

**ANALYSIS OF RUMEN AMMONIA & BLOOD UREA NITROGEN**  
**Updated September 2013**

Reference:

- Smith, F.E. and Murphy, T.A. Analysis of Rumen Ammonia & Blood urea Nitrogen March 10, 1993
- Berthelot MPE: Violet 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 Marbach, E.P. Modified reagents for determination of urea and ammonia. Clin Chem. 8:130 (1962)

Personal Protective Equipment:

1. Lab coat
2. Safety glasses/goggles
3. Latex gloves

Reagents:

1. Urease Buffer (BUN only)
  - a.) Dissolve 7.11g Sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) in 400-500ml boiling ( $\text{CO}_2$  free) distilled  $\text{H}_2\text{O}$
  - b.) Add 5.0 g Disodium ethylenediamine tetraacetate ( $\text{Na}_2\text{C}_{10}\text{H}_{14}\text{O}_8\text{N}_2 \cdot 2\text{H}_2\text{O}$ ) (EDTA)
  - c.) Bring volume to 1 liter with boiling distilled  $\text{H}_2\text{O}$
  - d.) pH should be 7 (adjust if necessary)
2. Buffered Urease Solution (BUN only) (0.4 mg/ml)
  - a.) Add 20 mg Urease (Sigma U1500 Type III 27,000 units/g) to 50 ml Urease buffer and dissolve
  - b.) Store at 2-10°C, **stable for one month**
  - c.) Concentration may need to be adjusted
3. Standards for BUN's
  - a.) Purchase from Sigma (Urea Nitrogen Standard Solution Catalog No. 535-30)
  - b.) Use standard to make levels: 30 mg/dl  
15 mg/dl  
7.5 mg/dl  
3.75 mg/dl  
0 mg/dl ( $\text{H}_2\text{O}$  only)
4. Phenol Color Reagent (2 liters)
  - a.) Dissolve 0.1g sodium nitroprusside ( $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO} \cdot 2\text{H}_2\text{O}$ ) in 1 liter of distilled  $\text{H}_2\text{O}$
  - b.) Add 20.0 g dry phenol ( $\text{C}_6\text{H}_5\text{OH}$ ). Mix and dissolve
  - c.) Bring volume up to 2 liters with distilled  $\text{H}_2\text{O}$
  - d.) Store in brown glass bottle at 2-10°C. Stable for 6 months when protected from light
5. Alkaline Hypochlorite Reagent (2 liters)
  - a.) Dissolve 10g sodium hydroxide (NaOH) in about 1300ml distilled  $\text{H}_2\text{O}$
  - b.) Add 75.7g Disodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ ) and dissolve with mild heating and mixing

- c.) After cooling, add 100ml of commercial bleach (“Clorox” – 5.25% sodium hypochlorite).  
MAKE SURE BLEACH IS FRESH! Mix
- d.) Bring volume up to 2 liters with distilled H<sub>2</sub>O
- e.) Filter through Whatman #1 filter paper and store in polyethylene bottle at 2-10°C protected from light. Stable for 6 months

6. Ammonia Standard Solution

- a.) Stock solution is 32mg/dl
- b.) To make with ammonium chloride (NH<sub>4</sub>Cl):

$$\begin{array}{l}
 N=14.01 \\
 H= 1.01 \quad \underline{NH_3} \quad \underline{17.04} = 31.86\% \\
 Cl=\underline{35.45} \quad \quad \quad \underline{NH_4Cl} \quad 53.49 \\
 53.48 \text{ (Formula weight)} \\
 0.3186X=0.032g \\
 X=0.10045g \text{ NH}_4\text{Cl}
 \end{array}$$

**So to make 1 liter use 1.0045g NH<sub>4</sub>Cl**

- c.) Dissolve 1.0045g NH<sub>4</sub>Cl in 800ml distilled H<sub>2</sub>O
- d.) Drop pH down to 2 with dilute (2N) HCl
- e.) Bring volume up to 1 liter with distilled H<sub>2</sub>O
- f.) Use Stock Solution to make levels: 32 mg/dl
  - 16 mg/dl
  - 8 mg/dl
  - 4 mg/dl
  - 2 mg/dl
  - 1 mg/dl
  - 0 mg/dl (H<sub>2</sub>O only)

**NOTE: Be sure to handle chemicals in designated fume hood.**

Principle:

Ammonia reacts with alkaline hypochlorite and phenol in the presence of a catalyst (sodium nitroprusside) to form indophenol (blue) (Berthelot reaction). The concentration of ammonia is directly proportional to the absorbance of indophenol which is measured spectrophotometrically.

