National Forage Testing Association -Forage Analysis Procedures

Nitrogen Determination by Kjeldahl (Block Digestion)

Scope:

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

Basic Principle:

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

The "block" method consists of:

Digestion of the sample in sulfuric acid with a catalyst, which results in conversion of nitrogen to ammonia
Determination of ammonia, either

a) colorimetrically on an autoanalyzer or
b) by steam distillation and titration .

Several catalysts are available for digestion, including mercury, copper, and copper/titanium. Choice of catalyst will depend on the difficulty of breakdown of the peptides in the sample protein to be analyzed and environmental problems associated with the disposal of the waste containing the catalyst.
The ammonia can be determined colorimetrically by heating with salicylate and hypochlorite to produce blue color which is proportional to the ammonia concentration. The color is intensified by adding sodium nitroprusside. Tartrate is added to the buffer to prevent precipitation of calcium and magnesium.
If the ammonia is determined by titration, it can be distilled into either of two types of trapping solutions which require different titrants :

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

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

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

Equipment:

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

Reagents:

Digestion:

- Sulfuric acid, 95-98%, reagent grade
- Mercury catalyst tablets (or alternative catalyst) – Selenium catalyst is used in our laboratory
- Lysine monohydrochloride, reagent grade, dried at 110oC for four hr

Distillation/titration quantification Base (one of the following)

- Sodium hydroxide, 45% w/w solution (for all catalysts except mercury) Dissolve 2250 g low N NaOH and dilute to 5 L
- Sodium hydroxide - Potassium sulfide solution (for mercury catalyst) Dissolve 400 g low N NaOH in water and, while still warm, dissolve 30 g potassium sulfide (K2S) in solution and dilute to 1 L

Trapping solution (one of the following)

- Boric acid solution, 4% dissolve 400 g boric acid (H3BO3) in distilled water containing 70 mL 0.1% alcoholic solution of methyl red and 100 mL 0.1% alcoholic solution of bromocresol green dilute to 10 L with distilled water

- Hydrochloric acid standard solution, 0.5N Prepare 0.5N standard acid solution by diluting 430.1 mL 36.5 to 38% HCl to 10 L with distilled water.

Titrating solution (one of the following)

- For boric acid trapping solution Prepare 0.2 N standard hydrochloric acid solution by diluting 172 mL 36.5 to 30% HCl to 10 L with distilled water.

- For standard acid trapping solution Prepare 0.1N sodium hydroxide (NaOH) solution.

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

Safety Precautions:

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

- The sulfur oxide fumes produced during digestion are hazardous to inhale.
- Digests must be cool before dilution water is added to avoid a violent reaction during which the acid can shoot out of the flask. Likewise, the diluted digest must be cool before sodium hydroxide is added to avoid a similarly violent reaction.

Procedure: Digestion

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

Protein, %	Sample, g
6 to 24	1.5±0.1
25 to 40	1.0±0.1
41 to 50	0.8±0.1
51 to 60	0.7±0.1
61 to 90	
+90	0.5±0.1

Weigh sample equal to 50 mg N Include reagent blank and high purity lysine as check of correctness of digestion parameters. Weigh a second subsample for laboratory dry matter determination.

- **2**) Place in a fume hood. Add sufficient catalyst tablets to supply 9 g K2SO4 and 0.42 g HgO (or appropriate amount of alternative catalyst). Then add 15 mL sulfuric acid.
- **3**) Place tubes in block digester preheated to 410oC. (Digester must be equipped with an exhaust system and/or placed in an acid fume hood.) Digest about 45 min.
- **4**) Remove tubes and let cool about 10 min in a fume hood. Time will depend upon airflow around tubes. Direct rapid spray or stream of deionized water to the bottom of each tube to dissolve acid digest completely (total volume of 50 to 75 mL if using distillation/titration quantification).

Option A: Colorimetric quantification (automated ammonia determination)

- **5**) Let cool, dilute to volume, and mix thoroughly. Transfer portion of each sample solution to analyzer beaker.

- **6**) Place standards in tray in increasing order of concentration, followed by group of samples. Analyze lowest concentration standard in duplicate, discarding first peak. Follow each group of samples with standard references to correct for possible drift.
- **7**) Plot mg N/250 mL vs average peak height of the two standards and determine mg N/250 mL for each sample.

