

Accuracy of the estimates of ammonia concentration in rumen fluid using different analytical methods

[Exatidão das estimativas de concentração de amônia em fluido ruminal utilizando diferentes métodos analíticos]

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ABSTRACT

The accuracy of two different methods in measuring the ammonia nitrogen (N-NH₃) concentration in rumen fluid were evaluated: a catalyzed indophenol colorimetric reaction (CICR) and the Kjeldahl distillation (KD). Five buffered standard solutions containing volatile fatty acids, true protein, and known ammonia concentrations (0, 3, 6, 12, and 24 N-NH₃ mg/dL) were used to simulate rumen fluid. Different ratios (10:1, 7.5:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:7.5, and 1:10) of a potassium hydroxide solution (KOH, 2 mol/L) to standard solutions were evaluated by the KD method. The accuracy of each method was evaluated by adjusting a simple linear regression model of the estimated N-NH₃ concentrations on the N-NH₃ concentrations in the standard solutions. When the KD method was used, N-NH₃ was observed to be released from the deamination of true protein (P<0.05), and an incomplete recovery of N-NH₃ was observed (P<0.05), except for 7.5:1 and 5:1 ratios of KOH solution to standard solutions (P>0.05). The estimates of the N-NH₃ concentration obtained by the CICR method were found to be accurate (P>0.05). After the accuracy evaluation, ninety-three samples of rumen fluid were evaluated by the CICR and KD methods (using the 5:1 ratio of KOH solution to rumen fluid sample), assuming that the CICR estimates would be accurate. The N-NH₃ concentrations obtained by the two methods were observed to be different (P<0.05) but strongly correlated (r = 0.9701). Thus, it was concluded that the estimates obtained by the Kjeldahl distillation using a 5:1 ratio of KOH solution to rumen fluid sample can be adjusted to avoid biases. Furthermore, a model to adjust the N-NH₃ concentration is suggested.

Keywords: ammonia nitrogen, indophenol colorimetric reaction, Kjeldahl distillation

RESUMO

Avaliou-se a exatidão de dois métodos para mensuração da concentração de nitrogênio amoniacal (N-NH₃) em fluido ruminal: reação colorimétrica catalisada por indofenol (RCCI) e destilação de Kjeldahl (DK). Cinco soluções-tampão contendo ácidos graxos voláteis, proteína verdadeira e concentrações conhecidas de amônia (0, 3, 6, 12 e 24 N-NH₃ mg/dL) foram utilizadas para simular o fluido ruminal. Diferentes relações (10:1; 7,5:1; 5:1; 2,5:1; 1:1; 1:2,5; 1:5; 1:7,5 e 1:10) entre a solução de hidróxido de potássio (KOH, 2 mol/L) e as soluções-padrão foram avaliadas no método DK. A exatidão de cada método foi avaliada por intermédio do ajustamento de um modelo de regressão linear simples das concentrações estimadas de N-NH₃ sobre as concentrações reais de N-NH₃ nas soluções-padrão. Com a utilização da DK observou-se que N-NH₃ foi liberada a partir da deaminação de proteína verdadeira (P<0,05). Adicionalmente, a utilização da DK promoveu recuperação incompleta da N-NH₃ (P<0,05).

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com exceção das razões entre a solução de KOH e as soluções-padrão de de 7,5:1 e de 5:1 ($P>0,05$). As estimativas de concentração de amônia obtidas por intermédio do método de RCCI foram exatas ($P>0,05$). Após a avaliação da exatidão, noventa e três amostras de fluido ruminal foram avaliadas por intermédio dos métodos de RCCI e de DK (neste caso usando a razão entre solução de KOH e fluido ruminal de 5:1). Nesta avaliação assumiu-se que as estimativas obtidas com a RCCI seriam exatas. Observou-se que as concentrações de N-NH₃ obtidas pelos dois métodos foram diferentes ($P<0,05$), mas fortemente correlacionadas ($r = 0,9701$). Concluiu-se que as estimativas obtidas com a DK utilizando-se razão entre a solução de KOH e a amostra de fluido ruminal de 5:1 poderiam ser ajustadas para correções de vieses. Um modelo para realização desse ajustamento foi sugerido.

