Put beakers onto preheated refluxing apparatus at 1 minute intervals.

8. Samples should come to a boil in 4 to 5 minutes. 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.

9. After the samples begin to boil (approximately 5 minutes after placing on hot plates), add one of the selected volumes of Amylase Test Solution (from lowest to highest) to each of the respective beakers.

10. Reflux for 10 minutes. One hour refluxing is not needed to evaluate amylases.

11. At 1 minute intervals remove each beaker from the refluxing apparatus.

12. Add the repeat volume of Amylase Test Solution to the respective beakers, swirl to mix and rinse down the sides of the beaker using a minimum amount of room temperature neutral detergent solution.

13. Let the amylase react with the beaker on the benchtop for 60 seconds (until after the next sample is removed from the hotplate and enzyme is added to it).

14. Place the beaker in the ice bath to cool for 5 minutes.

15. Remove each beaker and place it on the benchtop until all beakers have been cooled.

16. Arrange beakers in order of increasing enzyme concentration, preferably on a white sheet of paper or other light background colour surface.

17. Quickly add 0.5ml of Burke's iodine solution to each beaker, then swirl each beaker in turn.

18. Set timer to alarm in 90 seconds.

19. After 90 seconds evaluate the efficacy of raw corn starch (grits) hydrolysis using the following scale:

    a) Purple = not adequate enzyme

    b) Amber = not adequate enzyme

    c) Yellow = adequate enzyme.

20. Calculate the amount of enzyme needed in 2ml of standardised amylase solution to remove interfering starch.

Comments:

- The volume of the amylase test solution or extraction (Vs) that indicates no starch (yellow colour) is used to calculate the standardised amylase solution.

- It is best not to look at the beakers while waiting for the 90 seconds to elapse, instead look away from the beakers and after the alarm sounds make a quick decision (before 120 seconds 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.

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

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

2. Weigh each of the following into separate Berzelius beakers in duplicate: 0.1g of beta-glucan, 0.5g arabinogalactan, or 0.5g pectin.

3. *Follow the usual aNDF procedure using the standardised amylase solution determined previously, EXCEPT: add the amylase to one of the duplicate beakers but not the other*.

4. To aid filtering of these difficult materials, add 0.25g of glass wool or glass microfibre filters (Whatman GF/D, 4.25cm) to the crucibles prior to initial drying of the crucible before obtaining the tared weight (W_1).

5. Make sure wash water is boiling.

6. 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 fibre degrading activity.

Comments:

- Each new source or lot of enzyme should be standardised. 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 standardised 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 pipette or syringe, precalibrated to 2ml, can be used to dispense the enzyme regardless of its initial activity.

Two ml of dilute enzyme is used to minimise the error associated with a drop of solution that may be retained on the syringe or pipette, 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 2ml of standardised amylase solution to be added during the aNDF procedure.

$$E = 100 \times (Vs \times C) / 2$$

Where:

E = Enzyme powder (g) or liquid (ml) needed to make 100ml of standardised amylase solution

Vs = Minimum volume of test solution or extract resulting in yellow colour with iodine (no starch)

C = Concentration of Amylase Test Solution (g powder/100ml or ml liquid/100ml)

2ml = Volume to be added in aNDF procedure

100ml = Volume of standardised amylase solution to be prepared

Reference:

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

Method – 1.8A(a): Determination of Amylase Neutral Detergent Fibre - Ankom

Scope: This procedure describes the use of Ankom equipment to determine Neutral Detergent Fibre in feed samples which have been ground to pass a 1 mm screen.

Principle: Neutral Detergent Fibre (NDF) is considered to represent plant cell-wall constituents including hemicellulose, cellulose, lignin, cutin and silica. The sample is weighed into filter bags and refluxed in hot neutral detergent solution for 1 hour. It is then rinsed, dried and weighed. The amount of sample remaining is the NDF and is expressed as a percentage dry matter (%DM).

Apparatus:

ANKOM$^{200/220}$ Fibre Analyser

Bag Suspender with Baskets

Analytical Balance

Computer

Spatula

Filter Bags, ANKOM #57

Measuring Cylinder, Glass 1 L

Measuring Cylinder, Glass 10 mL

Electric Urn, 6 L capacity or ZIP Boil instant hot water service or equivalent

Beakers, Glass 250 mL and 2 L

Oven, 105^{o}C

Heat Sealer

Timer

Gloves

Reagents:

1. Neutral Detergent Solution (NDS), prepared as follows (all chemicals reagent grade):

Note: A total of 2.0 L of NDS should be used during all digestions regardless of the number of samples. The following formula gives weights required for 1.0L.

Note 2: If bulk solutions are made and stored prior to use, care MUST be taken to ensure that all components are mixed back into the solution before any solution is decanted. Failure to do so may result in significant error.

a) Place 18.61 ± 0.01 g of EDTA (disodium ethylenediaminetetraacetate, $C_{10}H_{14}N_2Na_2O_8$ x $2H_2O$) and 6.81 ± 0.01 of sodium tetraborate decahydrate ($Na_2B_4O_7$ x $10H_2O$), in a beaker and add some distilled water and heat until dissolved.

b) Add 30.0 ± 0.1 g sodium lauryl sulphate ($C_{12}H_{25}OSO_3Na$), 10.0 ± 0.1 mL of triethylene glycol and 4.56 ± 0.01 g disodium hydrogenphosphate (Na_2HPO_4).

c) Add water and heat until dissolved. Mix and dilute to 1000 mL. Check pH which should be in the range 6.9 - 7.1.

2. Sodium sulphite, anhydrous
3. Alpha-amylase, 17,400U/mL, Heat-stable, ANKOM #FAA standardised as per Appendix 1.8R(1).
4. Distilled water (dH_2O)
5. Acetone

Procedure:

Note: *Analyses are performed only on samples previously dried at* 55 *to 60°C (maximum) and ground to pass a 1 mm screen. Operators should wear gloves when handling filter bags.*

1. Weigh the filter bag, record weight (W_1) and then zero balance. The bags have negligible moisture content and do not need to be pre-dried.

2. Weigh 0.50 ± 0.05 g, of well-mixed laboratory dry sample directly into filter bag. Record weight to at least 0.1 mg (0.001 g) accuracy (W_2).

3. Seal the bag closed within 1 cm of the open edge using the heat sealer. Weigh and seal one empty bags to be run as blanks during analysis.

4. Spread sample uniformly inside the filter bag. This should be done by shaking and lightly flicking the bag to eliminate clumping.

5. Weigh a second sub-sample for laboratory dry matter determination.

6. Pre-extract lipid from all samples with acetone. Place bags into a glass container with a lid. Pour enough acetone into container to cover bags and secure top. Shake the container 10 times and allow bags to soak for 10 min. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air dry.

7. The Bag Suspender is composed of nine individual baskets, one centre post and one spring. Place three bags per basket, maximum 24 bags per run and stack baskets on centre post, each basket rotated 120 degrees from previous one. The ninth basket remains empty and acts as a top for the eighth basket.

8. Add 2000 mL of ambient temperature Neutral Detergent Solution (NDS) into the ANKOM digestion vessel. Add 20 g Sodium Sulphite to solution in vessel and mix briefly with stirring rod. Add 4 mL Alpha-Amylase (ANKOM #FAA) during reflux.

NOTE: A total of 2000 mL of NDS should be used during all digestions regardless of the number of samples.

9. Place the Bag Suspender with samples into the digestion vessel containing the NDS. Place the Metal Weight on top of the ninth basket to keep the Bag Suspender submerged.

10. Turn Agitation and Heat ON. Confirm agitation visually and then secure vessel top. Reflux samples for 75 minutes total run-time.

11. While samples are being digested, heat distilled water to 90-100ºC in the urn.

12. When digestion time is up, turn Heat and Agitation OFF, open the exhaust valve and exhaust hot solution.

Warning: The solution in the digestion vessel is under pressure. The exhaust valve must be opened to release the pressure before the vessel top can be opened. Ensure the effluent hose is positioned and secured for safe disposal.

13. After the solution has been emptied from the digestion vessel, close the exhaust valve and open the lid.

14. Add 2000 mL of hot rinse water containing 4 mL of heat stable alpha-amylase (ANKOM #FAA) and turn Agitator ON but leave the Heat OFF. Close the lid but do not tighten.

15. Agitate the bags in rinse water for 5 min then exhaust water through the exhaust valve.

16. Repeat hot water and amylase rinses a further one more time for a total of two (2) rinses adding 4 mL of heat stable alpha-amylase.

17. Continue with a further two (2) rinses adding hot water only.

18. Remove Bag Suspender from vessel. Remove filter bags from bag suspender and gently press out excess water.

19. Place bags in 250 mL glass beaker and add enough acetone to cover bags (approximately 200 mL).

20. Allow bags to soak for 5 min then remove and lightly press out excess acetone.

21. Spread bags out and air dry.

22. Dry bags for a minimum of 4 hr in an oven with desiccator pouches at 105ºC ± 2ºC.

23. When bags are dry, cool until room temperature (at least 40 min).

24. When bags are cool, weigh and record weight (W_3).

Calculations: Percent Neutral Detergent Fibre on Dry Matter basis (NDF %DM)

Calculate percent NDF as follows:

$$\textbf{\% NDF (DM basis)} = \frac{(W_3 - (W_1 \times C_1)) \times 100 \times 100}{W_2 \times \text{Lab DM\%}}$$

Where: W_1 = Bag tare weight (g)

W_2 = Sample weight (g)

W_3 = Final bag weight after digestion (g)

C_1 = Blank bag correction = Final weight Blank Bag (g) / Initial weight Blank Bag (g)

References:

Goering HK and van Soest PJ (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). *In.* "Agriculture Handbook No. 379", Agricultural Research Service, United States Department of Agriculture (USDA), Washington, D. C. pp. 1-20.

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Mertens DR (1992). Critical conditions in determining detergent fibers. *In*. "Proceedings of the National Forage Testing Authority Forage Analysis Workshop", Sep 16-17, Denver, CO, pp. C1-C8. *Available in*: Appendix F, "Forage Analyses Procedures", National Forage Testing Association, Omaha, NE, pp. 132-139.

Method – 1.8A(b): Determination of Amylase Neutral Detergent Fibre - Fibercap System

(Application Sub-Note ASN 3803)

Note: This method calculates % NDF either with or without a correction for ash within the fibre fraction. Results should be expressed without the ash correction for comparison with the reference method 1.8R.

Scope: This method is applicable for the determination of amylase neutral detergent fibre (aNDF) in all types of forages and feeds.

Principle: Neutral Detergent Fibre (NDF) is considered to represent plant cell-wall constituents, ie. hemicellulose, cellulose and lignin, cutin and silica. Detergent is used to solubilise the proteins and sodium sulphite also helps remove some nitrogenous matter; EDTA is used to chelate calcium and remove pectins at boiling temperatures; triethylene glycol helps to remove some non-fibrous matter from concentrate feeds; and heat-stable amylase is used to remove starch. The amount of sample remaining is the NDF and is expressed as a percentage dry matter (%DM). The Foss Tecator Application Note, AN 380 - "Fibre Determination Using The Fibertec 2021 Fibercap System" should be used in conjunction with this method.

Apparatus:

FiberCap™ capsules
Foss green FibreCap capsules, part No. 60024515.
Analytical Balance
A balance capable of weighing to four decimal places
Desiccator
FiberCap™ Analyser
The FiberCap apparatus including 2022 Hot Plate and a 6 or 18 place carousel and the appropriate sized glass vessel.
Drying Oven
Air ventilated oven capable of operating at 105°C.
Electric Kettle or ZIP heater

Reagents:

1. Acetone, technical grade.

2. Neutral Detergent Solution (NDS), prepared as follows:

Note: The following formula gives weights required for 1.0L.

Note 2: If bulk solutions are made and stored prior to use, care MUST be taken to ensure that all components are mixed back into the solution before any solution is decanted. Failure to do so may result in significant error.

a) Place 18.61 g of EDTA (disodium ethylenediaminetetraacetate, $C_{10}H_{14}N_2Na_2O_8$ x $2H_2O$) and 6.81 g of sodium tetraborate decahydrate, ($Na_2B_4O_7$ x $10H_2O$), in a beaker and add approximately 700 mL distilled water and heat until dissolved.

b) Add 30 g sodium lauryl sulphate, ($C_{12}H_{25}OSO_3Na$), 10ml of 2-ethoxyethanol ($C_4H_{10}O_2$) and 4.56 g disodium hydrogenphosphate, (Na_2HPO_4).

c) Add water and heat until dissolved. Mix and dilute to 1000 mL. Check pH which should be in the range 6.9 - 7.1.

Notes: All chemicals reagent grade. Inspect solution prior to use. Solution can precipitate if more than one month old.

3. Sodium Sulphite.

4. Amylase solution – Termamyl 300 l, type DX, available from Foss Tecator.

Note: Foss Tecator now recommends the use of a working α-amylase solution (WAS), standardised so that 2 additions of 2 mL of WAS will remove 0.5 g of starch, Appendix 1.8A(b)(1).

Procedure:

Sample Preparation:

1. Grind the samples by using a suitable laboratory mill (for example, Tecator Cyclotec), with a screen that will give a particle size <1 mm.

Dry capsules before use: The capsules can gain weight by adsorption of moisture. Before use, dry capsule with lid in an oven at 105ºC for at least 30 min, desiccate, cool for at least 5 min prior to weighing sample.

2. Weigh capsule + lid (W_1), tare and weigh 0.5 g (± 0.05 g), of well-mixed laboratory dry sample to an accuracy of ± 0.1 mg (W_2) into each capsule, secure lids and place in capsule tray on tray stand.

Pre-extraction of lipid:

3. Pre-extract lipid from all samples with acetone. Add enough acetone into a beaker to ensure that there is sufficient volume to completely cover the samples, but not the lids (approximately 120 mL). Place the tray stand with the capsules in the beaker and agitate for 30 seconds. Lift the tray stand out of the solution and drain the acetone from the capsules. Repeat wash two more times with fresh acetone. Remove tray holder and allow capsules to drain and air-dry in a fume hood.

Hot Extraction:

4. Pre-treat the sample in the capsules by adding 120 mL hot water (~80°C) and 0.1 mL Termamyl [**or** (n)*2 mL Working Amylase Solution where (n) = number of samples being analysed, Appendix 1.8A(b)(1)] to a beaker. Place the tray stand with the capsules in the beaker and gently agitate to mix well. Stand for 15 minutes at room temperature.

5. Drain the solution out of the capsules and wash once with cold water and drain.

6. Place capsule tray with capsules in place in the carousel and put on the stopper to lock capsules in place.

7. Place extraction vessel with 350 mL NDS solution on the hot plate. Add 3.5 g sodium sulphite. Turn on full heating on the hotplate.

8. Turn the hotplate to maximum heat, after 4-5 min add 0.1 mL Termamyl [**or** (n)*2 ml Working Amylase Solution where (n) = number of samples being analysed, Appendix 1.8A(b)1].

9. Place condenser on top of the extraction vessel and open cold water tap (0.4 L/min) for the reflux system.

10. When the reagent starts to boil (determined by the presence of small air bubbles breaking the surface of the liquid), lower the heating. Partially lower the extraction carousel unit into the boiling reagent sufficient to immerse the samples, gently agitate to thoroughly disperse samples and then fully lower the carousel into the reagent.