Option B: Distillation/titration quantification

- **5**) Place NaOH-K2S (for mercury catalyst) or NaOH (for alternative catalyst) in alkali tank of steam distillation unit. Make sure that sufficient NaOH is dispensed from unit to neutralize all acid in tube (about 50 mL) before conducting distillation.
- **6**) Place 250 mL tritration flask containing trapping solution and indicator on the receiving platform, with tube from the condenser extending below the surface of the trapping solution. The trapping solution will be either:
  a) about 25 mL 4% boric acid containing indicator
  b) appropriate volume (approximately 15 mL), accurately measured to nearest 0.1 mL (VHCl) standard HCl solution and sufficient water to insure that the end of the condenser tube is submerged. Also add 3 to 4 drops methyl red indicator.
- **7**) Attach digestion tube containing diluted, cooled digest to distillation unit.
- **8**) Dispense appropriate volume of base solution.
- **9**) Steam distill until 100-125 mL distillate collects.
- **10**) Remove titrating flask from unit, rinsing condenser tip with water.
- **11**) (For boric acid trapping solution) Titrate trapping solution with 0.2 N HCl to neutral gray endpoint. Record volume of acid (VHCl) required to nearest 0.01 mL. Titrate reagent blank (VB) similarly. Color change is green to gray to purple.
- **11a**) **-or-** (For HCl trapping solution) Titrate excess HCl with standard sodium hydroxide solution to orange endpoint. (Color change from red to orange to yellow). Record volume (VNaOH) of sodium hydroxide to titrate acid to nearest 0.01 mL. Titrate reagent blank (B) similarly.

Comments:

- Include a reagent blank and at least one sample of high purity lysine hydrochloride in each day's run as check of correctness of digestion parameters. If digestion is not complete, make appropriate adjustments. A standard, such as NIST Standard Reference Material No. 194, ammonium phosphate (NH4H2PO4), certified 12.15% N should also be included. Following is a list of some standards:

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

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

- Reagent proportions, heat input and digestion time are critical factors - do not change.
- Ratio of salt to acid (wt:vol) should be 1:1 at end of digestion for proper temperature control. Digestion may be incomplete at lower ratio; nitrogen may be lost at higher ratio. Each gram of fat consumes 10 mL sulfuric acid and each gram of carbohydrate consumes 4 mL sulfuric acid during digestion.
- Catalyst mixtures are commercially available in powdered or tablet form. Dispensers are available for convenient delivery of powdered catalyst mixtures.
- When using mercury catalyst, sodium thiosulfate can be added independently, rather than in the 45% NaOH, before distillation; however it must be added immediately before distillation to avoid production of H_2S gas. If added independently, add 15 mL of 8% $Na_2S_2O_3$ solution.
- Mercury containing Kjeldahl waste cannot be disposed directly to a sanitary sewer.
- Alternative catalysts are available, although not listed in AOAC Official Methods of Analysis. Two examples are: copper catalyst tablets (each tablet contains 0.35 g K_2SO_4 and 0.1 g $CuSO_4$) and selenium catalyst tablets (each tablet contains 3.5 g K_2SO_4 and 0.035

g Se). When using these catalysts, increase digestion time to approximately 60 min.

Calculation: Percent Nitrogen (N)

For standard sodium hydroxide titrant:

%N (DM basis) ={[(VHCl × NHCl) - (B × NNaOH) -(VNaOH × NNaOH)] × 1.4007} / (W × Lab DM/100)

- VNaOH = mL standard NaOH to titrate sample
- VHCl = mL standard HCl pipetted into titrating flask for sample
- NNaOH = Normality of NaOH
- NHCl = Normality of HCl
- B = mL standard NaOH needed to titrate 1 mL standard HCl minus VBK
- VBK = mL standard NaOH needed to titrate reagent blank carried through method and distilled into 1 mL standard HCl
- 1.4007= milliequivalent weight of nitrogen X 100
- W = weight of sample in grams

For standard HCl titrant:

%N (DM basis) = (VA - VB) × NHCl × 1.4007 / (W × Lab DM/100)

- VA = Volume, in mL, of standard HCl required for sample
- VB = Volume, in mL, of standard HCl required for blank
- NHCl = Normality of acid standard
- 1.4007= milliequivalent weight of N X 100
- W = sample weight in grams

Calculation: Percent Crude Protein (CP)

CP (DM basis)= % N (DM basis) ✕ F

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

Quality Control:

Include a reagent blank, one sample of high purity lysine hydrochloride, and one or more quality control (QC) samples in each run, choosing QC samples by matching analyte levels and matrices of QC samples to the samples in the run. Include at least one set of duplicates in each run if single determinations are being made. An acceptable average standard deviation among replicated analyses for crude protein ranges from about ±0.10

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

Reference:

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

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

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

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

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