Palavras-chave: destilação de Kjeldahl, nitrogênio amoniacal, reação colorimétrica de indofenol

INTRODUCTION

Several parameters have been suggested for evaluating the availability of dietary nitrogenous compounds in the rumen. To date, the concentration of the ammonia nitrogen (N-NH₃) has been used as a qualitative reference to understand the adequacy of the rumen environment according to the microbial activity on fibrous carbohydrates (Detmann *et al.*, 2009). This strategy is possibly associated with the fact that N-NH₃ is the preferred nitrogen source for the growth of fibrolytic microorganisms (Russell, 2002).

Thus, considering the relevance of N-NH₃ in ruminant nutrition, the methods used to evaluate its concentration in the ruminal fluid ought to provide accurate estimates.

Chaney and Marbach (1962) have proposed a method for N-NH₃ evaluation in biological fluids that is based on a catalyzed indophenol colorimetric reaction (CICR), and this method is intensely used in microbiological assays (e.g., Thomas and Russell, 2004). Alternatively, Fenner (1965) has established the theoretical basis for N-NH₃ evaluation in rumen fluid by steam distillation in the presence of a potassium hydroxide solution. This basis has been adapted for use in the Kjeldahl distillation (KD) procedure, which has been used in several ruminant nutrition assays (e.g., Detmann *et al.*, 2009; Souza *et al.*, 2010; Costa *et al.*, 2011). Nonetheless, no study has compared the accuracy of these methods in the estimation of N-NH₃ contents.

Therefore, the objective of this work was to evaluate the accuracy of N-NH₃ concentration estimates in rumen fluid with the KD and the CICR methods.

MATERIAL AND METHODS

The experiment was conducted at the Animal Nutrition Laboratory of the Animal Science Department at the Universidade Federal de Viçosa in Viçosa, Brazil.

To evaluate the accuracy of the methods, five standard solutions containing different N-NH₃ concentrations were used to evaluate the accuracy of the estimates obtained from the two methods. Those five solutions were produced to simulate rumen fluid characteristics.

The basal solution was constituted by a buffer solution whose pH had previously been adjusted to 6.8 by flushing with CO₂ (McDougall, 1949). Acetic acid (Sigma-Aldrich 320099; 60mmol/L), propionic acid (Sigma-Aldrich 402907; 30mmol/L) and butyric acid (Sigma Aldrich B103500; 10mmol/L) were added to the solution to represent the volatile fatty acids (VFA) present in the rumen fluid. Additionally, casein hydrolysate (Fluka 2209; 3g/L) was added to simulate the true protein content of the rumen fluid, and thimerosal (Sigma 8784; 50mg/L) was used to prevent microbial growth. The solution was divided into five aliquots, and known quantities of ammonium chloride (NH₄Cl, PA, ACS, Vetec 113) were added to provide 0, 3, 6, 12, and 24 mg N-NH₃/dL.

The evaluation of the N-NH₃ concentration by the CICR method was performed according to the descriptions of Chaney and Marbach (1962). Aliquots of 10µL of the standard solutions were poured into glass tubes, and 1.5mL of a phenol solution (50g/L of phenol and 0.25 g/L of sodium nitroprusside) plus 1.5 mL of sodium hypochlorite solution (16.9mL/L of sodium hypochlorite and 25g/L of sodium hydroxide)

were added. The tubes were stirred by vortexing and kept in a water bath at 39°C for 15 minutes. The absorbance was then read at 630nm in a spectrophotometer UV/Visible BEL Photonics 2000 UV. A standard curve was generated according to Chaney and Marbach (1962).

To evaluate the N-NH₃ concentration according to the KD method, 5-mL aliquots of standard solutions were poured into glass tubes that were coupled in a Kjeldahl distillator (Tecnal® TE 036/1). Potassium hydroxide solution (KOH, 2mol/L; Fenner, 1965) was then added, and the material was distilled in a boric acid solution (40g/L). The solution obtained from the distillation (approximately 100mL) was titrated with hydrochloric acid (HCl; 0.005N). Methyl red and bromocresol green were added to the boric acid solution and used as indicators. Different ratios of the KOH solution to the standard solutions were evaluated (10:1, 7.5:1, 5:1, 2.5:1, 1:1, 1:2.5, 1:5, 1:7.5, and 1:10)

The N-NH₃ concentration was estimated as follows:

$$N - NH_3 = \frac{V \times 0.005 \times f \times 14 \times 100}{A} \quad (1),$$

where N-NH₃ = the ammonia nitrogen concentration (mg/dL), V = the volume of hydrochloric acid (mL), f = the factor for the correction of the hydrochloric acid concentration obtained with a Na₂CO₃ solution (0.005 N), 14 = the atomic weight of nitrogen, and A = the aliquot volume (mL).

