USING AN AMMONIUM PROBE TO PREDICT RUMINAL AMMONIA CONCENTRATIONS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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This study examined the possibility of developing a prediction equation for ruminal ammonia through comparing ruminal ammonia concentrations to ruminal ammonium levels. Ruminal ammonia concentrations were measured the standard way, using a catalyzed indophenol colorimetric reaction, and ammonium levels were measure using the HQ440D Laboratory Ammonium (NH_4^+) Ion Meter Package with ISENH4181 Ion Selective Electrode from HACH. In addition to these base measurements, the pH of each sample was measured to determine if the acid/base equilibrium was the driving factor behind the relationship. The ammonia concentration and ammonium concentrations were compared to determine what type of relationship there is between them in a ruminal environment. Since the ruminal ammonia levels are so low, 1-12 mmol, the pH does not appear to have any effect on the relationship. The initial data set showed a linear relationship between the two, but after running more trials, it was seen that a different equation was produced each time. The equations from all four trials had different slopes and different Y-intercepts. Without being able to determine a consistent relationship between ruminal ammonia and ammonium, a prediction equation was not able to be made.

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CHAPTER 1

INTRODUCTION

The question of interest in this research is whether or not it is possible to create a prediction equation that can be used to determine ruminal ammonia concentration based off of ammonium concentration. Being able to determine ruminal ammonia in this way would require less work and resources than the current method, which is a catalyzed indophenol colorimetric reaction. This current method is both sensitive and reproducible, meaning this method can detect as low as 0.3 p.p.m of ammonia in the color developed solution and the standard deviation was 0.03 p.p.m at a level of 1 p.p.m of ammonia in the color developed solution (Bolleter et.al, 1961). The goal of this experiment is to be able to have similar levels of sensitivity and reproducibility. Once the concentration of ruminal ammonia is found, one can determine if they are over or under feeding protein. In the world of marine biology, ammonia concentrations are extremely important. This is because ammonia is toxic to marine life, even in low concentrations. Because of this, lots of work has been done to allow the ammonia concentration in water to be easily calculated based off of a measure of total free nitrogen (Emerson et.al, 1975). These calculations become much more complicated when the measurements are not being made in freshwater, so equations that take into account salinity have been found. Salinity effects the acid/base equilibrium because a variety of inorganic salts can act as either an acid or a base when they dissociate in solution. These equations show how a variety of things affect ammonia concentration. This is extremely important to us, because the environment of the rumen is very far from being similar to water. The purpose of this experiment will be to figure out how the rumen environment effects the ammonia/ammonium equilibrium, and if these differences can be taken into account within a prediction equation.

CHAPTER 2

METHODS

Sample Collection

Rumen fluid was collected from one ruminally cannulated steer and preserved by adding 1 mL of 1N HCl to 9 mL of rumen fluid, which was then frozen to be used later. With the rumen fluid being collected from one steer, it was necessary to manually adjust the ammonia concentrations of the rumen fluid. The ammonia assay was run on the base rumen fluid, and the ammonia concentration was found to be 7.28 mmol. From this, it was decided the best way to vary the ammonia concentrations was to mimic the ammonia levels in the standards of the ammonia assay. The base rumen fluid was then diluted down to 1 mmol, 2 mmol, 4 mmol, and 6 mmol ammonia concentrations using 0.1N HCl. In order to get the upper end of the spectrum, ammonium sulfate was added to the rumen fluid to get to 8 mmol, 10 mmol and 12 mmol ammonia concentrations. These adjusted rumen fluid samples were then refrozen and stored to be used later. The initial collection of rumen fluid was used for the first two trials. A second collection of rumen fluid was necessary for the third and fourth trials. The second collection of rumen fluid was found to have an ammonia concentration of 0.5 mmol. This rumen fluid was then adjusted to the same values as above by adding various amounts of ammonium sulfate to each 25mL sample.

pH Measurement

The pH probe was calibrated according to the probe manual and then the pH of each adjusted sample was measured and recorded.