11. Boil gently for 60 min.

12. While samples are refluxing, bring 1.5 L of water to boiling.

13. After 5 min of gentle boiling check that the capsules are seated and "breathing". Avoid boiling so hard that liquid covers the capsule lids, as it is important that the lid remains dry so that the capsule can "breathe". This ensures good mixing of sample and reagent inside the capsule. If sample is stuck to the capsule lid, use a pipette and wash it down with some reagent.

14. At the end of extraction, remove the condenser and place it in the holder on the back of the hot plate. Remove the extraction vessel and carousel from the hotplate.

Note: If solution is present at the lid membrane, it might be difficult to drain the capsule. Tap the whole extraction carousel against a hard surface or dry the lid membrane with a piece of soft paper. In all cases return the carousel to the extraction flask and "spin" rotate to remove all of the liquid from the capsules and discard the liquid.

15. Fill the extraction vessel with 350 mL boiling water. Wash by partially lowering the extraction carousel into the water ensuring that the capsules refill, gently agitate the carousel and raise it to empty the capsules and lower to refill. Do not use so much water that the lids come below the liquid level. Empty the capsules and extraction vessel. Repeat the washing procedure twice more, using fresh hot water each time.

16. Add 350 mL of hot water (~80°C) and 0.1 mL Termamyl [**or** (n)*2 mL Working Amylase Solution where (n) = number of samples being analysed, Appendix 1.8A(b)(1)]. Return the carousel to the extraction vessel and agitate. Allow to soak for 15 min.

17. Wash two times in fresh boiling water.

Post-extraction of lipid:

18. Move the capsule tray with the capsules to the tray holder.

19. Removal of lipid from the fibre residue is made by washing twice with Acetone. Add enough acetone into a beaker to ensure that there is sufficient volume to completely cover the samples, but not the lids (approximately 120 mL). Place the tray stand with the capsules in the beaker and agitate for 30 seconds. Lift the tray stand out of the solution and drain acetone from the capsules. Allow capsules to drain and air-dry in a fume hood

Drying and Ashing:

20. Dry capsules for a minimum of 4 hr in an oven for 5hr at 105ºC ± 2ºC.

21. Cool the capsules to room temperature (at least 40 min) in a desiccator and weigh with a precision of ± 0.1 mg (W_3).

Note: If ash correction is not to be applied ignore Steps 21–23. Once blank values have been determined, proceed to the calculation.

22. Place the capsules in pre-dried and pre-weighed (W_4) ashing crucibles (45 x 60 mm) or tall glass beakers. It is important that the crucible used is high enough so that all of the ash is retained inside the crucible as a standing capsule can fall during ashing. An alternative procedure is to use aluminium foil cups for ashing.

23. Ash the sample in the ashing crucibles for at least 4 hours at 550-600ºC

24. Cool the ashing crucibles slowly to room temperature for minimum of 1 hr, place them in a desiccator and weigh with a precision of ± 0.1 mg (W_5).

Blank determinations:

Run a blank through the entire procedure, for each new batch of capsules purchased as the capsules can loose a small amount of weight during reaction with the reagents. A correction factor (C) to compensate for this loss is used in the formula for calculation of analytical results.

Typically the correction factor (C) is >0.9990, corresponding to an approximate weight loss of 3 mg for a capsule during processing.

$$C = \frac{\text{blank capsule weight after extractions (g)}}{\text{blank capsule weight at start (g)}}$$

During the final ashing step some ash weight is obtained from the capsule itself (D). It is recommended that an ash evaluation be made for each new batch of capsules purchased. The ash weight contribution from the capsule is typically <3 mg.

Calculations:

Without ash correction:

$$\textbf{\% NDF} = \frac{(W_3 - (W_1 \times C)) \times 100 \times 100}{W_2 \times \%\text{Lab Dry Matter}}$$

With ash correction:

$$\textbf{\% NDF} = \frac{(W_3 - (W_1 \times C) - (W_5 - W_4 - D)) \times 100 \times 100}{W_2 \times \%\text{Lab Dry Matter}}$$

Where:

W_1 = Initial capsule weight (g)

W_2 = Sample weight (g)

W_3 = Capsule plus residue weight (g)

W_4 = Empty ashing crucible weight (g)

W_5 = Residue ash plus ashing crucible weight (g)

C = Blank correction for capsule solubility

D = Capsule ash weight (g)

References:

Goering HK and van Soest PJ (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). *In.* "Agriculture Handbook No. 379", Agricultural Research Service, United States Department of Agriculture (USDA), Washington, D. C. pp. 1-20.

Undersander D, Mertens DR, Thieux N (1993). Method 5. Neutral Detergent Fiber – Amylase Procedure. *In*. “Forage Analyses Procedures”, National Forage Testing Association, Omaha, NE, pp. 86-94.

Mertens DR (1992). Critical conditions in determining detergent fibers. *In*. “Proceedings of the National Forage Testing Authority Forage Analysis Workshop”, Sep 16-17, Denver, CO, pp. C1-C8. *Available in*: Appendix F, “Forage Analyses Procedures”, National Forage Testing Association, Omaha, NE, pp. 132-139.

FOSS TECATOR Application Sub-Note ASN 3805 - The Determination of Amylase Treated Neutral Detergent Fibre Using The Fibercap System.

FOSS TECATOR Application Note AN 380 - Fibre Determination Using The FIBERTEC 2021/2023 FIBERCAP System.

Appendix 1.8A(b)(1): Standardising Alpha-Amylase Activity for Neutral Detergent Fibre Determination

Standardise heat-stable α-amylase (Termamyl 120L/300L) stock solutions so 2 additions of 2 mL Working Amylase Solution (WAS) will remove starch from 0.5 g of raw cornstarch.

1. Weigh 0.5 g (± 0.005 g) of ground, dried hominy corn into each of six beakers.

2. Prepare ice bath for cooling the beakers (must contain enough ice to maintain temperature below 1ºC), and prepare tempering bath (a shallow pan containing enough water at exactly 20ºC to exceed the depth of solutions in the beakers.)

3. Add 50 mL of Neutral Detergent Solution (NDS), swirl beaker, and place on a hot plate in one-minute intervals.

4. After NDS begins to boil (approximately 5 min), add one of six doses of stock solution, (geometric progression, e.g. 0, 0.025, 0.05, 0.10, 0.20 and 0.40 mL, exact doses will depend on the source of amylase) to beakers in ascending order.

5. Reflux for 10 min, remove at one-minute intervals, add a second dose of amylase (matching the first), swirl, and rinse sides of beaker using a minimum of room temperature NDS.

6. React 60 seconds and filter through glass wool into a 100 mL glass beaker. Prepare a blank by adding 2 of the intermediate doses (e.g. 0.05 and 0.10 mL= 0.15 mL) in 40 mL of room temperature NDS in a 100mL glass beaker.

7. Place beakers, except blank, in ice bath. Remove from ice bath after 5 min (temperature of solution should be approximately 21ºC) and place all beakers in tempering bath (20ºC).

8. After solutions are 20ºC ± 0.5ºC (may take 5 min or more) remove beakers from the tempering bath and arrange in order of increasing enzyme doses on a white background.

9. Quickly add 0.5 mL of Burke´s Iodine Solution* to beakers and mix.

   *2g KI, 1g I_2 and 100ml H_2O

10. Do not look at the beaker, after 90 seconds view through the solutions from above and make a quick decision (before 120 seconds) about the colour of each solution using the following scale:

    Purple solution = not adequate enzyme
    Pink amber or amber solution = not adequate solution
    Pale yellow solution = adequate enzyme
    (Compare to blank, brown tint of enzyme solution should not be confused with pink-amber or amber).
    If colour differences are unclear, place beakers in tempering bath for 5 min and repeat steps 8-10.

11. After the lowest dose that is pale yellow (V_2) and the next lowest dose (V_1) that is pink-amber or amber are identified using a geometric progression, do a final standardisation using the dose below the pink-amber one (V_{-1}) and a linear progression of doses ($0.25*V_1$) between V_1 and V_2, (e.g., if 0.05 mL treatment is amber (V_1) and 0.10 mL treatment is pale yellow (V_2) use doses of 0.025, 0.05, 0.0625, 0.075, 0.0875, and 0.10 mL) of stock solution in the final standardisation.

12. The lowest dose that is pale yellow, and follows an inadequate dose with a pink-amber or amber solution, represents the volume of amylase stock solution (V_s) that is used to make amylase working solution.

13. Record the date, batch or lot of amylase, the doses tested, amount of iodine solution used, and colour and final temperature (before iodine addition) of each dose in a reagent log book.

14. Determine the number (n) of samples to be analysed in the following 5 days or less. To add 2 mL of amylase working solution twice for each sample requires a total volume of amylase working solution of (n) * 4 mL. Mix (n)*2*(Vs) mL of amylase stock solution with $n*[4-2*(V_s)]$ mL of dH_2O. Store working amylase solution in refrigerator using a stoppered container no longer than 5 days.

15. Confirm the adequacy of the amylase working solution by repeating the standardisation procedure using corn grits with 0, 2 and 4 mL of the working solution (each added at boiling and after removal from the refluxing apparatus). If there is no appreciable differences in colour between 2 and 4 mL of working solution, then two additions of 2 mL is adequate for the aNDF method.

Notes:

- Time and temperature are critical for proper assessment of the adequate amylase dose. The solutions must be 20°C and the decision about color must be made within 90 to 120 seconds after addition of the iodine solution. Pink-amber or amber colours fade quickly and waiting longer than 120 sec before making a decision will result in a dosage that is too low.

- Initial doses of stock solution or extract may have to be adjusted, and standardization rerun. If the maximum dose (0.4 mL) results in purple or pink-amber colour, extend the geometric progression of doses starting at 0.4 ml and rerun initial standardisation. If the minimum dose (0.025 mL) is yellow, decrease the geometric progression to between 0 and 0.025 mL and rerun initial standardisation.

- Each new source or lot of enzyme should be standardised, and if a single lot is being used over a period of time it should be checked every six months for activity. Excess enzyme is not beneficial and can be detrimental. Concentrated enzyme solution is not recommended as a working solution because a one-drop error when dispensing enzyme contains significant activity that can affect results. Many amylase extracts are crude mixtures that may contain fibrolytic and proteolytic activities. Heat-stable amylase solution must be used in hot liquids (>80°C) to inactivate contaminating enzymes and minimize fibre loss.

References:

Goering HK and van Soest PJ (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). *In.* "Agriculture Handbook No. 379", Agricultural Research Service, United States Department of Agriculture (USDA), Washington, D. C. pp. 1-20.

FOSS TECATOR Application Sub-Note ASN 3805 - The Determination of Amylase Treated Neutral Detergent Fibre Using The Fibercap System.

FOSS TECATOR Application Note AN 380 - Fibre Determination Using The FIBERTEC 2021/2023 FIBERCAP System.

Method – 1.9R: Determination of Acid Detergent Fibre by Refluxing

National Forage Testing Association - Forage Analysis Procedure 4.1

Scope: This procedure is applicable for the determination of acid detergent fibre (ADF) in all types of forages. The value obtained is influenced by the sequence of analysis for fibre components as explained in Appendix 1.9R(1). It has been agreed by the QEC that sequential analysis of ADF is the preferred method. **This method cannot be modified to facilitate sequential ADF.**

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^{o}C

Reagents:

1. Acid detergent solution: Prepare 1 litre of 1.00N Sulphuric acid ±0.005N. Normality must be verified by titration with a primary base standard (NFTA Method 3.1.2) before adding CTAB.

2. A solution approximately 1.0 N sulphuric acid can be made by adding 51.04 g (27.7ml) of concentrated reagent grade sulphuric acid (95 - 98% purity) to 972.3ml water (AOAC 935.70). Allow to cool before titrating. Titrate by NFTA method 3.1.2 or standardisation procedure given in Method – 1.9A(a) and add water (if normality too high) or sulphuric acid (if normality too low) to adjust normality to 1.00N ±0.005N. Add 20g cetyl trimethylammonium bromide (CTAB), technical grade, to 1 litre of 1.00N H_2SO_4. Stir to aid solution.

3. Acetone, reagent grade.

Safety Precautions:

- Always add sulphuric acid to water. Wear face shield and heavy rubber gloves. If acid is splashed on skin, wash immediately with copious 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 vapours 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 fibre residue before placing in the drying oven.

Procedure:

1. Samples should be microwave dried or oven dried at 55 to 60^{o}C (maximum) to 85% dry matter, then ground to pass a 1mm screen.

2. Dry 50ml fritted glass crucibles overnight at 100^{o}C and hot weigh (W_1), recording weight to nearest 0.1 mg. (Hot weigh techniques described in NFTA method 2.2.2.2.).

3. Thoroughly mix and weigh sample (W_2) (approximately 0.9 to 1.1g, record weight accurate to 0.1mg) into Berzelius beaker. Weigh a second sub-sample for laboratory dry matter determination.

4. Add 100ml acid-detergent solution at room temperature. Place beaker on heater under the cold water condenser.

5. Heat to boiling in 5 - 10 minutes; reduce heat to avoid foaming as boiling begins. Reflux 60minutes from onset of boil, adjusting boiling to slow, even level.

6. After about 30 minutes, 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 fibre particles into the crucible.

8. Soak twice with boiling (95-100^{o}C) water by breaking up mat and filling crucible each time with vacuum off and allowing to soak a minimum of 15 to 30 seconds (2 minutes 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 - 40ml acetone by filling crucible each time with vacuum off, allowing a minimum of 15 to 30 seconds (2 minutes recommended) before vacuuming dry. Repeat acetone washings until no more colour is removed, breaking up all lumps so that solvent wets all particles of fibre.

10. Dry 3 hours or overnight in forced-air oven (100^{o}C) and weigh hot, recording weight (W_3) to nearest 0.1mg.

Comments:

- Sulphuric acid for acid detergent fibre solution must be standardised 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 minutes from the 60 minutes 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 sulphuric acid and high temperature can char the sample and result in low ADF values. If black discolouration 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 50ml of distilled water and record the time required to drain completely without vacuum. This should be about 180 seconds. If filtration takes more than 240 seconds, crucibles need cleaning. If cleaning does not improve the filtration rate, the crucible should be discarded. If filtering takes less than 120 seconds, check crucible for cracks or holes in the fritted disk. If filtering takes less than 100 seconds, 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 fibre 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 Fibre (ADF)

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

Where: W_1 = tare weight of crucible in grams

W_2 = initial sample weight in grams

W_3 = dry weight of crucible and dry fibre 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 (s) among replicated analyses for acid detergent fibre 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.

Reference:

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

Appendix 1.9R(1): Differences in the ADF analytical results when ADF is determined directly or sequentially after NDF

The direct determination of ADF can sometimes lead to inflated values due to contamination with pectin or biogenic silica. This problem is outlined by Hall (2003) in a paper on the University of Florida website.

Pectin is a soluble fibre that is not digested by mammalian enzymes but can be fermented by rumen microbes. It is soluble in NDF solution but remains unextracted or is precipitated by the ADF solution (Bailey and Ulyatt 1970). However the retention of pectin in ADF does not appear to be quantitative. Contamination of ADF with pectin can be a problem with some forages (particularly legumes) and high pectin byproducts such as citrus pulp. This problem can be overcome by determining ADF sequentially after NDF.

Biogenic silica, the silica absorbed by plants and deposited in their tissues, is another source of contamination of ADF. It is present in a range of forages including rice straw, sugarcane bagasse and grasses. It is soluble in NDF solution but is quantitatively recovered in the ADF (Van Soest 1994), so determining ADF sequentially after NDF will overcome contamination. Alternatively, ashing the ADF residue and expressing the fibre result on an ash-free basis will also overcome the problem. In any event Hall (2003) recommends that all NDF and ADF data be expressed on an ash-free basis to avoid problems of the inflation of the results by the plant mineral fraction or by soil contamination.

