

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. Reper 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
4. Perform procedure in designated fume hood

Reagents:

1. Urease Buffer (BUN only)
 - a.) Dissolve 7.11g Sodium phosphate (Na_2HPO_4) in 400-500ml boiling (CO_2 free) distilled H_2O
 - 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 H_2O
 - d.) pH should be 7 (adjust if necessary)
2. Buffered Urease Solution (BUN only) (0.4 mg/ml)
 - a.) Add 20mg 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 (H_2O 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 H_2O
 - 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 H_2O
 - 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 10.0 g sodium hydroxide (NaOH) in about 1300ml distilled H_2O
 - 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 100 ml of commercial bleach ("Clorox" – 5.25% sodium hypochlorite). MAKE SURE BLEACH IS FRESH! Mix
 - d.) Bring volume up to 2 liters with distilled H₂O.
 - 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 32 mg/dl
 - b.) To make with ammonium chloride (NH₄Cl):

$$\begin{array}{rcl}
 \text{N} & = & 14.01 \\
 \text{H} & = & 1.01 \\
 \text{Cl} & = & 35.45 \\
 \hline
 & & 53.48 \text{ (Formula weight)}
 \end{array}
 \qquad
 \begin{array}{rcl}
 \text{NH}_3 & & 17.04 \\
 \hline
 \text{NH}_4\text{Cl} & & 53.49
 \end{array}
 = 31.86\%$$

$$0.3186X = 0.032\text{g}$$

$$X = 0.10045\text{g NH}_4\text{Cl}$$

So to make 1 liter use 1.0045g NH₄Cl

- c.) Dissolve 1.0045g NH₄Cl in 800ml distilled H₂O
- d.) Drop pH down to 2 with dilute (2N) HCl
- e.) Bring volume up to 1 liter with distilled H₂O
- f.) Use Stock Solution to make levels: 32mg/dl
16mg/dl
8mg/dl
4mg/dl
2mg/dl
1mg/dl
0mg/dl (H₂O only)

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 H₂O 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 H₂O 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 H₂O 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)

Standard	Absorbance
0 mg/dl	0.048
3.75	0.14
7.5	0.23
15	0.415
30	0.75

$$Y = mx + b$$

$$b = -2.26$$

$$m = 42.7$$

$$r = .9997$$

Standard	Absorbance
0 mg/dl + Serum	0.265
3.75 +Serum	0.37
7.5 +Serum	0.46
15 +Serum	0.625
30 +Serum	0.92

$$y = mx + b$$

$$b = -13.2$$

$$m = 46.3$$

$$r = 0.998$$

Standard	Absorbance
0 mg/dl +Urea	0.258
3.75 +Urea	0.35
7.5 +Urea	0.445
15 + Urea	0.625
30 +Urea	0.963

$y = mx + b$
 $b = -11.2$
 $m = 42.6$
 $r = 0.9997$

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

Validation (for Rumen Ammonia)

Standard*	Absorbance
0 mg/dl	0.003
1	0.047
2	0.092
4	0.192
8	0.392
16	0.762
32	1.496

$y = mx + b$
 $b = -0.108$
 $m = 21.37$
 $r = .9999$

* = Used 40µl standard + 40µl H₂O

Standard	Absorbance
0 mg/dl +Rumen Fluid	0.121
1 +Rumen Fluid	0.187
2 +Rumen Fluid	0.234
4 +Rumen Fluid	0.328
8 +Rumen Fluid	0.509
16 +Rumen Fluid	.895
32 +Rumen Fluid	1.631

$y = mx + b$
 $b = -2.898$
 $m = 21.33$
 $r = .9999$