Catalyzed Indophenol Colorimetric Reaction (Ammonia Assay)

The standard operating procedure for ammonia analysis of rumen fluid was followed for all samples. The operating procedure is adopted from experiments by Broderick et al, (1980).

Ammonium Probe Measurement

The ammonium probe was calibrated each day that measurements are made in order to ensure that the values are accurate. The calibration method can be found in the manual of the probe being used, as each probe is slightly different. The ammonium probe requires 25 mL of sample in order to give a reading, so for the diluted sample trials 5 mL of each sample was added to 20 mL of 0.1N HCl, and for the undiluted sample trials 25 mL of straight sample was used. The particular probe and meter that was used in this experiment was the HQ440D Laboratory Ammonium (NH_4^+) Ion Meter Package with ISENH4181 Ion Selective Electrode from HACH, and it required an ionic strength adjuster to be added to the now 25 mL of sample just before the measurement was made. If there is sediment in the rumen fluid, it is necessary to allow it all to settle to the bottom before the ionic strength adjuster is added to the solution so that the sediment does not affect the probe. While taking the measurement with the probe, it is necessary to gently swirl the probe in the sample to make sure there is no air bubble at the bottom of the probe.

CHAPTER 3

RESULTS

Data Set for Ammonia Assay

The results for the ammonia assays were used as something to compare the ammonium probe values to in order to create the prediction equation. The samples were adjusted in order to get variability in the ammonia concentrations so as to be able to test the probe at various ammonia concentrations. While the data collected from the ammonia assay was not directly looked at, it served as a baseline to compare the probe values to. The first trial had samples ranging from 0.965 mmol NH_3 to 11.65 mmol NH_3, the second trial had samples ranging from 0.965 mmol NH_3 to 12.81 mmol NH_3, the third trial had samples ranging from 0.683 mmol NH_3 to 11.08 mmol NH_3, the fourth trial had samples ranging from 2.46 mmol NH_3 to 12.88 mmol NH_3. Table 1 below shows the ammonia values tested during each trial.

Table 1. Data set for Ammonia Assay

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**
1 mmol Adjustment	0.965	0.965	0.683	
1 mmol Duplicate	0.986	0.921	0.705	
2 mmol Adjustment	2.05	2	1.68	2.46
2 mmol Duplicate	2.19	2.07	1.63	2.49
4 mmol Adjustment	4.11	4.76	3.43	4.48
4 mmol Duplicate	4.16	4.59	3.54	4.66
6 mmol Adjustment	6.27	5.13	4.57	6.98
6 mmol Duplicate	6.17	5.3	4.48	6.8
8 mmol Adjustment	7.88	9.14	12.25	8.61
8 mmol Duplicate	8.43	9.13	12.1	8.55
10 mmol Adjustment	9.89	12.49	9.37	10.91
10mmol Duplicate	10.19	12.81	9.72	10.94
12 mmol Adjustment	11.04		11.07	12.93
12 mmol Duplicate	11.65		11.08	12.88

Data Set for Ammonium Probe

The ammonium probe data is the integral part of making the prediction equation. The first trial had values ranging from 0.859 mmol NH_4 to 11.33 mmol NH_4, the second trial had values ranging from 1.89 mmol NH_4 to 15.56 mmol NH_4, the third trial had values ranging from 7.08 mmol NH_4 to 32.0 mmol NH_4, and the fourth trial had values ranging from 5.54 mmol NH_4 to 16.74 mmol NH_4. Table 2 below shows all the ammonium values measured for each trial.

Table 2. Data Set for Ammonium Probe

	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**
1 mmol NH3 Adjustment	0.859	1.89	7.08	
1 mmol NH3Duplicate	0.837	1.93	6.92	
2 mmol NH3 Adjustment	1.72	2.59	9.54	5.54
2 mmol NH3 Duplicate	1.68	2.94	9.26	5.56
4 mmol NH3 Adjustment	3.61	5.73	12.44	7.91
4 mmol NH3 Duplicate	3.52	5.75	12.36	7.88
6 mmol NH3 Adjustment	6.91	8.07	14.81	10.39
6 mmol NH3 Duplicate	6.81	8.07	14.62	10.31
8 mmol NH3 Adjustment	9.48	12.22	26.52	12.18
8 mmol NH3 Duplicate	9.48	12.59	25.92	12.15
10 mmol NH3 Adjustment	10.89	15.41	26.67	14.96
10mmol NH3 Duplicate	10.81	15.56	26.37	14.96
12 mmol NH3 Adjustment	11.41		32	16.89
12 mmol NH3 Duplicate	11.33		31.11	16.74