Recent evidence with silages highlighted the importance of determining ADF sequentially after NDF (Kaiser and Kerr 2003). The 70 silage samples covered a wide range of crops and pastures with ADF (determined sequentially) covering the range 21.7-41.8 % of true DM. On average, ADF was 4.11 % units higher when measured directly (compared to sequentially) and this difference was consistent over the full ADF range (slope of regression = 0.99). Given the mean ADF for the silages was 33 % this represents a large error.

It is clear that for research, where an accurate separation of the fibre fraction into its components is needed, determining ADF sequentially after NDF is the most appropriate method.

The AFIA QEC has agreed that sequential ADF analysis will be the accepted method.

References:

Bailey, R.W. and Ulyatt, M.J. (1970). *N.Z. J. Agric. Res.* **13**: 591.

Hall, M.B. (2003). *Interpreting Feed Analyses: Uses, Abuses, and Artifacts.* Internet publication www.dps.ufl.edu/hall/IntFd/Anl.htm

Kaiser, A.G. and Kerr, K.L. (2003). *More Accurate Laboratory Tests for Assessing Silage Quality,* Final Report for DRDC Project DAN 100 and RIRDC Project DRD-4A, 50pp.

Van Soest, P.J. (1994). *Nutritional Ecology of the Ruminant,* 2nd ed. (Cornell University Press: Ithaca, NY).

Method – 1.9A(a): Determination of Acid Detergent Fibre - Ankom

Scope: This procedure describes the use of Ankom equipment to determine Acid Detergent Fibre (ADF) in all types of forages and feeds. The value obtained is influenced by the sequence of analysis for fibre components as explained in Appendix 1.9R(1). It has been agreed by the Quality Evaluation Committee (QEC) that sequential analysis of ADF is to be conducted following the analysis of NDF.

Principle: Acid Detergent Fibre (ADF) is considered to consist chiefly of cellulose and lignin. The sample is weighed into filter bags and refluxed in hot acid detergent solution for 60 min. It is then rinsed, dried and weighed. The amount of sample remaining is the ADF and is expressed as a percentage of dry matter (%DM).

Apparatus:

ANKOM$^{200/220}$ Fibre Analyser

Bag Suspender with Baskets

Analytical Balance

Computer

Spatula

Filter Bags, ANKOM #57

Measuring Cylinder, Glass 2 L

Electric Urn, 6 L capacity or ZIP Boil instant hot water service or equivalent

Beaker, Glass 250 mL

Oven, 105^{o}C

Heat Sealer

Timer

Gloves

Reagents:

1. Acid detergent solution: Prepare 3 L of 1.00 N sulphuric acid (H_2SO_4) ±0.005 N.

   Note: 2.0L of ADS must be used in each batch regardless of the number of bags used.

   The following is a formula for making up 1 L of ADS solution. Scale up to the volume you wish to make as necessary.

   A solution approximately 1.0 N H_2SO_4 can be made by adding 51.04 g (27.7mL) of concentrated reagent grade H_2SO_4 (95 - 98% purity) to 972.3 mL $_dH_20$ (AOAC 935.70). Allow to cool before titrating.

   Titrate by NFTA method 3.1.2 (or alternatively use the acid standardisation procedure given below) and add $_dH_20$ (if normality too high) or H_2SO_4 (if normality too low) to adjust normality to 1.00 N ±0.005 N.

   Finally, add 20.0 g cetyl trimethylammonium bromide (CTAB), technical grade, to 1.0 L of 1.00 N H_2SO_4. Stir to aid solution.

Normality **MUST** be verified by titration with a primary base standard (see acid standardisation section below) before adding CTAB. Normality must be verified if solution is more than 30 days old.

2. Distilled Water
3. Acetone
4. Mixed Indicator solution: Use equal volumes of solutions 1% Bromocresol green and 1% Methyl red solutions.

Procedure:

1. **Samples should have previously been analysed for NDF using AFIA Method – 1.8A(a): Determination of Amylase Neutral Detergent Fibre – Ankom.**

2. The Bag Suspender is composed of nine individual baskets, one centre post and one spring. Place three bags per basket, maximum 24 bags per run and stack baskets on centre post, each basket rotated 120 degrees from previous one. The ninth basket remains empty and acts as a top for the eighth basket.

3. Add 2000 mL of ambient temperature Acid Detergent Solution (ADS) into the ANKOM digestion vessel.

4. Place the Bag Suspender with samples into the digestion vessel containing the ADS. Place the Metal Weight on top of the ninth basket to keep the Bag Suspender submerged.

5. Turn Agitation and Heat ON. Confirm agitation visually and then secure vessel top. Run samples in the unit for a total run-time of 60 min.

6. While samples are being digested, heat distilled water to 90-100^{o}C in the urn.

7. When digestion time is up, turn Heat and Agitation OFF, open the exhaust valve and exhaust hot solution.

Warning: The solution in the digestion vessel is under pressure. The exhaust valve must be opened to release the pressure before the vessel top can be opened. Ensure the effluent hose is positioned and secured for safe disposal.

8. After the solution has been emptied from the digestion vessel, close the exhaust valve and open the lid.

9. Add 2000 mL of hot rinse water and turn Agitator ON but leave the Heat OFF. Close the lid but do not tighten.

10. Agitate the bags in rinse water for 5 min then exhaust water through the exhaust valve.

11. Repeat hot water rinse four (4) more times for a total of five (5) rinses or continue rinsing until neutral pH levels are achieved.

12. Remove Bag Suspender from vessel. Remove filter bags from bag suspender and gently press out excess water.

13. Place bags in 250 mL glass beaker and add enough acetone to cover bags (approximately 200 mL).

14. Allow bags to soak for 5 min then remove and lightly press out excess acetone.

15. Spread bags out and air dry.

16. Dry bags for a minimum of 4 hr in desiccant pouches placed in an oven at 105^{o}C ± 2^{o}C.

17. When bags are dry, cool until room temperature (at least 40 min).

18. When bags are cool, weigh and record weight.

Calculations: Percent Acid Detergent Fibre on dry matter basis (ADF %DM)

Calculate percent ADF as follows:

$$\textbf{\% ADF (DM basis)} = \frac{\{W_3 - (W_1 \times C_1)\} \times 100 \times 100}{W_2 \times \text{Lab DM\%}}$$

Where: W_1 = Bag tare weight (g)

W_2 = Sample weight (g) - determined from method 1.8A(a)

W_3 = Final bag weight after digestion (g)

C_1 = Blank bag correction = Final weight Blank Bag (g) / Initial weight Blank Bag (g)

Acid Standardisation

1. The concentration of the standard acid must then be checked by titration against a predetermined solution of sodium carbonate. This is done by drying approx. 10 g of anhydrous sodium carbonate (Na_2CO_3) at 200° C for 2 hours, storing in desiccator.

2. Dilute 10.00 ± 0.05 mL of the 1.0N H_2SO_4 solution volumetrically in 100mL. This will be used as the titrating acid.

2. Weigh 0.10 ± 0.01 g of Na_2CO_3 recorded 0.0001, into five conical beakers and dissolve with approx 30 mL of water.

3. Add 3–4 drops mixed indicator. Titrate to neutral grey, with standard acid, and record volume.

4. Boil gently for 3 mins, should return to green colour. Cool rapidly under running tap water. Continue titrating to neutral grey and note titrant volume.

Note 1: 0.1 g will require approx 18 mL of 0.1 N H_2SO_4

$$\text{Normality, N} = \frac{18.868 \times \text{weight Na2CO3}}{\text{Vol.1} + \text{Vol. 2}} \text{ x 10 (original dilution)}$$

If the Molarity is outside of the range 1.00 ± 0.005 N , dilute solution to required normality by following formula:

$V = V_2 \ x \ N_2/N_1$

N_2 = normality of stock
V_2 = volume of stock solution
V_1 = volume to which stock solution should be diluted to obtain desired normality (N1).

References:

Goering HK and van Soest PJ (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). *In.* "Agriculture Handbook No. 379", Agricultural Research Service, United States Department of Agriculture (USDA), Washington, D. C. pp. 1-20.

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ANKOM Technology – ANKOM^{200} “Acid Detergent Fiber in Feeds - Filter Bag Technique”, version 10-21-05.

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Method – 1.9A(b): Determination of Acid Detergent Fibre - Fibercap System

(Application Sub-Note ASN 3804)

Note: This method calculates % ADF either with or without a correction for ash within the fibre fraction. Results should be expressed without the ash correction for comparison with the reference Method 1.9R.

Scope: This procedure describes the use of the Fibercap System to determine Acid Detergent Fibre (ADF) in all types of forages and feeds. The value obtained is influenced by the sequence of analysis for fibre components as explained in Appendix 1.9R(1). It has been agreed by the Quality Evaluation Committee (QEC) that sequential analysis of ADF is to be conducted following the analysis of NDF.

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 and is expressed as a percentage of dry matter (%DM). The Foss Tecator Application Note, AN 380 - "Fibre Determination Using The Fibertec 2021 Fibercap System" should be used in conjunction with this method.

Apparatus

FiberCap™ capsules
Foss green FibreCap capsules, part No. 60024515.
Analytical Balance
A balance capable of weighing to four decimal places
Desiccator
FiberCap™ Analyser
The FiberCap apparatus including 2022 Hot Plate and a 6 or 18 place carousel and the appropriate sized glass vessel.
Drying Oven
Air ventilated oven capable of operating at 105°C.
Electric Kettle or ZIP heater

Reagents:

1. Acetone, technical grade.

2. The following is a formula for making up 1 L of ADS solution. Scale up to the volume you wish to make as necessary.

   A solution approximately 1.0 N H_2SO_4 can be made by adding 51.04 g (27.7mL) of concentrated reagent grade H_2SO_4 (95 - 98% purity) to 972.3 mL ${}_dH_20$ (AOAC 935.70). Allow to cool before titrating.

   Titrate by NFTA method 3.1.2 (or alternatively use the acid standardisation procedure given in the ankom - ADF procedure) and add ${}_dH_20$ (if normality too high) or H_2SO_4 (if normality too low) to adjust normality to 1.00 N ±0.005 N.

   Finally, add 20.0 g cetyl trimethylammonium bromide (CTAB), technical grade, to 1 L of 1.00 N H_2SO_4. Stir to aid solution.

Normality **MUST** be verified by titration with a primary base standard (see acid standardisation section below) before adding CTAB. Normality must be verified if solution is more than 30 days old.

Procedure:

1. **Samples should have previously been analysed for NDF using AFIA Method – Method – 1.8A(b):Determination of Amylase Neutral Detergent Fibre - Fibercap System.**

Hot Extraction:

2. Place capsule tray with capsules in place in the carousel and put on the stopper to lock capsules in place.

3. Place extraction vessel with 350 mL ADS on the hot plate.

4. Partially lower the extraction carousel unit into the cold reagent sufficient to immerse the samples, gently agitate to thoroughly disperse samples and then fully lower the carousel into the reagent.

5. Turn the hotplate to maximum heat. Place condenser on top of the extraction vessel and open cold water tap (0.4 L/min) for the reflux system.

6. When the reagent starts to boil, lower the heating to approximately 5. Boil gently for 60 minutes. Always measure boiling from the time when the solution has reached the boiling point (determined by the presence of small air bubbles breaking the surface of the liquid).

7. After 5 minutes of gentle boiling check that the capsules are seated and “breathing”. Avoid boiling so hard that liquid covers the capsule lids, as it is important that the lid remains dry so that the capsule can “breathe”. This ensures good mixing of sample and reagent inside the capsule. If sample is stuck to the capsule lid, use a pipette and wash it down with some reagent. Meanwhile preheat approximately 1.5 L of water to boiling.

8. While samples are refluxing, bring 1.5 L of water to boiling

9. At the end of extraction, remove the condenser and place it in the holder on the back of the hot plate. Remove the extraction vessel and carousel from the hotplate. Remove the carousel from the extraction vessel and empty the vessel and capsules of liquid.

Note: If solution is present at the lid membrane, it might be difficult to drain the capsule. Tap the whole extraction carousel against a hard surface or dry the lid membrane with a piece of soft paper. In all cases return the carousel to the extraction flask and “spin” rotate to remove all of the liquid from the capsules and discard.

10. Fill the extraction vessel with 350 mL boiling water. Wash by partially lowering the extraction carousel into the water ensuring that the capsules refill, gently agitate the carousel and raise it to empty the capsules and lower to refill. Do not use so much water that the lids come below the liquid level. Empty the capsules and extraction vessel. Repeat the washing procedure four (4) more times, using fresh hot water each time for a total of 5 washes.

De-fatting:

11. Move the capsule tray with the capsules to the tray holder.

12. De-fat the fibre residues by washing twice with acetone. Add 120 mL solvent in a beaker. Ensure that there is sufficient volume to completely cover the samples, but not the lids. Place the tray stand with the capsules in the beaker and agitate for 30 sec. Lift the stand out of the solution and drain the solvent from the capsules. Allow capsules to drain and air-dry in a fume hood.

Drying and Ashing:

13. Dry capsules for a minimum of 4 hr in an oven at 105^{o}C ± 2^{o}C.

14. Cool the capsules to room temperature (at least 40 min) in a desiccator and weigh with a precision of ± 0.1 mg (W_3).

Note: If ash correction is not to be applied ignore Steps 15 – 17. Once blank values have been determined proceed to the calculation.

15. Place the capsules in pre-dried and pre-weighed (W_4) ashing crucibles (45 x 60 mm) or tall glass beakers. It is important that the crucible used is high enough so that all of the ash is retained inside the crucible as a standing capsule can fall during ashing. An alternative procedure is to use aluminium foil cups for ashing.

16. Ash the sample in the ashing crucibles for at least 4 hours at 550-600ºC.

17. Cool the ashing crucibles slowly to room temperature by placing them in a desiccator for a minimum of 45 minand weigh with a precision of ± 0.1 mg (W_5).

Blank determinations:

Run a blank through the entire procedure, for each new batch of capsules purchased as the capsules can loose a small amount of weight during reaction with the reagents. A correction factor (C) to compensate for this loss is used in the formula for calculation of analytical results.

Typically the correction factor (C) is >0.9990, corresponding to an approximate weight loss of 3 mg for a capsule during processing.

$$C = \frac{\text{blank capsule weight after extractions (g)}}{\text{blank capsule weight at start (g)}}$$

During the final ashing step some ash weight is obtained from the capsule itself (D). It is recommended that an ash evaluation be made for each new batch of capsules purchased. The ash weight contribution from the capsule is typically <3 mg.

Calculations:

Without ash correction:

$$\textbf{\% ADF} = \frac{(W_3 - (W_1 \times C)) \times 100 \times 100}{W_2 \times \%\text{Lab Dry Matter}}$$

With ash correction:

$$\textbf{\% ADF} = \frac{(W_3 - (W_1 \times C) - (W_5 - W_4 - D)) \times 100 \times 100}{W_2 \times \%\text{Lab Dry Matter}}$$

Where:

W_1 = Initial capsule weight (g)

W_2 = Sample weight (g) - determined from method 1.8A(b)

W_3 = Capsule plus residue weight (g)

W_4 = Empty ashing crucible weight (g)

W_5 = Residue ash plus ashing crucible weight (g)

C = Blank correction for capsule solubility

D = Capsule ash weight (g)

References:

Goering HK and van Soest PJ (1970). Forage fiber analyses (apparatus, reagents, procedures and some applications). *In.* "Agriculture Handbook No. 379", Agricultural Research Service, United States Department of Agriculture (USDA), Washington, D. C. pp. 1-20.