Five replicates for each standard solution were evaluated by both the CICR method and KD method at different KOH:standard solution ratios.

After accuracy evaluation, a laboratorial assay was performed to compare the estimates obtained by the methods in rumen fluid samples.

Ninety-three rumen fluid samples were obtained from cattle under grazing conditions, receiving nitrogen supplementation (true protein and/or non-protein nitrogen) during 2008. Those samples were taken at different times during the day and filtered through three layers of cheesecloth. For each sample, a 100-mL aliquot was then separated, fixed with 2.5mL H₂SO₄ (1:1) and frozen at -20°C (Costa et al., 2011).

After thawing, a 50-mL aliquot of each sample was poured into a centrifuge tube, 0.5mL of a trichloroacetic acid solution (100g/L) was added, and the sample was slowly stirred. The material was allowed to settle at room temperature for 30 minutes after which, the material was centrifuged at 1,000 × g for 10 minutes. The supernatant was analyzed in duplicate by the CICR method and KD method using the KOH:rumen fluid ratios of 5:1 and 1:10.

The accuracy of the N-NH₃ concentrations obtained by the CICR and KD methods was evaluated by adjusting a simple linear regression equation of estimated values (dependent variable) on the N-NH₃ content in standard solutions (independent variable); the statistical analysis was conducted under the null hypotheses below:

$$H_0 : \beta_0 = 0 \quad (2a),$$

$$H_0 : \beta_1 = 1 \quad (2b),$$

where β_0 = intercept, and β_1 = slope.

The N-NH₃ contents were considered to be accurate when both of the null hypotheses were not rejected.

Additionally, the N-NH₃ contents in the rumen fluid samples were compared by adjusting a simple linear regression equation of the values estimated by the KD method (dependent variable) on the values estimated by the CICR method (independent variable). The statistical analysis was conducted under the null hypotheses expressed by equations (2a) and (2b). The estimates were considered to be similar if both of the null hypotheses were not rejected.

All of the statistical procedures were performed using the PROC REG of SAS (*Statistical Analysis System*, version 9.1) ($\alpha = 0.05$).

RESULTS

With the KD method, the intercept estimates were observed to be different from zero ($P < 0.05$) for all of the ratios of KOH solution to standard solutions (Table 1). Additionally, as the KOH proportion decreased, the estimates of the N-NH₃ concentrations in the blank standard solution (0 mg N-NH₃/dL) and the intercept estimates also decreased (Table 2 and Figure 1).

Accuracy of the estimates...

Table 1. Estimates of the linear regression parameters for the relationship between the N-NH₃ concentrations obtained by two methods (dependent variable, Y) and the true concentrations of N-NH₃ in the standard solutions (independent variable, X)

Method	Regression parameter ⁴		s _{X,Y} ⁵	r ²	P Value ⁶	
	Intercept	Slope			H ₀ : β ₀ = 0	H ₀ : β ₁ = 1
KD ^{1, 2}						
10:1	2.601±0.045	0.932±0.004	0.156	0.9996	<0.001	<0.001
7.5:1	2.489±0.179	0.962±0.018	0.590	0.9928	<0.001	0.053
5:1	2.284±0.242	0.976±0.025	0.798	0.9875	<0.001	0.358
2.5:1	2.097±0.034	0.905±0.003	0.116	0.9998	<0.001	<0.001
1:1	1.479±0.070	0.917±0.006	0.242	0.9991	<0.001	<0.001
1:2.5	1.053±0.053	0.888±0.004	0.179	0.9995	<0.001	<0.001
1:5	0.808±0.052	0.887±0.004	0.179	0.9995	<0.001	<0.001
1:7.5	0.693±0.055	0.891±0.005	0.186	0.9994	<0.001	<0.001
1:10	0.569±0.120	0.891±0.009	0.409	0.9975	<0.001	<0.001
CICR ³						
	0.688±0.369	0.995±0.029	1.264	0.9806	0.077	0.878

¹Kjeldahl distillation. ²Using different ratios of the KOH solution to the standard solutions (mL/mL). ³ Catalyzed indophenol colorimetric reaction. ⁴Estimate±standard error. ⁵Standard deviation of the relationship between X and Y. ⁶See more details about the hypotheses' test in Equation 2.