Prediction Equation

The ammonia assay values and the ammonium probe values were then compared to establish the relationship between the two and a prediction equation was made based off of the individual trials. The equation from the first trial was $y = 1.0994x - 0.3696$ with an R^2 value of 0.9804**,** the equation from the second trial was $y = 1.2079x + 0.7529$ with and R^2 value of 0.9829, the

equation from the third trial was y = 2.0512x + 5.6139 with an R^2 value of 0.989, and the equation from the fourth trial was y = 1.0887x + 2.8803 with an R^2 value of 0.9991. Figure 1 below shows the graphs and subsequent equation for the trials with dilute samples and Figure 2 below shows the graphs and subsequent equation for the trials with undiluted samples.

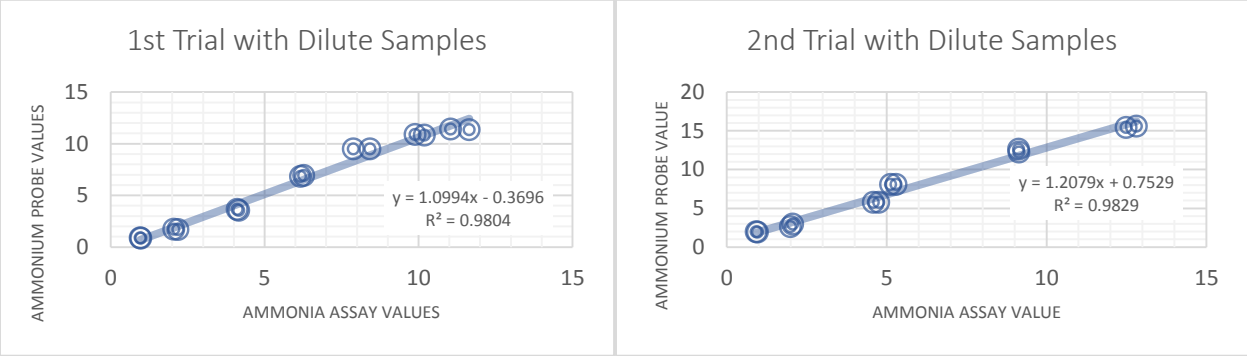

Figure 1. Graphs and equations for Dilute Trials

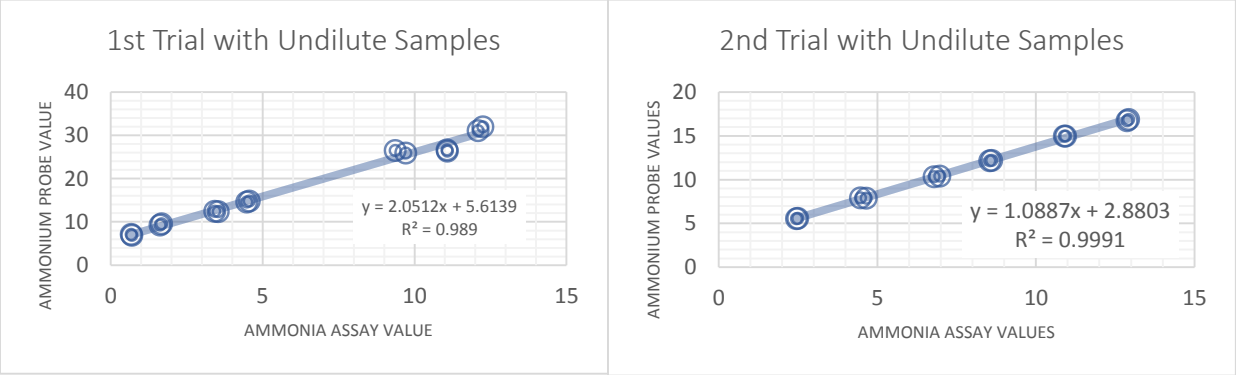

Figure 2. Graphs and Equations for Undilute Trials

CHAPTER 4

CONCLUSION

From the results discussed above, it can be seen that there was significant amounts of variability in the ammonium probe values between each individual trial. The ammonium probe values of samples that were adjusted to the same ammonia value should have been relatively close to one another. This leads to the belief that there is something either in the sample that is interfering with the measurements, or there is an inconsistency within the measuring methods. The initial thought was that the pH of the samples could have been a cause for the variability, but with all of the samples having a pH below 7.25 nearly all of the ammonia was in the form of NH_4. Another issue that was run into, is that adding the ionic strength adjuster (ISA) makes the standards and samples unstable (Paparone, 2017), and therefore neither can be used or measured more than once with any accuracy. This made it impossible to recheck the samples whose values seemed to be off compared to the rest. Unlike pH measurements, temperature differences are not easily accounted for with ion measurements, so any samples that are not the same temperature will subsequently differ in their readings (Paparone, 2017). All of these issues point to there being a flaw in the measuring method. Ammonium probe values were taken on a variety of different days, which could have led to significant temperature differences, especially when considering that samples had to be thawed out. The ammonium probe used takes a temperature measurement while it is measuring the ammonium levels and after looking back at the temperatures of the samples, there is some variability from trial to trial. This shows that not all the samples were allowed to fully thaw to room temperature before measurements were made. The samples for one trial were measured at the same time, so there is little intra-trial variability, but since each trial was measured on different days this led to the significant inter-trial variability. In order to make