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FOSS TECATOR Application Sub-Note ASN 3804 - The Determination Of Acid Detergent Fibre Using The Fibercap System.

FOSS TECATOR Application Note AN 380 - Fibre Determination Using The FIBERTEC 2021/2023 FIBERCAP System.

Version 1
Date 31/08/05

Method – 1.10R: Determination of Ash

National Forage Testing Association - Forage Analysis Procedure 7

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 oxidise all organic matter. Ash is determined by weighing the resulting inorganic residue.

Equipment:

Porcelain crucibles, 30ml vol, with covers numbered with furnace ink

Analytical electronic balance, accurate to 0.1mg

Muffle furnace with pyrometric controller

Desiccator with vented lid

Oven 105˚C

Reagents:

Nil

Safety Precautions:

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

Procedure:

1. Remove crucibles with cover which have been dried for at least 2 hours at 100˚C from oven to desiccator. Cool, and record weight of crucibles with cover to 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. Place crucibles in a cold furnace and ash at 600˚C for 2 hours 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 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. Air movement is necessary to cool crucibles.

- Slide the desiccator lid open. Do not place the lid on 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 the 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.

- The desiccator lid should be left open for minimal time.

- Desiccant should be checked and dried periodically. Replace desiccant twice annually or more frequently depending on use. Use of desiccant with colour 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 fibre analysis, set furnace at 500˚C and ash until carbon-free and grey ash colour (3 to 5 hours). Lower ashing temperatures require longer ashing times.

- Higher temperatures will melt glass and ruin filter crucibles. A practical maximum service temperature for pyrex glass is 510˚C and annealing temperature is 560˚C.

Calculations: Percent Ash

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

Where: W_1 = tare weight of crucible in grams

W_2 = initial weight of crucible and sample in grams

W_3 = dry 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 (s) 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.

References:

National Forage Testing Association - Forage Analysis Procedures 7 – Total ash in forages.

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

Method – 1.11R: Determination of Water-Soluble Carbohydrates – Water Extraction - Anthrone

Scope: This procedure details the extraction and subsequent determination of water-soluble carbohydrates in forage samples.

Principle: Soluble carbohydrates are extracted from forage with water and filtered. The resulting filtrate is heated with anthrone in sulphuric acid to form a blue-green complex. The absorbance of the extract is measured on a UV spectrophotometer at 620nm and the absorbance used to calculate the concentration of water soluble carbohydrates. See Appendix 1.11R(1) for a discussion of the anthrone method.

Reagents

1. Deionised Water
2. Sulphuric Acid, Approximately 98% w/w H_2SO_4.
3. Anthrone Reagent
   a) Add carefully, with stirring, 760mL sulphuric acid to 330mL of water.
   b) Cool and add 1.00g of thiourea and 1.00g of anthrone.
   c) Stir until dissolved and then store in the refrigerator when not in use. Discard any unused reagent after one (1) week.
4. Glucose Stock Solution (0.8mg/mL)
   a) Dissolve 0.400g of anhydrous D(+)-glucose in water and dilute to 500mL. Prepare immediately before use.
5. Glucose Working Standard Solutions

For spectrophotometers that are linear up to an absorbance of 2 (0, 0.08, 0.16, 0.32, 0.48 and 0.64mg/mL)

   a) Pipette 10 and 20mL glucose stock solution into two 100mL volumetric flasks and dilute to the mark. Pipette 20, 30 and 40mL glucose stock solution into three 50mL volumetric flasks and dilute to the mark. Prepare immediately before use.

For spectrophotometers that are linear up to an absorbance of 1 (0, 0.04, 0.08, 0.12, 0.16 and 0.2mg/mL)

   b) Pipette 5, 10, 15, 20 and 25mL glucose stock solution into six 100mL volumetric flasks and dilute to the mark. Prepare immediately before use.

Apparatus

Analytical Balance

End-over-end rotator

50mL Falcon Tubes

Volumetric flasks 100mL, 50mL, 500mL

Measuring Cylinder, 500mL, 1L

Electronic Variable Volume Pipette (eg Brand Electronic Handystep)

Manual Variable Volume Pipette (eg Brand Handystep)

Pipette tips- 50mL

Precision Pipette(s) capable of delivering 250μL and 1mL

Disposable Pipette Tips

Test Tubes, Glass 150 x 12.5mm or screw top test tubes of similar size

Test Tube Racks x 3

Glass marbles >12.5mm diameter for the top of the test tubes or screw caps

Vortex Mixer

Magnetic Stirrer and Magnetic Fleas

Spectrophotometer (620nm): Either a traditional cell-type with 10mm cells or a 96-well multiplate reader with microplates

Schott Bottles 100mL with lid

Conical Flasks, 250mL

Beakers, 5 x 50mL, 250mL, 500mL and 1L

Filter Funnels, plastic

Filter Paper, 12.5cm Whatman No 1 or equivalent

Water Bath, 100ºC

Weighing Boats

Tea Towels x 2

Spatulas

Digital timer(s)

Procedure

1. Ensure sample has been ground to pass through a 1mm sieve.
2. Weigh 0.1g of sample to an accuracy of ±0.1mg (W) into a 50mL falcon tube. Carry a blank and control through the process. Prepare at least one duplicate sample.
3. Add 50mL of water (E), cap and place on the end-over-end rotator for one (1) hour.
4. After the hour has elapsed, filter the solution into conical flask, discarding the first few mLs. Retain the filtrate for analysis.

Note: This extract may need to be further diluted later if the absorbance obtained falls outside the range of the standard graph. This dilution factor (D) must be applied as in the calibration below.

5. Pipette 1mL of the glucose working standard solutions, samples and blanks in duplicate into labelled test tubes.
6. Prepare a timed procedure so that each tube is in the boiling water bath for 20min only. For example, start a 20min timer once the first tube is placed in the boiling water bath and add each successive tube every 20seconds. At the end of the 20min, remove the first tube and place in the cool water bath, then remove each successive tube every 20seconds.
7. Complete this step rapidly: Add 5mL of anthrone reagent to the first tube using a manual variable volume pipette and 50mL tip. Immediately vortex the tube, place it in the boiling water bath, add a glass marble to the top of the tube (or cap with screw cap) and press start on a 20min timer.
8. Add anthrone reagent to each successive tube as in step 7, but following the procedure determined in step 6.
9. After 20min, remove the first tube and place in a cool water bath. Remove each successive tube using the procedure determined in step 6. Cool the tubes for a further 10min after the removal of the last tube.
10. Measure the absorbance at 620nm of each solution using either a) or b) below:

    a) Traditional Spectrophotometer with 10mm cells.

b) Multiplate reader with 96- well plates. Ensure a blank plate reading is taken before adding the samples.

Calculations

1. Construct a standard calibration curve of absorbance versus glucose standard (0 – 0.64mg). The absorbance of the zero and maximum glucose standards are approximately 0.03 and 1.8 on a Labsystems Multiskan respectively.
2. Use the calibration curve to determine the mg of glucose equivalent to the absorbances of the samples and blank.
3. Use the following equation to calculate the percentage of water soluble carbohydrates (WSC) as glucose equivalent.

$$\%WSC = \frac{G \times E \times D}{W} \times \frac{100}{\%Lab\ Dry\ Matter} \times \frac{100}{1}$$

Where:

W = Sample weight (mg)

G = mg glucose read from graph

E = Extract volume (50mL)

D = Dilution factor

If no dilution factor is required, the above equation can be simplified to:

$$\%WSC = \frac{G \times 500000}{W \times \%Lab\ Dry\ Matter}$$

Quality Control:

1. A blank determination and a control sample are carried through the sample preparation and colour development stages. If the absorbance of the sample blank is greater than the lowest standard, the analysis is repeated. If the control result is outside one standard deviation from the mean of previous control results, the batch may need to be adjusted or repeated.
2. A calibration curve is determined for each batch of extracts.
3. From each batch of samples, at least one sample is run in duplicate.
4. If duplicate results are not within 2 percentage units, the analysis is repeated.
5. The average of duplicate results is reported.

REFERENCES

FeedTest Laboratory Method Manual, DPI Vic, Pastoral and Veterinary Institute, Hamilton, 1999, Method 2.11 Determination of Water Soluble Carbohydrates.

Ministry of Agriculture, Fisheries and Food, Technical Bulletin 27 ‘The Analysis of Agricultural Materials,’ London, Her Majesty’s Stationery Office, 1973.

Appendix 1.11R(1): Notes on the Anthrone Method for Carbohydrate Analysis and the Alkaline Ferricyanide Method for Reducing Sugars.

Anthrone Method

One of the methods in this section utilises an anthrone reagent for the estimation of water-soluble or total carbohydrates in plant material. Anthrone methods have generally been displaced by phenol-sulphuric or more specific methods (Avigad 1990). The use of non-specific condensation methods has also decreased in food chemistry and human nutrition in favour of specific assays for sugars based on separation by GC or LC or on specific enzymes. However, anthrone methods have remained popular in forage analysis, but there are many variations of the method and the colour yield from specific monosaccharides is often not known for the modifications used.

Food chemists abandoned the use of anthrone in concentrated sulphuric acid as the solution is unstable and must be freshly prepared if high and variable blanks are to be avoided (see Southgate 1991). Re-crystallisation of the anthrone improves this variability but not the need to always work with freshly prepared reagent solutions (re-crystallisation from a 3:1 mixture of benzene and petroleum ether (60-80 bp.) or successively from benzene then ethanol, followed by vacuum drying is recommended (see Perrin *et al.* 1980).

Stable solutions in 66% v/v sulphuric acid with the addition of thio-urea were recommended as more stable, but the carcinogenic nature of thio-urea makes these modifications hard to recommend. The use of a 2% w/v stock solution of anthrone in ethyl acetate and the separate addition of concentrated sulphuric acid (Avigad 1990) solves the problem of unstable stock solutions but results in a precipitate forming during the reaction that must be dissolved by vigorous mixing.

If the Australian fodder industry wishes to continue the use of anthrone-based methods, an acceptable procedure will need to be agreed on and the colour response of the monosaccharides encountered will need to be established.

A better approach may well be to establish analytical methods for the individual sugars of importance.

Alkaline Ferricyanide Method

The alkaline ferricyanide reducing sugar method is proposed for the other method in this section. The use of a reducing sugar method assumes that all carbohydrates have been hydrolysed to reducing monosaccharides. The hydrolysis of poly and oligo saccharides with acid is complex and can result in the loss of some monosaccharides or the incomplete hydrolysis of others (Sturgeon 1990). There are many reducing sugar methods available, and most are based on the reducing action of sugars in alkaline solution on metallic salts. A full review is given by Binkley (1970). In the ferricyanide method, ferricyanide is oxidised to ferrocyanide and the excess ferricyanide determined in various ways.

The alkaline ferricyanide method was originally proposed in 1923 (Hagedorn and Jensen 1923) and although still used in AACC Methods for Flour Diastatic Activity, it is now not commonly used for sugar analysis in other fields. It has the same problems as copper-based reducing sugar methods and the anthrone carbohydrate methods, such as the absence of stoichiometric relationships, dependence on empirically adjusted reaction conditions and varying results with different monosaccharides. In addition a number of substances can interfere with the results. Its use in an autoanalyser will allow standardisation of reaction conditions, however the relative amount of ferricyanide used will still vary between sugars, which is a real problem where the carbohydrates being analysed contain varying amounts of different monosaccharides.

If a reducing sugar method is required, the PAHBAH (parabenzoic acid hydrazide) method is recommended (Blakeney and Mutton 1980). This method is relatively free from interference and has a lesser range of colour yields. Again, as with anthrone, if the Australian fodder industry wishes to use a ferricyanide reducing sugar method, an acceptable procedure will need to be agreed on and the colour response of the monosaccharides encountered will need to be established.

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Avigad, G. (1990). Disaccharides. In Dey, P.M. and Harbone, J.B. Methods in Plant Biochemistry, Vol 2, Carbohydrates. Academic Press.

Binkley, W.W. (1970). Reducing sugar methods. In Chapter 45. Aminoff, D., Binkley, W.W., Schaffer R. and Mowry, R.W. Analytical Methods for Carbohydrates. In Pigman, W. and Horton, D. (Eds.) The Carbohydrates, Chemistry and Biochemistry 2nd ed. Vol Iib, Academic Press, New York.

Blakeney, A.B. and Mutton, L.L. (1980). A Simple colorimetric method for the determination of sugars in fruit and vegetables. J. Sci. Food Agric. 31: 889-897.

Perrin, D.D., Armarego, W.L.F. and Perrin, Dawn R. (1980). Purification of Laboratory Chemicals, 2nd Edition. Pergamon Press.

Hagedorn, H.C. and Jensen, B.N. (1923) Zur Mikrobestimmung des Blutzuckers mittels Fericyanid. Biochem. Z. 135: 46-53.

Southgate, D.A.T. (1991). Determination of Food Carbohydrates. Elsevier Applied Science.

Sturgeon, R.J. (1990). Monosaccharides. In Dey, P.M. and Harbone, J.B. Methods in Plant Biochemistry, Vol 2, Carbohydrates. Academic Press.

Method – 1.11A: Determination of Water-Soluble Carbohydrates – Water Extraction – Alkaline Ferricyanide

Scope: This method documents the analysis of water soluble carbohydrate in plant material, by use of a flow injection analyser (FIA). The range and use of this method extends to all vegetative matter. Cold water extraction is widely used for the analysis of forages for silage production to indicate the quantity of sugars available for microbial fermentation.

Principle: In this method, cold water soluble carbohydrates are extracted from the sample. Cold water is used with benzoic acid to kill any microbes in the extracted sample and stop any resulting consumption of soluble carbohydrates before analysis. The filtered extracts are then analysed in a FIA using the alkaline ferricyanide decolouration method. The method involves the hydrolysation of the extracted carbohydrates to invert sugar by 1N hydrochloric acid and heat (90^{o}C). The invert sugar is then dialysed into an alkaline stream of potassium ferricyanide and heated (90^{o}C). The invert sugar reduces the yellow ferricyanide to colourless ferrocyanide. The decrease in colour at 420nm is directly proportional to the amount of sugar present in the extract. Interfering coloured components are eliminated by incorporating a dialyser membrane into the system.

See Appendix 1.11R(1) for a discussion of the alkaline ferricyanide method.

Equipment:

Analytical Balance(0.1 mg, capacity ≥ 100 g)

Top-pan Balance (0.1 g, capacity ≥ 2 kg).

Magnetic Heater/Stirrer, variable speed.

Stirring Magnet.

Reciprocal Shaker.

Volumetric Flasks (5 L, 1 L, 200 mL, 100 mL).

Glassware:

Beakers (100 mL, 5 L).

Measuring cylinder (100 mL).

Storage Bottle Schott (1 L).

Storage Bottle amber glass (1 L).

Boxes to hold plastic tubes

Dispenser (20 mL) accurate ±0.1mL

Plastic Tubes with Lids (30 mL)

Lachat Flow Injection Analyser (FIA) 8000 series or equivalent

FIA manifold, tubing and optical filter as per details in Figure 1

Dialyser membrane (Elkay) Type C (available from Analytical Instruments) porosity 2-6nm.