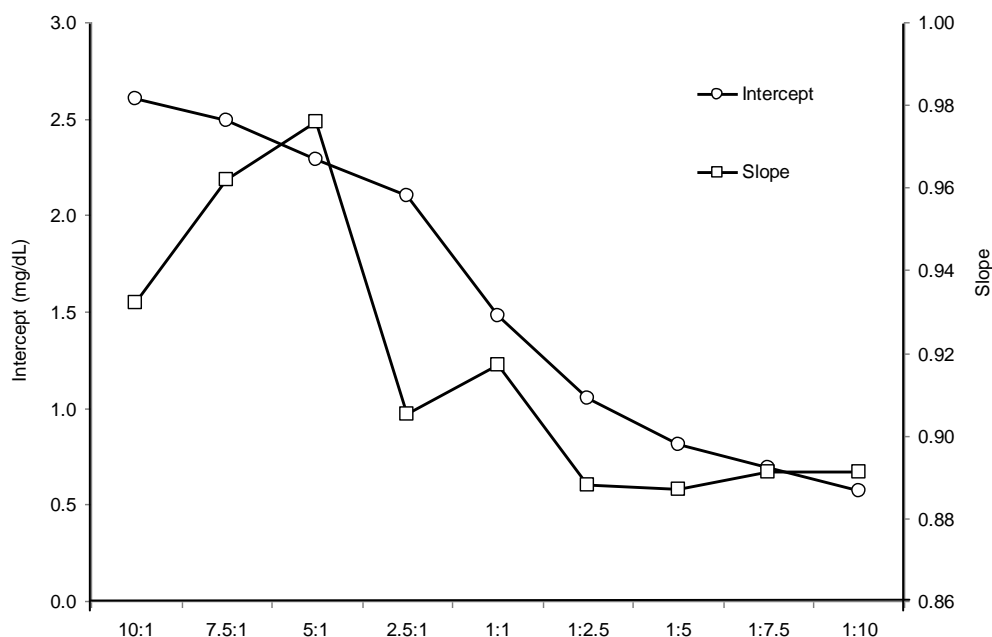


Figure 1. Estimates of the intercept and slope for the adjusted linear functions of the ammonia nitrogen concentrations obtained by the Kjeldahl distillation of standard solutions using different ratios of potassium hydroxide solution to standard solutions.

For the results obtained using the KD method, the slopes were found to be different from one ($P < 0.05$), except for at the 5:1 and 7.5:1 ratios of KOH solution to the standard solutions (Table 1 and Figure 1).

The estimates of the N-NH₃ concentration obtained by the CICR method were found to be accurate ($P > 0.05$; Table 1).

When rumen fluid samples were evaluated using the KD method, N-NH₃ was not detected at the 1:10 ratio of the KOH solution to the standard solutions.

The relationship between the N-NH₃ concentrations obtained by the CICR and KD (5:1 ratio) methods presented an interception that was different from zero and slope different from

one ($P < 0.05$). Therefore, the results obtained with these methods differed from each other (Figure 2).