a prediction equation for ruminal ammonia using the ammonium probe, a full set of new trials would have to be run. Instead of taking ammonium probe measurements as each individual set of trial samples has undergone the ammonia assay, all ammonium probe measurements, for every sample, should be made at the same time. Before the measurements are made, it should be made sure that each sample has fully come to room temperature. It would also be beneficial to have an excess of each adjusted sample, so as to be able to run multiple trials on the same sample to take into account that samples cannot be rerun once the ISA has been added. With all of this in mind, it would be possible to come up with a prediction equation, but the equation would only be useful if used under the exact same conditions. This means that a lab could come up with their own prediction equation which would allow them to speed up the process of gathering ruminal ammonia concentrations, but that equation would likely not be accurate for any other lab. In order to come up with an equation that works under a variety of situations one would have to determine the relationship between the temperature changes and the changes in the ammonium probe measurements as this is the most likely condition to change.

REFERENCES

Baetz, A. L., T. D. Faidley, and M. J. Allison. Automated enzymatic method for the determination of ammonia: application to rumen fluid, gut fluid, and plasma. Applied and Environmental Microbiology 38:212–215, 1979.

Bolleter, W. T., C.J. Bushman, & P.W. Tidwell. Spectrophotometric determination of ammonia as indophenol. Analytical Chemistry, *33*(4):592-594, 1961.

Broderick, G.A. and J.H. Kang, 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media1. Journal of dairy science, 63(1), pp.64-75.

Emerson, K., R.C. Russo, R.E. Lund, & R.V. Thurston. Aqueous ammonia equilibrium calculations: effect of pH and temperature. Journal of the Fisheries Board of Canada, *32*(12): 2379-2383, 1975.

Paparone, Frank. 2017. What You Should Know About ISE Measruements of Ammonia. Cole-Parmer. www.coleparmer.com/tech-article/ise-measurements-of-ammonia.

Souza, N.K.P., E. Detmann, S.C. Valadares Filho, V.A.C Costa, D.S Pina, D.I. Gomes, A.C Queiroz, & H.C. Mantovani. 2013. Accuracy of the estimates of ammonia concentration in rumen fluid using different analytical methods. Arquivo Brasileiro de Medicina Veterináriae Zootecnia, *65*(6): 1752-1758. https://dx.doi.org/10.1590/S0102-09352013000600024