AMMONIA NITROGEN IN RUMEN FLUID AND AMMONIA NITROGEN RELEASE
Up-dated September 2013

I. References:

Berthelot MPE: Viotet d'aniline. Repert Chim Appl. 1:284 (1859).

Broderick, G.A. and Kang, J.H. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J Dairy Sci. 63:64 (1980).

Chaney, A.L. and Marback, E.P. Modified reagents for determination of urea and ammonia. Clin. Chem. 8:130 (1962).

Smith, F.E. and T.A. Murphy. March 10, 1993.

II. Principle:

Ammonia (H_3N) reacts with alkaline hypochlorite and phenol (C_6H_6O) in the presence of a catalyst (Sodium Nitroprusside – $C_5H_4FeN_6Na_2O_3$) to form indophenol (blue) (Berthelot reaction). The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrophotometrically at 550nm.

III. Personal Protective Equipment:

A. Lab coat
B. Safety glasses/goggles
C. Latex gloves

IV. Reagents:

A. McDougal's Buffer

1. Sodium Bicarbonate ($B_4Na_2O_7$) 180 g
2. Sodium Phosphate (Dibasic) (HNa_2O_4P) 54.0 g
3. Potassium Chloride (KCl) 10.8 g
4. Sodium Chloride (NaCl) 9.0 g
5. Magnesium Sulfate ($MgSO_4$) 2.16 g
6. Calcium Dichloride ($CaCl_2$) 2.88 g
7. Bring to18 liter in carboy
8. Store in the refrigerator at 2-8°C

B. Alkaline Hypochlorite Reagent

1. Dissolve 10 g Sodium Hydroxide (NaOH) in about 1300 mL dH_2O
2. Add 40.1 g anhydrous Na_2HPO_4 (or 75.7 g Disodium Phosphate – $Na_2HPO_4 * 7H_2O$) and dissolve with mild heating and mixing.
3. Cool and add 100 mL **fresh** Clorox bleach (Clorox is 5.25% Sodium Hypochlorite)
4. Mix and q.s. to 2 liters with dH_2O
5. Filter through #1 Whatman filter paper and store in an amber polyethylene bottle in refrigerator at 2-8°C. Reagent is stable for about **one** month.

C. Phenol Color Reagent

1. Dissolve 0.1 g sodium nitroferricyanide ($Na_2Fe(CN)_5NO*2H_2O$) in 1 liter dH_2O
2. Add 23 mL 88% liquid phenol (in flame room) or 20 g dry phenol (C_6H_5OH)

3. Mix and q.s. to 2 liters with dH_2O
4. Store in an amber polyethylene bottle in refrigerator at 2-8°C. Reagent is stable for about **one** month.

D. Ammonia Standards
1. Diluted from a 32 mg/dL commercial stock solution. Or can be made by dissolving 1.0045g NH_4Cl in 800mL dH_2O.
2. Reduce pH to 2 with 2N HCl
3. q.s. to 1 liter with dH_2O to make 32 mg/dL stock solution
4. Use stock to make levels:
   a. 30 mg/dL
   b. 15 mg/dL
   c. 7.5 mg/dL
   d. 3.75 mg/dL
   e. 0 mg/dL

E. Rumen Fluid
1. Strain through 4 layers of cheesecloth and keep warm in a thermos until use. Try to keep anaerobic.

V. Procedure:

A. Obtain crude protein values for all samples to be tested
B. Weigh samples into 50 ml. in vitro tubes according to the following calculation: (2/avg. nitrogen value of sample to be tested) = mg of sample in each tube
C. Runs will be divided into 18 and 24 hr. incubations. 3 tubes per time period for each sample (6 tubes total/sample).
D. Tubes are randomized and placed in the large in vitro bath (38.5 ^{o}C) for inoculation. Each tube will receive 30 ml of a 50/50 mixture of pre-warmed and carbonated (CO_2) McDougal's Buffer and rumen fluid strained through 4 layers of cheesecloth, then separated in separatory funnels. Mix should be carbonated throughout inoculation procedure and each tube must also be separately carbonated to assure anaerobic conditions. Tubes are capped with rubber stoppers (vented with 1/8" hole) and mixed by swirling (see Modified In-Vitro Procedure).
E. After incubation, add 2 ml (l mL two times) dilute HCl (1 part concentrated HCl/ 3 parts water) to each tube twice to kill microbes
F. Pipette 3 mL from each tube and centrifuge for 10 min at 1500 rpm
G. Pipette 40 µL of supernatant, 40 µL dH_2O, 2.5 ml Phenol Color Reagent, and 2 ml Alkaline Hypochlorite into pre-labeled tubes
H. Vortex and heat at 37°C for ten minutes
I. Prepare a blank (40 microliters water) and ammonia standards (40 microliters each). Run these (in duplicate) through the same reaction procedure used in step 4.
J. Once heated, samples will be stable. Pipette 300 µL into wells of a microtiter plate. Read and record absorbance at 550nm.

VI. Calculations:

Calculate standard curve using linear regression.

x = absorbance y = concentration

Note: Normal rumen ammonia levels are expected to be in the range of 1-25 mg/dL.