Sample Collection:

1. Rumen Fluid
  - a.) Strain rumen fluid through 2 layers of cheesecloth (Grade 50 or finer)
  - b.) Add 2ml of fresh 25% metaphosphoric acid to 8ml of strained rumen fluid. Cap and invert to mix
  - c.) Store samples in freezer (-20°C) if not analyzing right away
  - d.) Prior to Analysis, centrifuge samples at 11-12,000 x g for 20 minutes. Use supernatant for analysis
2. Blood
  - a.) Centrifuge whole blood to collect serum or plasma
  - b.) Store in freezer until analysis

Procedure

**For BUN start here**

1. Vortex serum (or plasma)
2. Using “Digiflex” automatic pipette, dispense 20 µl sample or standard plus 20 µl H<sub>2</sub>O into pre-

- labeled test tubes. Run in duplicate
3. Add 500µl of Urease solution to each tube. Use a repeating pipette (Eppendorf)
  4. Incubate in a 37°C water bath for 10 minutes. Skip to step #7

**For Rumen Ammonia start here**

5. Vortex rumen fluid
6. Using “Digiflex” automatic pipette, dispense 40µl rumen fluid or standard plus 40µl H<sub>2</sub>O into pre-labeled test tubes. Run in duplicate
7. Pipette 2.5ml phenol reagent into each tube
8. Pipette 2.0ml alkaline hypochlorite reagent into each tube. Vortex
9. Incubate in a 37°C water bath for 10 minutes
10. Pipette 300µl from each tube into wells of a microtiter plate
11. Read absorbance on plate ready at 550 nanometers. (Any wavelength between 500 and 660nm can be used). If color is too intense, a lower wavelength can be used or samples can be diluted 1:1 with H<sub>2</sub>O and read again
12. Calculate standard curve using linear regression:
  - x = absorbance y = concentration
  - a. Substitute sample absorbances (x) into equation. For rumen ammonia samples, multiply result by 1.25 to correct for the dilution with metaphosphoric acid.
  - b. Normal rumen ammonia levels expected to be in the range of 1-25 mg/dl. Normal ruminant BUN’s expected to be in the range of 8-25mg/dl

Validation (for BUN)

| <u>Standard</u> | <u>Absorbance</u> |            |
|-----------------|-------------------|------------|
| 0 mg/dl         | .048              | Y = mx + b |
| 3.75            | .14               | b = -2.26  |
| 7.5             | .23               | m = 42.7   |
| 15              | .415              | r = .9997  |
| 30              | .75               |            |

| <u>Standard</u> | <u>Absorbance</u> |            |
|-----------------|-------------------|------------|
| 0 mg/dl + Serum | .265              | y = mx + b |
| 3.75 + Serum    | .37               | b = -13.2  |
| 7.5 + Serum     | .46               | m = 46.3   |
| 15 + Serum      | .625              | r = .998   |
| 30 + Serum      | .92               |            |

| <u>Standard</u> | <u>Absorbance</u> |           |
|-----------------|-------------------|-----------|
| 0 mg/dl +Urea   | .258              | y=mx + b  |
| 3.75 +Urea      | .35               | b = -11.2 |
| 7.5 +Urea       | .445              | m = 42.6  |
| 15 +Urea        | .625              | r = .9997 |
| 30 +Urea        | .963              |           |

Control (Sigma Accutrol Catalog No. A2034)  
 Value should be between 11-17 mg/dl. We got 16.3

Validation (for Rumen Ammonia)

| <u>Standard*</u> | <u>Absorbance</u> |              |
|------------------|-------------------|--------------|
| 0 mg/dl          | .003              | $y = mx + b$ |
| 1                | .047              |              |
| 2                | .092              | $b = -0.108$ |
| 4                | .192              | $m = 21.37$  |
| 8                | .392              | $r = .9999$  |
| 16               | .762              |              |
| 32               | 1.496             |              |

\* = Used 40 $\mu$ l standard + 40 $\mu$ l H<sub>2</sub>O

| <u>Standard</u>      | <u>Absorbance</u> |              |
|----------------------|-------------------|--------------|
| 0 mg/dl +Rumen Fluid | .121              | $y = mx + b$ |
| 1 +Rumen Fluid       | .187              |              |
| 2 +Rumen Fluid       | .234              | $b = -2.898$ |
| 4 +Rumen Fluid       | .328              | $m = 21.33$  |
| 8 +Rumen Fluid       | .509              | $r = .9999$  |
| 16 +Rumen Fluid      | .895              |              |
| 32 +Rumen Fluid      | 1.631             |              |