Reagents:

1. Benzoic acid, 0.2% w/v, extracting solution.
   a) Weigh 10.0± 0.02 g benzoic acid into a 100 mL beaker.
   b) Add 20-25 mL of 95% ethanol and dissolve by stirring with a glass rod.
   c) Add approximately 2.5 L H_2O into a 5 L beaker. Place a stirring magnet inside the beaker and place on a magnetic heater/stirrer. Begin stirring at a reasonable speed.
   d) Very slowly add the dissolved benzoic acid solution down a glass rod into the vortex of the stirring H_2O.
   e) Once all of the benzoic acid solution is added, wash excess "scum" from 100 mL beaker using wash bottle of H_2O, continue stirring until dissolved.
   f) At room temperature full dissolution can take many hours. Dissolution can be aided by heating the solution to approximately 60°C.
   g) Once dissolved allow to cool then transfer to 5L volumetric flask and make to volume with H_2O. Make solution up fresh if not completely used in 1 year.

2. Ethanol (95%)

3. Water – Type II (H_2O) (degassed)

4. Hydrochloric acid hydrolysing solution 1N
   (a) Add 100± 2mL of 36% HCl (or alternately, 113± 2mL of 32% w/w) to 600 mL of degassed type II water while stirring.
   (b) When miscible and cool, quantitatively transfer to 1 L volumetric flask and make to volume. Store in bottle with ground glass stopper. Make solution up fresh if not completely used in 1 year.

5. Potassium Ferricyanide $K_3Fe(CN)_6$ in 1N NaOH
   (a) Dissolve 0.77±0.005 g (accurately weighed) potassium ferricyanide - $K_2Fe(CN)_6$, and 40.0± 0.02g of sodium hydroxide in about 600 mL degassed type II water, while stirring.
   (b) Transfer quantitatively to a 1 L volumetric flask and make to volume with H_2O.
   Store in an amber glass bottle – refrigerate. Solution is stable for 1 week.

6. Stock Standard – 5.0% Sucrose $C_6H_{12}O_6$ Solution.

   Dissolve 5.00g sucrose in extracting solution, make to 100 mL in a volumetric flask. Prepare fresh when required

7. Working Standards Sucrose Solutions
   Take all aliquots from 5.0 % stock std solution as follows;

0.50 mL into100 mL	=	0.025 % Sucrose
1.00 mL into 100 mL	=	0.05 % Sucrose
3.00 mL into 100 mL	=	0.15 % Sucrose
7.00 mL into 100 mL	=	0.35 % Sucrose
15.0 mL into 100 mL	=	0.75 % Sucrose
25.0 mL into 100 mL	=	1.25 % Sucrose

Make up all working standards to volume with extracting solution in volumetric flasks. Make fresh at start of each run.

Procedure:

Preparation

1. Weigh ~0.5 ± 0.01 g (record accurately) of plant sample into 30 mL plastic container. Include some QC standards in each batch and a blank in each batch. Refer to Quality Control section for more detail.

2. Add 20.0 ± 0.1 mL 0.2% benzoic acid extracting solution.

3. Shake for 1 hour on reciprocal shaker.

4. Allow to settle (about 30 min), decant solution and filter (pour into test tube and push seraclear filter into the test tube, pouring the filtered solution out of the centre and into an analysis tube).

5. Analyse immediately or store filtered samples in fridge to await analysis. Analyse within 24 hours, otherwise new samples must be prepared. Stored samples must be capped to prevent contamination and evaporation.

Analytical Finish

1. Figure 1 shows the operation of the reducing sugars manifold for use on the FIA. Refer to the specific operating guide of the instrument for routine use and troubleshooting where necessary.

2. Ensure that the dialyser membrane is changed at the beginning of each run and/or every 500 samples. Peristaltic pump tubing should be checked for excessive stretch or wear prior to each run. It is important that all type II water used in the method is degassed prior to use.

3. Run the prepared extractions through the FIA. Set up a batch containing all sample solutions prepared and calibrate using the prepared calibration solutions.

4. Prime the auto-dilutor, and preview the baseline. When the baseline has stabilised the batch can be started. Review the calibration curve once the standards have been run, and confirm the correlation coefficient is an acceptable value (>0.99)

5. At the completion of the batch ensure that any necessary repeat samples are reanalysed, and the values of the QC standards are within their respective control limits.
(see Quality Control Section)

Calculation:

Use the following calculation to determine WSC as equivalent % (w/w) sucrose.

$$\textbf{\% Water Soluble Carbohydrates (WSC)} = \frac{(\text{raw-blank}) \times 20}{\text{Sample Weight}}$$

Report result values correct to 2 significant figures.

Quality Control:

- Include one or more quality control (QC) samples in each run, choosing QC samples by matching WSC levels and matrices to the samples in the run.

- A sample blank must be prepared with each extraction run, and used to calculate the final result.

- Include at least one set of duplicates within the batch, and preferably run one set of duplicates for every fifteen successive samples. Duplicates should be below 5% relative error or should not differ by more than 0.5 % w/w sucrose.

- Plot the results of the QC samples on a control chart and examine the chart for trends.

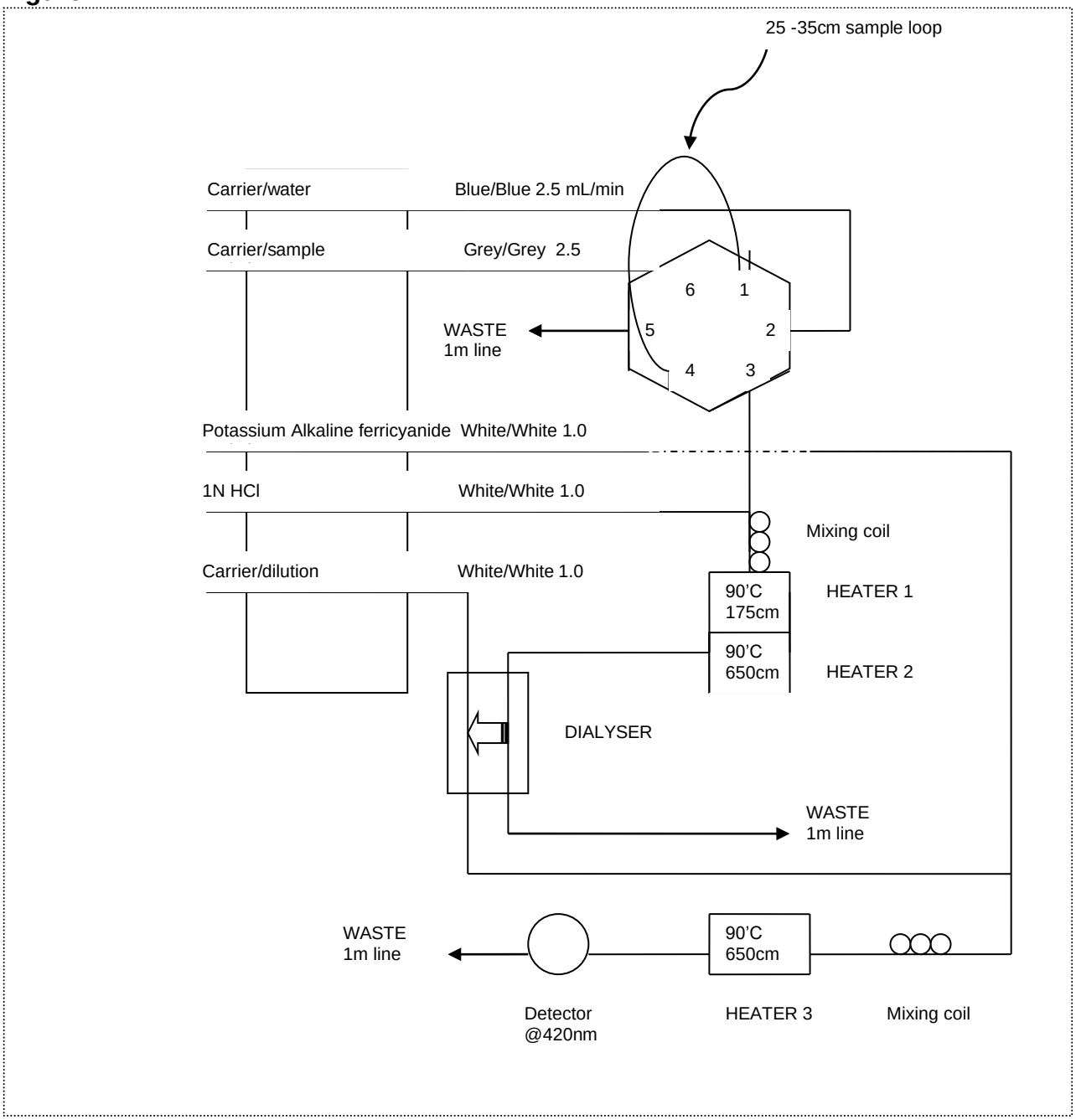
- Results outside of the upper or lower warning limits, ±2 s.d. (95% confidence limits), are evidence of possible problems with the results. 2 consecutive readings outside the limits indicate loss of control, and the run should be repeated.

- Results outside the control limits ±3 s.d. (99% confidence limits) indicate loss of control, and the run should be repeated.

References:

Technicon Industrial Method Number 302-73A
"Determination Of Reducing Sugars by Flow Injection Analysis" QuikChem Method 26-201-00-1-B (Lachat Instruments)

Figure 1


25 -35cm sample loop
Carrier/water
Blue/Blue 2.5 mL/min
Carrier/sample
Grey/Grey 2.5
6
1
5
2
4
3
WASTE
1m line
Potassium Alkaline ferricyanide White/White 1.0
1N HCl
White/White 1.0
Mixing coil
Carrier/dilution
White/White 1.0
90'C
175cm
HEATER 1
90'C
650cm
HEATER 2
DIALYSER
WASTE
1m line
WASTE
1m line
90'C
650cm
Detector
@420nm
HEATER 3
Mixing coil

Method – 1.12R: Determination of Silage pH

Scope: This method can be applied to the measurement of pH in silages and other forages.

Principle: Distilled water is added to a chopped silage sample and the pH recorded using a pH meter.

Equipment:

pH Meter

pH Electrode

250mL Beaker

Commercial bowl chopper or scissors

Reagents:

1. Commercially available 4.0 and 7.0 colour coded buffer solutions (solutions must be checked for current expiry date)

2. Potassium Chloride (KCl) AR grade for storage

Procedure:

1. **Calibrate pH Meter**
   Turn the pH meter on and allow to stabilise.

   Calibrate pH meter using 4.0 and 7.0 buffers according to the instrument manual.

   Once calibrated, rinse electrode thoroughly using distilled water.

   Place electrode into a beaker of distilled water until required. The pH meter is now ready to read samples.

2. **Chop the silage sample**
   More consistent results are obtained if the silage sample is finely chopped (particle size less than 1cm, and preferably less than 0.6cm). This can most easily be achieved by chopping the material in a rotating bowl chopper (e.g. Hobart Model 8185 food processor). This equipment is commonly used in the food processing industry and comprises a bowl which rotates in the horizontal plane, delivering the forage to knives which chop in the vertical plane. Alternatively the sample can be cut finely using scissors.

3. **Preparation for pH measurement**
   Weigh out 20 g (+/-0.2g) fresh chopped silage into a small container (beaker) and cover with 100 ml distilled water.

4. **Record pH**
   After allowing the sample to stand for 30 minutes at room temperature, place the electrode into the silage solution and read the pH to two decimal places after allowing the pH meter to equilibrate for 30 seconds (It is important at this stage to ensure the electrode is immersed in the solution and is not obstructed by the forage matter being tested. One way to ensure this is by using a small knife or something similar and creating a well in the centre of the material where the solution can accumulate. The electrode can then be placed in this solution).
   Ensure that the pH electrode is rinsed thoroughly with distilled water between measurements.
   Store the electrode in 0.1M KCl solution following use.

Comments:

- Silage is a fermented feed and the silage pH can be used as an indicator of silage fermentation quality. For lower DM silages the pH of well preserved silages is usually in the range 3.6-4.0. Higher pH values may indicate a poor silage fermentation. In drier silages bacterial growth, and hence the fermentation, is restricted so the resulting silage pH is higher. For an interpretation of silage pH and its use as an indicator of silage fermentation quality refer to Kaiser and Piltz (2003).

- Silage is a perishable product and should be stored in a sealed bag (anaerobically) at low temperature. If the sample is not to be analysed immediately it should be frozen.

- If the sample is exposed to air it will start to decompose. An early indication of this process is a rise in pH.

References:

Kaiser, A.G. and Piltz, J.W. (2003). 12. Feed testing: assessing silage quality. In: *"Successful Silage"*, eds A.G. Kaiser, J.W. Piltz, H.M. Burns and N.W. Griffiths (Dairy Research and Development Corporation and NSW Agriculture: Australia), 24pp.

Method – 1.13R: Determination of the Energy Required to Shear Dry or Dried Fodder

Scope: This is a procedure devised by Baker *et al.* (1993) for the determination of a biomechanical property of a composite plant sample, measured as the energy required to shear (now known as shear energy). This method is suitable for dry or dried feeds only, including pastures, hays, fodders and forages. A similar method for measurement of shear energy of fresh, green hydrated plant samples is described by Henry *et al.* (1996); that method is not included here.

Application: The shear energy of a forage or fodder relates to the effort that ruminant animals use to comminute forages and fodders, principally during rumination. In turn it relates to reluctance by the animal to consume that forage or fodder, and this is known as constraint to forage or fodder consumption.

Principle: Replicate portions of a representative test sample are equilibrated to constant moisture content. Each portion of the test sample is compressed into a flat disk and then a hole is sheared through it using a punch-and-die apparatus. Force-displacement data are collected throughout the shear process and integrated to calculate the energy required to shear. The shear energy is the energy required to shear through the disk corrected for the area of the sheared surface.

Apparatus and facilities required:

The apparatus required are described by Baker *et al*. (1993).

An 'environment' room with a constant temperature and humidity is required. The tests must be conducted at a constant temperature of 20(±2)°C and at a constant humidity of 65%.

A universal testing machine, capable of testing and acquiring force-displacement data in compression and shear up to 10,000 N (±1% force accuracy) with a cross-head speed of 2 to 5 mm/min.

A press (hydraulic jack) capable of compression to 10 tonnes. The press must have a gauge so that the portions of the samples are compressed to the same extent.

A compression chamber. This is a cylindrical, hardened-steel chamber no less than 80mm in height, with an internal diameter of 30mm and a solid, detachable base. Clearance between the compression chamber and the punch should be 0.04mm.

A punch-and-die apparatus. The internal diameter of the die is 20mm, capable of accommodating a 30mm diameter compressed pellet. Clearance between the punch and the die should be 0.05mm. The punch must have a cutting-edge angle of 90°.

Safety precautions:

A universal testing machine and a hydraulic press operate at extremely high forces.
Do not insert fingers or any body part into instruments.
Wear appropriate footwear and eye protection.
Appropriate safety training in use of these machines is essential.

Sampling and sample preparation:

Laboratory samples must be air-dried, freeze-dried or oven-dried. If the samples are oven dried, the drying must be at no greater than 75°C for 72 hours.

Laboratory samples must be equilibrated for at least 48h to a relative humidity of 65% at 20°C before analysis for shear energy and all tests must be conducted in this environment.

Laboratory samples must be chopped to 5-8mm in length prior to analysis for shear energy.

Laboratory samples must be free of contaminants such as sand, soil, grit etc.

Procedure:

Weigh to the nearest 0.01g 2g of portions of the test sample, which have previously been equilibrated at a relative humidity of 65% at 20°C. Record the weights of the portions of the test samples.

Place a portion of the test sample into the compression chamber and, using the universal testing machine, compress it at a constant speed of 5mm/min until a total force of 5kN is reached. Record force-displacement data throughout compression process. Release the pressure.

Place compression chamber in the press (hydraulic jack) and compress the test portion further to a total force of 100kN. Release the press.

Remove the compressed test portion from the compression chamber and place it into the punch-and-die apparatus.

Using the universal testing machine, shear the pellet at a constant speed of 2mm/min, recording the force-displacement data. Allow the punch to completely pass through the pellet before ceasing the test.

In general, five replicate portions from each sample should be tested.

Calculations:

The shear energy of the test portion, expressed as the energy expended per unit area of the sheared surface, is calculated to 1 decimal place using the following equation:

$$\text{shear energy (kJ/m}^2\text{)} = e / a$$

Where

e = the energy required to shear the pellet, measured by integrating the force-displacement data from the moment of peak force to the completion of shearing.

a = area of the sheared surface

$= t \times c$

Where

t = thickness of the pellet at the moment of peak force, which can be calculated from the time taken to complete the shear (at a constant speed)

c = circumference of the punch

Baker, S.K., Klein, L., De Boer, E.S. and Purser, D.B. (1993). Genotypes of dry, mature subterranean clover differ in shear energy. Proceedings of the XVII International Grasslands Congress, New Zealand, 1993, pp. 592-593.

Henry, D.A., Baker, S.K. and Purser, D.B. (1996). Measuring the compression and shear energies of green plant material. Anim. Prod. Aust. 21: 497.

Method – 1.14R Determination of Crude Fat (Ether Extract)

National Forage Testing Association - Forage Analysis Procedure 8

Scope: This method is used to calibrate and monitor the NIR determination of the fat content in feeds and to determine fat content on ground feed samples which cannot be determined by the NIR calibration. It details the extraction of fat from forages, grains, pellets, mixed feeds and byproducts in the range of 0.5% to 90% of Dry Matter.

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

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

Equipment:

Fat extraction apparatus

Extraction thimbles, 22 x 80 mm

Fat beakers, pyrex, with ground lip, engraved with a number, 50 x 85 mm

Drying oven, 105^{o}C gravity convection

Analytical balance, sensitive to 0.1 mg

Desiccator and tongs

Boiling Stones, JJP Type A

Reagents:

Petroleum Spirit B.R. 40-60^{o}C, Unichrom HPLC Grade

Safety Precautions:

- There should be no open flames nearby. Avoid inhaling spirit vapours. Store spirit in metal containers. Handle open containers (reagent cans and fat beakers) in a hood. Conduct the extractions in a well ventilated area.

- Electrical equipment is to be grounded. Extractors should be spark-proof.

- Make sure all spirit 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 the 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 beakers to be used for fat determination for at least 1 hr at 100°C. Cool the appropriate number of fat beakers in a desiccator. Weigh and record the weight to the nearest 0.1 mg (W_2).

Extraction

3. Line up the fat beakers in front of the extractor and match the thimbles with their corresponding fat beakers.

4. Slip the thimble into a thimble holder and clip the holder into position on the extractor.

5. Add petroluem spirit to each fat beaker.

6. Place the beaker into the ring clamp and tightly clamp the beaker onto the extractor.

7. Raise the heaters into position.

8. Turn on the heater switch, the main power switch, and the condenser water.

9. After the spirit has begun to boil, check for leakage. If there is leakage, check the tightness of the clamp and if necessary replace the gasket(s).

10. Extract as per apparatus method.

11. 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.

Spirit Distillation and Weighting of Fat Residue

12. Remove the thimble from the holder and allow spirit to air dry in fume hood with extraction fan until any remaining spirit has evaporated.

13. Place the beakers in a 102°C gravity convection oven.

14. Dry for a maximum of 30 min. Excessive drying may oxidize the fat and give high results.

15. Cool in a desiccator, weigh and record the weight to the nearest 0.1 mg (W_3).

16. Repeat drying and weighing until two successive weighings do not differ by more than 0.1% from the previous weighing. NOTE: Extraction, drying and weighing have to immediately succeed each other.

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

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

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 (s) among replicated analyses for crude fat 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.

References:

National Forage Testing Association - Forage Analysis Procedures 8 – Crude Fat (Ether Extract) in Forages.

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

FEEDTEST, DPI Vic, Hamilton, Victoria - Method Manual, Method 2.8 Determination of Fat in Feed Samples

SECTION 2

AFIA REFERENCE PROCEDURES

Method – 2.1R: Determination of *in vivo* Digestibility using Sheep

Scope: This procedure is applicable to all forages fed to ruminant animals

Principle: Sheep are used in this determination. Animals are individually housed and fed. During experimental feeding periods, animals are fed known amounts of feed each day and feed not consumed is collected and weighed to determine actual dry matter consumption. Faecal collection harnesses enable total collection of faecal output. Dry matter digestibility (DMD) is calculated as the % difference between the dry matter of the feed consumed and the dry matter of the faeces excreted. Organic Matter digestibility (OMD) can also be calculated if the ash content of feed and faeces are determined. Digestible Organic Matter in the Dry Matter (DOMD) can then also be calculated.

Experimental Procedure:

Note: Variations to this procedure are possible, depending on type of fodder and numbers of fodders to be evaluated. The following procedure relates to a specific design.

Hays were assessed as follows:

1. Design: 4 X4 Latin square (4 periods, 2 feeding levels, 2 sheep per diet per period) yielding 8 individual animal values per hay type at each level of feeding.

2. Feeding levels: *ad libitum* and restricted (2.3% of bodyweight).

3. Animals: Merino wether sheep approximately 8 months of age and fasted bodyweight of 32kg. Animals were fitted with faecal collection harnesses.

4. Experimental Cycle: 14 day equilibration period; 8 day measurement period.

5. Daily Measurements:
   a) Weight of feed offered
   b) Sub-sample of feed offered (%DM at 100^{o}C)
   c) Weight of feed refused (dried to constant weight at 60^{o}C)
   d) Total faecal output

6. Trial Measurements:
   a) Total dry matter fed out
   b) Total dry matter feed refusal
   c) Total dry matter faecal output (dried to constant weight at 100^{o}C)

7. Determinations:

   a) Dry matter digestibility (DMD) values were calculated as the percentage difference between the total dry matter hay eaten and the total dry matter faeces excreted for 8 sheep on each hay at both levels of feeding.

   b) *Ad libitum* dry matter intake (DMI) values were also calculated for 8 sheep on each hay.
   c) Means and standard errors were calculated for both measurements.

Chemical Analysis:

1. Hay:

During each digestibility trial, samples of each hay were taken daily during the 8 day collection for all 4 periods. Each day a sub-sample of each hay was weighed, dried overnight at 100^{o}C then reweighed to determine dry matter (DM) content. The remaining portions were pooled and mixed to produce 4 samples of all hays.

Representative sub-samples of these were ground in a cyclone mill to pass a 1mm screen and analysed for crude protein (CP) (Kjeldahl nitrogen x 6.25) and dry matter digestibility using a pepsin-cellulase enzymatic technique (Method – 1.7R).

Analytical values for pepsin-cellulase dry matter disappearance (PCDMD) were adjusted using a linear regression based on existing "standard" samples of known *in vivo* DMD.

2. Feed Refusals:

Samples of the dried feed refusals from all sheep throughout both trials were taken, ground and analysed for DMD in the same manner as for hay samples.

The values for DMD determined on the refusals were used to adjust the final figures for *in vivo* DMD on all the hays.

3. *In vivo* Hay Standards:

When the digestibility trials were completed, the hay samples from each period were bulked, thoroughly mixed and coarsely ground in a hammermill to pass a 4mm screen. This resulted in large quantities of hays which were then stored in airtight containers in an air-conditioned store pending distribution to other laboratories. Additional quantities of each hay were taken from the original bales and set aside for later processing if required.

Reference:

FEEDTEST, DPI Vic, Hamilton, Victoria - Method Manual, Method 2.7 Determination of Digestibility using the Pepsin - Cellulase Method.

Method – 2.2R: Calculation of Metabolisable Energy

Scope: This method calculates the metabolisable energy value (ME) of feeds for ruminants from digestibility data. The prediction equations are based on *in vivo* data for both digestibility and ME. For greater accuracy separate equations are used for different classes of feeds.

Principle: ME is predicted from *in vivo* digestibility, either measured with animals or estimated using an *in vitro* procedure such as the pepsin-cellulase enzymatic procedure (Method 1.7R). Where only DMD data are available, DOMD can be calculated using the equations in Appendix 2.2R(1). The calculated ME values are all expressed on a DM basis as megajoules (MJ) per kg DM. One MJ is one million (10^6) joules.

Procedure:

Three ME prediction equations below are recommended for use in Australia and replace the single equation used in the first edition of this manual:

1. Roughages other than silages:

ME (MJ/kg DM) = 0.203 DOMD (%) – 3.001

2. Silages:

ME (MJ/kg DM) = 0.16 DOMD (%)

3. Grains & concentrates:

ME (MJ/kg DM) = 0.858 + 0.138 DOMD (%) + 0.272 EE (%)

Comments:

- Metabolisable energy (ME) is that fraction of the total energy in a feed that can be used by an animal for maintenance and production. It is the difference between the gross energy supplied by the feed and the sum of the energy excreted in the faeces (undigested feed), in the urine and from the methane produced during digestion.
- The first and third equations were derived from actual ME data from the UK (MAFF, 1990). These are the most comprehensive data available from metabolism chamber experiments with ruminants offered either forages (particularly hays, fresh grasses/legumes and straws) or grains and concentrates. Work with silages in the UK showed that generalised ME prediction equations underestimated the ME of silages, so a separate equation that takes account of the energy value of the volatiles was recommended (AFRC 1993). This is the second equation listed above. A fuller discussion of the background to these equations is provided by Kaiser *et al.* 2005.
- The digestibility data need to be corrected for silage volatiles (see Appendix 1.3R(1)).

Calculation:

Step 1: Determine digestibility using Method 1.7R. If DOMD is not determined then it should be predicted from DMD using the calculation described in Appendix 2.2R(1).

Step 2: Calculate ME using the appropriate equation above, where DOMD is expressed as % digestible organic matter in the DM. In the concentrate equation, EE% is the ether extract (fat) and is expressed on a DM basis.

References:

AFRC 1993. " Energy and Protein Requirements of Ruminants", (CAB International: Wallingford, UK), 159 pp.

Kaiser, A., Freer, M., Flinn, P. and Black, J. 2005. Prediction of the metabolisable energy content of forages and concentrates from measurements of digestibility. Paper presented to the AFIA Quality Evaluation Committee meeting, Adelaide, 17 February 2005, 8pp.

Ministry of Agriculture Fisheries and Food 1990. "UK Tables of Nutritive Value and Chemical Composition of Feedingstuffs", eds D.I. Givens, J.R. Hopkins, C.A. Morgan, M.H. Shranks, J.H. Topps and J. Wiseman (Rowett Research Services Ltd: Aberdeen, UK), 420 pp.

Appendix 2.2R(1). Calculation of Organic Matter Digestibility from Dry Matter Digestibility

Digestibility is the difference between the amount of a feed component eaten and the amount of that component excreted in the faeces. It accounts for a major proportion of the metabolisable energy (ME) in a feed. Digestibility can be measured either directly in animals, or estimated from *in vitro* assays, for example Method 1.7R in this manual. The digestibility of a feed can be expressed in three ways:

Dry matter digestibility (DMD) % = 100 × (feed DM – faecal DM)/feed DM

Organic matter digestibility (OMD) % = 100 × ((feed DM-feed ash) – (faecal DM – faecal ash)) /(feed DM – feed ash)

Digestible organic matter in the DM (DOMD) % = 100 × ((feed DM – feed ash) – (faecal DM – faecal ash)) /feed DM

The ash within a feed does not provide energy to animals so predictions of the ME content based on DMD will not be accurate when the ash contents of the ingredients vary widely. Significant errors can arise with some feed samples. Estimates of ME based on OMD account for the ash content and should therefore more accurately reflect the actual ME value for that feed. However, when used to formulate diets for animals, ME is expressed as MJ/kg DM or /kg as-fed and not on the basis of kg OM. Consequently, the widely accepted practice is to predict ME from DOMD which takes account of the effects of varying ash contents and uses the same DM denominator as ME.

In order to predict ME from DOMD, it is necessary to undertake the ashing stage in the digestibility procedure (Method 1.7R), and also to determine the ash content of the sample itself. Whilst this is preferable, some laboratories may wish to measure DMD only, which avoids the ashing steps. However prediction equations are needed in this case to predict DOMD from DMD. These equations need to be robust, covering feeds with a wide range in ash content.

Principle: An estimate of digestibility can be obtained using the pepsin-cellulase enzymatic procedure (Method 1.7R). Previously, where only DMD was determined, DOMD was estimated using the SCA (1990) equation:

DOMD% = 0.95 DMD (%) – 0.9 (This equation is no longer used, it is for information only)

A major limitation with this equation is that it can only be applied to feeds with ash content in the range 9-12%. As many feeds have ash contents falling outside this range, the SCA equation is no longer recommended, and more robust equations are required to predict the DOMD for most if not all feeds.

Using the MAFF (1990) database which provides *in vivo* DMD and DOMD data for a wide range of feeds, the following regression equations were developed (see Kaiser *et al.* 2005).

Roughage/forage equation: This equation covers a diverse set of 58 roughages with ash contents in the range 4-18% and DOMD in the range 42-79%:

DOMD% = 6.83 + 0.847 DMD (%) (R^2 = 0.93; s.e. = 2.67)

Concentrate equation: This equation covers a diverse set of 40 concentrates, including cereal grains, grain legumes, fodder and sugar beet, various fruit and vegetable by-products, vegetable protein meals, and fish meal, with ash contents in the range 1-21% and DOMD in the range 48-92%.

DOMD% = 2.11 + 0.961 DMD (%) (R^2 = 0.96; s.e. = 2.75)

Calculation:

Step 1: Determine digestibility using Method 1.7R.

Step 2: Where ash content is not determined, and only DMD data are available, calculate DOMD (%) using the above only the roughage/forage equation or the concentrate equation. The SCA equation is no longer accepted by AFIA for determining DOMD.

References:

Kaiser, A., Freer, M., Flinn, P. and Black, J. 2005. Prediction of the metabolisable energy content of forages and concentrates from measurements of digestibility. Paper presented to the AFIA Quality Evaluation Committee meeting, Adelaide, 17 February 2005, 8pp.

Ministry of Agriculture Fisheries and Food 1990. "UK Tables of Nutritive Value and Chemical Composition of Feedingstuffs", eds D.I. Givens, J.R. Hopkins, C.A. Morgan, M.H. Shranks, J.H. Topps and J. Wiseman (Rowett Research Services Ltd: Aberdeen, UK), 420 pp.

Standing Committee on Agriculture 1990. "Feeding Standards for Australian Livestock. Ruminants." (CSIRO Publications: Melbourne, Australia), 266 pp.

SECTION 3

DRAFT METHODS

Draft methods have been accepted by the AFIA QEC for provisional inclusion in the Manual. Draft methods can be either upgraded to Reference or Approved methods or removed from the Manual at subsequent QEC meetings.

"Draft" methods for a specific analysis are numbered and followed by "D(a..n)" for cases where there is more than one Draft method.

Method – 3.1D: Determination of Starch

Scope: This procedure describes the use of Megazyme Total Starch kit for the determination of total starch in all vegetative matter including food, feed, plant and cereal products. Samples require pre-dissolution in 2M KOH solution.

Principle: Samples are pre-treated with 2M KOH solution at temperature to ensure the breakdown of resistant starch that may be present. Samples are then incubated at temperature in the presence of thermostable A-amylase to convert starch to maltodextrins. The addition of amyloglucosidase (AMG) converts maltodextrins to D-glucose which is then oxidised to D-gluconate to release hydrogen peroxide (H2O2) that is then measured by colourimetry.

Apparatus:

Glass test tubes (round bottomed: 16x120mm or 18x150mm)

Micro-pipettors, 100ul

Positive displacement pipettor

- with 50mL Combitip (to dispense 3mL aliquots of bacterial a-amylase solution
- with 50mL Combitip (to dispense 0.1mL aliquots of amyloglucosidase solution

Bench Centrifuge (required speed 3,000rpm)

Analytical Balance

Spectrophotometer set at 510nm

Vortex mixer

Thermostatted water bath set at 50.0oC

Boiling water bath with tube rack

Stop Clock

Spatula

Reagents:

1. Stock Solution A. Thermostable a-amylase (3,000 U/mL on Ceralpha reagent at pH 6.5 and 40oC or 1600 U/mL on Ceralpha reagent at pH 5.0 and 40oC).

2. Stock Solution B. Amyloglucosidase (3300 U/mL on soluble starch at ph 4.5 and 40oC).

3. Stock Solution C. Potassium phosphate buffer (0.26M, pH 7.4) p-hydroxybenzoic acid (0.22M) and sodium azide (0.4%w/v). NOTE: Sodium Azide is a poisonous chemical and should be treated accordingly.

4. Stock D. Glucose oxidase (>12,000U) plus perozidase (>650 U) and 4-aminoantipyrine (80mg). (Freeze dried powder)

5. Stock Solution E. D-Glucose standard solution (5mL, 1.0mg/mL) in 0.2% w/v benzoic acid.

6. Stock F. Standardised regular maize starch control.

7. Solution 1. : Dilute 1.0mL of Stock Solution A to 30mL with Reagent A. Divide into appropriately sized aliquots and store in polypropylene tubes at -20oC between use and keep cool during use if possible.

8. Solution 2. : Stock Solution B as is however it should be dispensed using a positive displacement dispenser capable of 0.1mL aliquots.

9. Solution 3. : Stock Solution C (volume to be advised) diluted to 1L with distilled water.

10. Solution 4. : Glucose Determination Reagent (GOPOD Reagent) - Dissolve Stock D (amount to be advised) in 20mL of Solution 3 and quantitatively transfer this to the bottle containing the remainder of Solution 3. Cover this bottle with aluminium foil to protect from light. Stable for approx 3 months at 2-5oC or >12 months at -20oC. If frozen, divide into aliquots to prevent freezing and thawing of all the reagent each time it is used.

11. Reagent A. Sodium Acetate Buffer (100mM, pH 5.0) plus calcium chloride (5mM). Add 5.8mL of glacial acetic acid (1.05g/mL) to 900mL of distilled water. Adjust the pH to 5.0 by the addition of 1M (4g/100mL) sodium hydroxide solution (approx 30mL is required). Add 0.74g of calcium chloride dehydrate and dissolve. Adjust the volume to 1L with distilled water and store at 4oC (stable for >6months at 4oC).

12. Reagent B. Sodium Acetate Buffer (1.2M, pH 3.8). Add 69.6 mL of glacial acetic acid (1.05g/mL) to 800ml of distilled water and adjust to pH 3.8 using 4M sodium hydroxide. Adjust the volume to 1L with distilled water. Stable for 12 months at room temperature.

13. Reagent C. Potassium Hydroxide solution (2M). Add 112.2g KOH to 900mL of deionised water and dissolve by stirring. Adjust volume to 1L and store in sealed container. Stable for 12 months at room temperature.

Controls and Precautions:

1. The time of incubation with GOPOD reagent is not critical, but should be at least 20min. The colour formed should be measured within 60min.
2. With each set of determinations, reagent blanks and glucose controls (100ub, quadruplicate) should be included.
   a) The reagent blank consists of 0.1ml distilled water + 3.0ml GOPOD reagent.
   b) The glucose control consists of 0.1ml glucose standard solution (100ug/0.1ml) + 3.0ml GOPOD reagent. The Factor "F" is calculated by dividing the amount of D-glucose analysed (100ug) by the absorbance obtained for this amount of D-glucose in the standard assay. The absorbance value will vary.
3. With each set of determinations, a standard flour or starch sample should be included.
4. With each new batch of GOPOD reagent, the time for maximum colour formation with 100ug of glucose standard should be checked. This is usually approximately 15 min.
5. Sample blanks can be determined using the same assay procedure with the following modifications:

a) In step 5 add 3 ml of distilled water instead of thermostable a-amylase.
b) In step 6, amyloglucosidase is replaced by distilled water.

Procedure

1. Mill sample to pass through a 0.5mm screen.

2. Weigh accurately 0.100g milled sample to a glass centrifuge tube (16x120mm; 17ml capacity).

3. Wet with 0.2ml of aqueous ethanol (80%v/v) to aid dispersion stir the contents on a vortex mixer.

IF sample contains >2% D-glucose and/or maltodextrins , otherwise proceed with step

4. Add 5.0 mL of aqueous ethanol (80 % v/v), and incubate the tube at 80-85°C for 5 min. Mix the contents on a vortex stirrer and add another 5 mL of 80% v/v aqueous ethanol.

5. Centrifuge the tube for 10 min at 1,800 g (approx. 3,000 rpm) on a bench centrifuge. Discard the supernatant.

6. Resuspend the pellet in 10 mL of 80 % v/v aqueous ethanol and stir on a vortex mixer. Centrifuge as above and carefully pour off the supernatant.

Then

7. Add a magnetic stirrer bar (5 x 15 mm) and 2 mL of 2 M KOH to each tube and re-suspend the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 1).

NOTE:
1. Do not mix on a vortex mixer as this may cause the starch to emulsify.
2. Ensure that the tube contents are vigorously stirring as the KOH solution is added. This will avoid the formation of a lump of starch material that will then be difficult to dissolve.

8. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer.

Immediately add 3ml of thermostable a-amylase (Solution 1) and incubate the tube in boiling water for 6min, stirring vigorously after 2, 4 and 6 min.

Note: It is essential to stir vigorously to ensure complete homogeneity of the slurry. Also, stirring after 2 min prevents the possibility of some of the sample expelling from the top of the tube as the alcohol evaporates.

10. Place the tube in a bath at 50oC: add 1.0ml Stock Solution B (amyloclucosidase) and stir tube on vortex mixer then incubate at 50oC for 30min.

11. Transfer the entire contents of the test tube to 100ml volumetric flask (with funnel to assist transfer). Use wash bottle to rinse the tube contents thoroughly. Adjust volume with distilled water. Mix thoroughly. Centrifuge an aliquot of this solution at 3,000rpm for 10min. Use the clear, undiluted filtrate for assay.

12. Transfer duplicate aliquots (0.1ml) of the diluted solution to the bottom of glass test tube (16x100mm).

13. Add 3.0ml of GOPOD reagent to each tube (including the d-glucose controls and reagent blanks) and incubate the tubes at 50oC for 20min.

14. D-Glucose controls consist of 0.1ml of D-glucose standard solution (1mg/ml) and 3.0ml of GOPOD reagent. Reagent blank solutions consist of 0.1ml of water and 3.0ml of GOPOD reagent.

15. Read the absorbance of each sample, the D-glucose control at 510nm against the reagent blank.

Calculations: Percent Starch on DM basis

Starch % = Abs x F x FV/0.1 x 1/1000 x 100/W x 162/180

Where Abs = Absorbance (reaction) read against the reagent blank
F = 100 (ug of D-glucose)/absorbance for 100ug of glucose
FV = Final volume
0.1 = volume of sample analysed
1/1000 = conversion from ug to mg
100/W = Factor to express “starch” as a percentage of flour weight
W = weight of sample in milligrams
162/180 = Adjustment from free D-glucose to anhydro D-glucose

Starch % w/w (dry matter basis) = Starch % x 100/(100-mositure (%w/w))

Reference:

TOTAL STARCH ASSAY PROCEDURE (AMYLOGLUCOSIDASE/
a-AMYLASE METHOD) K-TSTA 04/2009

Method – 3.2D: Determination of The Ammonia-N Content of Silages by Ion Specific Electrode

Scope: This method determines the ammonia-N content of silages to evaluate silage fermentation quality.

Principle: An acid extract is prepared from a chopped silage sample stored in a sealed container in a cold room for a period of at least 24 hours. Ammonia-N is absorbed into the solution as ammonium ion. The ammonia-N is determined using an ammonia electrode on a mV meter. For interpretation of results see Method 1.12R.
Apparatus:

A suitable millivolt meter or dedicated ion specific electrode meter fitted with an ammonia electrode.

Small electric stirrer.

Plastic sample cups, 85mL capacity or similar.

Variable Micro pipettor or dispensor to deliver 1mL.

Reagents:

All reagents are reagent grade and all water is reagent grade unless otherwise specified.

1. Standard ammonia solution, 1000mg NH_3-N/L in 0.3M Sulphuric acid. Transfer approximately 800mL of water to a 1L beaker. Working in a fume cupboard, add, slowly with constant stirring, 16.8mL of concentrated sulphuric acid. Allow the solution to cool to room temperature. Weigh 3.82g of NH_4Cl and transfer to the beaker and stir to dissolve. Transfer the solution to a 1L volumetric flask, make to volume with water and mix. Transfer to a suitably labelled plastic container.

2. Standard ammonia solution, 100mg NH_3-N/L in 0.3M Sulphuric acid. Transfer approximately 800mL of water to a 1L beaker. Working in a fume cupboard, add, slowly with constant stirring, 16.8mL of concentrated sulphuric acid. Allow the solution to cool to room temperature. Transfer, by pipette, 100mL of Standard ammonia solution, 1000mg NH_3-N/L to the beaker. Transfer the solution a 1L volumetric flask, make to volume with water and mix. Transfer to a suitably labelled plastic container.

3. Standard ammonia solution, 10mg NH_3-N/L in 0.3M Sulphuric acid. Transfer approximately 800mL of water to a 1L beaker. Working in a fume cupboard, add, slowly with constant stirring, 16.8mL of concentrated sulphuric acid. Allow the solution to cool to room temperature. Transfer, by pipette, 10mL of Standard ammonia solution, 1000mg NH_3-N/L to the beaker. Transfer to a 1L volumetric flask, make to volume with water and mix. Transfer to a suitably labelled plastic container.

4. Standard ammonia solution, 1mg NH_3-N/L in 0.3M Sulphuric acid. Transfer approximately 800mL of water to a 1L beaker. Working in a fume cupboard, add, slowly with constant stirring, 16.8mL of concentrated sulphuric acid. Allow the solution to cool to room temperature. Transfer, by pipette, 10mL of Standard ammonia solution, 100mg NH_3-N/L to the beaker. Transfer to a 1L volumetric flask, make to volume with water and mix. Transfer to a suitably labelled plastic container.

5. Sodium hydroxide solution, 10M. Weigh 40.0g of NaOH into a 250mL beaker. Dissolve the solid in about 80mL of water. Allow the solution to cool and make up to 100mL with water. Transfer to a suitably labelled plastic bottle.

6. Sodium hydroxide solution, 0.1M. Dilute 1mL of Sodium hydroxide solution, 10M to 100mL with water. Transfer to a suitably labelled plastic bottle.

7. Sulphuric Acid, 0.3M. Transfer approximately 1200mL of water to a 2L beaker. Working in the fume cupboard, add slowly and with constant stirring, 33.6mL of concentrated sulphuric acid to the beaker. Allow the solution to cool and transfer to 2L volumetric flask. Make to volume with water and mix. Transfer the solution to a suitably labelled plastic container.

8. Ammonia Electrode Filling Solution, 0.1M NH_4Cl. Weigh 0.5349g of ammonium chloride into a 100mL beaker. Add approximately 50mL of water and swirl to dissolve the solid. Transfer the solution to a 100mL volumetric flask, make to volume and mix. Transfer the solution to an appropriately labelled plastic container.

Procedure:

Sample Extraction

1. Weigh 20 g of chopped silage (for chopping procedure see silage pH method) into an open neck glass bottle with a screw cap.

2. Cover with 200ml 0.3M sulphuric acid and replace the cap. Stir gently.

3. Store the sample in a refrigerator for at least 24 hours (preferably 3 days) before determining the ammonia concentration.

4. Stir gently and allow the solids to settle somewhat. If the analysis cannot be carried out immediately freeze until ready for analysis.

Preparation of the Electrode

The electrode should be prepared for use about 1 hour prior to calibration. A new electrode and membrane may require a longer conditioning time. Refer to the electrode manufacturers instructions for details of electrode care and maintenance.

5. Add sufficient electrode filling solution to fill the electrode membrane assembly. Tap the membrane assembly to remove any air bubbles.

6. Rinse the sensing electrode with water.

7. Screw the membrane assembly on to the electrode ensuring that no air bubbles are trapped beneath the sensing electrode.

8. Place the electrode in the stand with the tip in Sodium hydroxide, 0.1M storage solution.

9. On completion of analysis return the electrode to the Sodium hydroxide, 0.1M storage solution. If the electrode will not be used again within 72 hours remove the membrane module, rinse it with water and store. Rinse the sensing electrode with water, refill the hydrating cap with water and fit it to the electrode.

Calibration of the Electrode

10. Transfer, by measuring cylinder, 45mL of each of the standard ammonia solutions to a series of labelled sample cups.

11. Place the lowest concentration standard in the measurement stand with the stirrer and electrode immersed in the solution.

12. Start the stirrer and set the speed to ensure thorough mixing without cavitation and ensuring

that the electrode remains immersed.

13. Add, using the dispensor 1mL of Sodium hydroxide 10M.

14. Record the millivolt readings obtained.

15. Plot the mV readings obtained against the ammonia nitrogen concentration on a logarithmic scale.

Measurement of Samples

16. Defrost sample extract and gently mix to ensure homogenous solution.

17. Transfer, by measuring cylinder, 45mL of sample solution to a sample cup.

18. Place the sample in the measurement stand with the stirrer and electrode immersed in the solution.

19. Start the stirrer and set the speed to ensure thorough mixing without cavitation and ensuring that the electrode remains immersed.

20. Add, using the dispenser, 1mL of Sodium hydroxide 10M.

21. Record the millivolt readings obtained.

22. Determine the Ammonia N concentration from the calibration curve.

Calculation

The Ammonia-N concentration of the sample is calculated from:

$$Af = \frac{A \text{ x } V}{W \text{ x } 1000}$$

where: Af = ammonia-N in fresh sample (g/kg)

A = ammonia-N in solution from calibration curve (mg/L)

V = volume of acid used to extract sample (mL)

W = weight of sample extracted (g)

Convert the result to an estimated true DM basis:-

$$Ad = \frac{(Af \times 1000)}{TDM}$$

where: Ad = ammonia-N concentration in dry sample (g/kg)
TDM = true DM content of the silage (g/kg)

Express the result as a % of total N (g/kg estimated true DM) previously determined by Kjeldahl digestion or combustion.

Acceptance of results:

The r^2 value obtained for the calibration should be 0.98 or better and the slope mV/10 fold change in concentration should be between 55 and 59. If the results do not meet these criteria repeat the calibration. If the results are still not acceptable refer to the manufacturers instructions for guidance on how to service the electrode.

References:

Standard Methods for the Examination of Water and Wastewater, Page, A.D. et al Editors, 20th Edition,1998.

Version 1
Date 01/08/13

Method – 3.3D: Determination of Nitrogen fractions

Scope: These procedures describe determination of the nitrogen fractions in forages and feeds according to procedures described by Licitra, Hernandez and Van Soest (1996). The object of determining nitrogen fractions is to further predict the nutritive value of a feed based on the premise that different nitrogen fractions have absorbed in different proportions and different rates.
The separation of protein and nitrogen fractions is frequently used by models such as the Cornell Net Carbohydrate Protein Model. Non-protein nitrogen is denoted as the A fraction while true protein is broken down into B_1, B_2, B_3, B_4, and C fractions based on decreasing solubility.

These fractions and descriptions, as described by (1) are described in the table below:

Fraction	**ABREV**	**Definition**	**Enzymatic Degredation**	**Classification**
Nonprotein N	NPN	Not precipitatable	Not Applicable	A
True protein	TP	Precipitate in tungstic acid	Fast	
True soluble protein	TSP	Buffer soluble but precipitatable (TP-IP)	Variable	B_1
Insoluble protein	IP	Insoluble in buffer	-	
Neutral detergent soluble protein	IP-NDIP	Difference between IP and protein insoluble in neutral detergent (ND)	Variable to slow	B_2
ND insoluble protein, but soluble in AD	NDIP-ADIP	Protein insoluble in ND but soluble in acid detergent	slow	B_3
Insoluble in acid detergent	ADIP or ADIN	Includes heat-damaged protein and nitrogen associated with lignin	Indigestible	C

Non-Protein Nitrogen and True Protein

Principle: Non-protein nitrogen (NPN) is traditionally the nitrogen passing into the filtrate after precipitation with a protein specific reagent. True protein (TP) on the other hand is calculated using the nitrogen contained in the residue on the filterpad.
Feedstuffs may contain a wide variety of low molecular weight nitrogenous substances. The determination of NPN depends upon precipitation of true protein by a suitable precipitant, filtration and determination of the insoluble nitrogen in the residue. The NPN is calculated as the difference between the total crude protein nitrogen and the value of the precipitated true protein nitrogen.

Apparatus:
Erlenmeyer flask (125 ml>,
Whatman #54 or 541 filter paper,
analytical balance,
pH meter,
filter funnels,
Kjeldahl apparatus. See method 1.4R Determination of Crude Protein by Kjeldahl Method

Reagents:

1. Sodium tungstate (Na,W0,2H,O) (100 g 1-l) solution in water, 0.30 M.
2. 0.5 M sulfuric acid (H,SO,).

Procedure

1. Weigh 0.50 ±0.05 g dry ground sample to 4 decimal places into a 125 ml Erlenmeyer flask.
2. Add 50 ml of cold deionised water.
3. Add 8 ml of 10% sodium tungstate solution
4. Let flask stand at 20-25°C for 30 min.
5. Bring pH to 2 by adding 10 ml of 0.5 M sulfuric acid (check pH with pH meter).
6. Let flask stand overnight at room temperature
7. Fold Whatman #54 or 541 filter paper and place in a conical funnel. Thoroughly wet paper with distilled water before adding any sample.
8. Filter by gravity; or with mild vacuum. If first filtrate is cloudy return to the filter funnel and refilter.
9. If vacuum is used, separate filter flasks must be used, so that any cloudy filtrate can be recycled through the funnel.
10. Transfer paper to Kjeldahl tube and determine residual nitrogen.

Refer to Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion) for instructions on determining nitrogen content of residue.

Calculations: For standard H_2SO_4 titrant, results are calculated using the following equations:

- **%True Protein Nitrogen (%TPN)** = $$\frac{(V_S - V_B) \text{ X } N\ (H_2SO_4) \text{ X } 14.007 \text{ X } 100}{W \text{ X } 1000}$$

Where: V_S = Volume, in ml, of standard H_2SO_4 required to titrate sample

V_B = Volume, in ml, of standard H_2SO_4 required to titrate blank

N (H_2SO_4) = Normality of the acid titrant

14.007 = equivalent weight of Nitrogen

W = sample weight in grams

- **% Nitrogen Dry Matter basis (%TPN DM) = %N X [100 / Lab DM%]**

Where: Lab DM% = Percent Laboratory Dry Matter of the sample analysed

- % **True Protein (% TP DM,) = %N X 6.25**
- **% Non Protein Nitrogen (%NPN DM, fraction A, expressed as CP) = CP - TP**

Comments:

- Value of NPN may be expressed as crude protein (N X 6.25) or as percent of total feed nitrogen.
- The tungstic acid value is variable depending upon conditions. Several physicochemical factors can be discerned in this variability. One is the length of time needed in the mildly alkaline tungstate extraction to solubilize protein. This could be a problem in dried feeds, as polymers like proteins need time to swell and dissolve. If this is inadequate, underestimation of soluble protein results.
- A second factor is the final pH and time of the acidic precipitation. Recommendation is to check pH in case of highly buffered feeds. Overnight precipitation decreases the NPN estimate and signifies more complete precipitation.
- Precipitates of soluble protein by tungstic acid are finely divided and sometimes difficult to filter. There is a danger that fine material may pass the filter and time of filtration can be lengthy. Filtration using vacuum can lose up to 10 percent of the soluble protein, which is recoverable if the first filtrate containing any cloudy matter is returned to the funnel. Thus individual filtration flasks must be used. If samples are filtered by gravity and the time is very long, funnels need to be covered to avoid evaporation which can lead to variable results. Centrifugation as an alternative procedure requires more steps in the preliminary preparation, especially in the case of forages that do not form definite pellets. To get a good pellet with forages require pre-treatment with ultrasonication under mild vacuum, to take out most of the air trapped in forage structure (P. Schofield personal communication). A swinging bracket centrifuge at 5000 rpm for 15 min. at 4°C is required. After decanting the supernatant, the pellet is resuspended in

distilled water (15 ml) and re-centrifuged.

Soluble nitrogen and protein (buffer soluble nitrogen)

Principle

Soluble protein is defined as true protein that is soluble in buffer at rumen pH.
This definition differs from others in that NPN components are excluded from the fraction. The procedure offered here is for total insoluble nitrogen which in combination with a measurement of NPN allows estimation of soluble true protein by difference.
Soluble crude protein (nitrogen) is a simplistic concept that evolved out of the observation that most soluble nitrogen components were rapidly degraded in the rumen, and therefore, reduced protein that could be passed to the lower tract. Krishnamoorthy et al. (1982)
The sample is weighed and then a borate-phosphate buffer is added to insure pH stabilization.. Incubation at physiological pH of nonsterile feeds provides the opportunity of microbial growth and utilization of nitrogenous feed components, as well as activation of indigenous enzymes present in the sample.

Apparatus

Erlenmeyer flask 125 ml,
Whatman #54 or 541 filter paper,
analytical balance,
waterbath,
vacuum source,
filter manifold fitted with conical funnels (50 ml),
Kjeldahl apparatus. See method 1.4R Determination of Crude Protein by Kjeldahl Method

Reagents

1. Borate-phosphate buffer, pH 6.7-6.8
   Add the following chemicals to 400mL deionised water and make up to 1.0L in a volumetric flask.
   12.20 g monosodium phosphate (NaI-I,PO,.H,O)
   8.91 g sodium tetraborate (Na,B,O,.lOH,O)
   100 ml tertiary butyl alcohol
2. Sodium azide 10% solution
   This solution must be prepared freshly each day. Dissolve 5.0g of sodium azide in 30mL of deionised water and make up to 50mL volumetrically.

Procedure

1. Accurately weigh 0.50 ±0.05 g to 4 decimal places ground dry sample into a 125 ml Erlenmeyer flask.
2. Add 50 ml borate-phosphate buffer.
3. Add 1.0 ± 0.05 ml of sodium azide solution.
4. Let stand at room temperature for 3 h.
5. Filter through Whatman #54 or #541 filter paper using mild vacuum.
6. Wash the residue with 250 ml cold distilled water.
7. Estimate N in residue by Kjeldahl. This gives the insoluble protein fraction. Soluble protein is calculated by difference from total crude protein. can be obtained by subtracting the NPN by tungstic acid procedure.

Refer to Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion) for instructions on determining nitrogen content of residue.

Calculations: For standard H_2SO_4 titrant, results are calculated using the following equations:

- **%Buffer Insoluble Nitrogen (%BIN)** = $$\frac{(V_S - V_B) \times N\ (H_2SO_4) \times 14.007 \times 100}{W \times 1000}$$

Where: V_S = Volume, in ml, of standard H_2SO_4 required to titrate sample

V_B = Volume, in ml, of standard H_2SO_4 required to titrate blank

N (H_2SO_4) = Normality of the acid titrant

14.007 = equivalent weight of Nitrogen

W = sample weight in grams

- **Correct for DM if required % Nitrogen Dry Matter basis (%BIN DM) = %BIN X [100 / Lab DM%]**

Where: Lab DM% = Percent Laboratory Dry Matter of the sample analysed

- **Buffer insoluble Protein (IP) = BIN-DM x 6.25**
- **True Soluble Protein (TSP, Fraction B_1) = TP - IP**

Notes: Extraction temperature has an effect on results. The procedure of choice is the incubation at room temperature (20-25°C) which involves the least work and equipment.

Determination of Neutral-detergent insoluble nitrogen (NDIN)

Principle:

The nitrogen associated with NDF is normally cell wall-bound protein which also includes the indigestible nitrogen found in the acid-detergent residue. The protein insoluble in the neutral-detergent solution, but soluble in acid-detergent is digestible, but slowly degradable and has been termed the B, fraction in the Cornell Net Carbohydrate Protein Model. Heating denatures B, proteins and may render them insoluble thus increasing the B, fraction as well as the C fraction obtained as the ADIN.

This procedure is based on processing samples through Ankom equipment using the Method – 1.8A(a): Determination of Amylase Neutral Detergent Fibre - Ankom described earlier in this manual, and then analysing the residue using the Kjeldahl procedure described in Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion). It is important that sodium sulfite is omitted from the NDF procedure, as this interferes with the procedure.

Apparatus.

Ankom equipment as per Method – 1.8A(a): Determination of Amylase Neutral Detergent Fibre - Ankom
forced-air oven,
Kjeldahl apparatus as per Method– 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion)

Reagents.

1. Neutral detergent solution according to Method – 1.8A(a): Determination of Amylase Neutral Detergent Fibre - Ankom
2. Acetone
3. Kjeldahl reagents according to Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion)

Procedure.

1. A 0.50 ± 0.05 g sample is weighed and digested in NDF solution, in an Ankom Fibre System according to Method – 1.8A(a): Determination of Amylase Neutral Detergent Fibre - Ankom **It is important to omit sodium sulfite from the method as discussed above.**
2. Bags may be weighed if required, or analysed directly by folding the bag containing the NDF residue and adding to a Kjeldahl digestion tube.
3. Nitrogen residue is then analysed according to Refer to Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion) for instructions on determining nitrogen content of residue. Ensure that 0.01N acid is used as the titrant to ensure more accurate results.

Calculations: For standard H_2SO_4 titrant, results are calculated using the following equations:

- **%Neutral detergent insoluble nitrogen (%NDIN)** = $\frac{(V_S - V_B) \text{ X N } (H_2SO_4) \text{ X } 14.007 \text{ X } 100}{W \text{ X } 1000}$

Where: V_S = Volume, in ml, of standard H_2SO_4 required to titrate sample

V_B = Volume, in ml, of standard H_2SO_4 required to titrate blank

N (H_2SO_4) = Normality of the acid titrant

14.007 = equivalent weight of Nitrogen

W = sample weight in grams

- **Correct for DM if required % Nitrogen Dry Matter basis (%NDIN DM) = %NDIN X [100 / Lab DM%]**

Where: Lab DM% = Percent Laboratory Dry Matter of the sample analysed

- **Neutral Detergent Insoluble Crude Protein (%NDICP) = NDIN-DM x 6.25**
- **Neutral Detergent Soluble Crude Protein (fraction B2) = %IP - %NDICP**
- **ND insoluble protein, but soluble in AD**

  %NDIPADIP (Fraction B_3) = %NDICP - %ADICP

Notes: It is important to push the filterbags down the bottom of the kjeldahl tubes to prevent foaming during the digestion step.

Determination of acid-detergent insoluble nitrogen (ADIN)

Principle:

It is not possible to completely extract all nitrogen from plant cell wall. A residual core appears to be resistant, indigestible and associated with lignin even in fresh forages that do not contain tannins. Heat-drying of forages at temperatures above 60C shows analytically significant increases in yield of lignin and fiber. The increased yield of acid-detergent fiber (ADF) can be accounted for largely by the production of artifact lignin via the non-enzymic browning reaction (Van Soest, 1965).

This procedure is based on processing samples through Ankom equipment using the procedure Method – 1.9A(a): Determination of Acid Detergent Fibre – Ankom described earlier in this manual, and then analysing the residue using the Kjeldahl procedure described in Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion).

Apparatus.

Ankom equipment as per Method – 1.9A(a): Determination of Acid Detergent Fibre Ankom

porcelein crucibles,

analytical balance,

forced-air oven,

Reagents.

1. Acid-detergent solution

2. Acetone

3. Kjeldahl reagents

Procedure.

4. A 0.50 ± 0.05 g sample is weighed and digested in ADF solution, in an Ankom Fibre System according to Method – 1.9A(a): Determination of Acid Detergent Fibre Ankom. **Use direct determination for non-sequential ADF.**
5. Bags may be weighed if required, or analysed directly by folding the bag containing the ADF residue and adding to a Kjeldahl digestion tube.
6. Nitrogen residue is then analysed according to Refer to Method – 1.4R: Determination of Crude Protein by the Kjeldahl Method (Block Digestion) for instructions on determining nitrogen content of residue. Ensure that 0.01N acid is used as the titrant to ensure more accurate results.

Calculations: For standard H_2SO_4 titrant, results are calculated using the following equations:

- **%acid detergent insoluble nitrogen (%ADIN)** = $$\frac{(V_S - V_B) \text{ X N } (H_2SO_4) \text{ X } 14.007 \text{ X } 100}{W \text{ X } 1000}$$

Where: V_S = Volume, in ml, of standard H_2SO_4 required to titrate sample

V_B = Volume, in ml, of standard H_2SO_4 required to titrate blank

N (H_2SO_4) = Normality of the acid titrant

14.007 = equivalent weight of Nitrogen

W = sample weight in grams

- **Correct for DM if required % Nitrogen Dry Matter basis (%ADIN DM) = %ADIN X [100 / Lab DM%]**

Where: Lab DM% = Percent Laboratory Dry Matter of the sample analysed

- **Acid Detergent Insoluble Crude Protein (ADICP, Fraction C) = ADIN-DM x 6.25**
- **Adjusted Crude Protein**
  - Calculation: **ADFCP/CP Ratio**

    Ratio = %ADFCP/%CP x 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 – [(ratio-7) x CP]/100

  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 fibre crude protein

Notes: It is important to push the filterbags down the bottom of the kjeldahl tubes to prevent foaming during the digestion step.

References:

1. Standardization of procedures for nitrogen fractionation of ruminant feeds, G. Licitra , T.M. Hernandez , P.J. Van Soest , Animal Feed Science Technology 57 (1996) 347-358
2. Krishnamoorthy, U., Muscato, T.V., Sniffen, C.J. and Van Soest, P.J., 1982. Nitrogen fractions in selected
3. NFTA Method A2. Adjusted Crude Protein