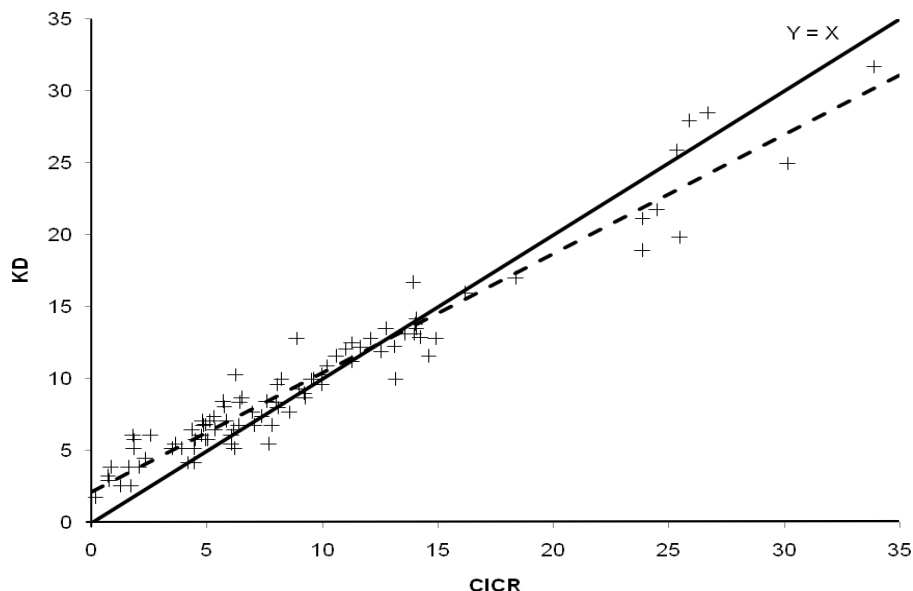


Figure 2. Relationship between the ammonia nitrogen concentrations (mg/dL) in rumen fluid samples estimated by the catalyzed indophenol colorimetric reaction (CICR) and Kjeldahl distillation (KD; 5:1) methods [$\hat{Y} = 2.109 \pm 0.254 + 0.828 \pm 0.022 \times X$; $s_{XY} = 1.47$; $r^2 = 0.9410$; the dashed line represents the least squares straight line].

DISCUSSION

The quantity of the KOH solution used in the KD method must be sufficient to neutralize even extremely high concentrations of VFA; yet, the solution should remain weak enough to prevent the deamination of major amino acids (Fenner, 1965). The neutralization of VFA is assumed to be necessary to release the volatile bases. In other words, it is necessary to convert NH_4^+ (that presents low volatility) into NH_3 , the volatile form of ammonia that can be easily steam-carried to the boric acid solution. In contrast, an excessive quantity of KOH can cause the hydrolysis of peptide bonds and the deamination of true protein, which would artificially increase the N-NH₃ concentration.

The pattern of intercept estimates in the KD method (Table 1) indicates that KOH promoted the release of ammonia nitrogen from amino acids, and the deamination intensity seems to be directly associated with the proportion of KOH in the distilled solution (Figure 1).

When evaluating the standard solutions, the slope of the adjusted functions can be interpreted as the recovery of N-NH₃ from the medium (Equation 2b). Except for the 5:1 and 7.5:1 ratios, an incomplete N-NH₃ recovery ($P < 0.05$) was found with the KD method (Table 1). The utilization of lower proportions of KOH suggested a gradual decrease in the recovery (Figure 1), which seemed to indicate an ineffective neutralization of the acids in the medium. However, the highest ratio (10:1) also caused an incomplete recovery (Table 1 and Figure 1), which could not be the result of a lack of VFA neutralization. The 10:1 ratio of the KOH solution to the standards caused the highest level of deamination, which was supported by the highest intercept estimate (Table 1). During the function adjustment, a high intercept estimate could force down the slope, which would explain the slope lower than one and the incomplete N-NH₃ recovery.

The colorimetric evaluation of the N-NH₃ concentration in biological fluids presents great

specificity and is based on the reaction of ammonia with phenol and sodium hypochlorite to produce indophenol, which is intensely blue in an alkaline medium (Bolleter *et al.*, 1961; Chaney and Marbach, 1962). In particular, the non-significant intercept (Table 1) reinforced the high specificity of the CICR method because the other nitrogenous compounds present in the medium did not directly interfere with the N-NH₃ quantification.

The comparative evaluation of the methods was performed under the assumption that the CICR method is accurate, which was supported by the results obtained with the standard solutions (Table 1). To evaluate the KD method, the 5:1

ratio of the KOH solution to the rumen fluid samples was adopted because it presented the highest estimate of N-NH₃ recovery between the ratios that produced a complete recovery with the standard solutions (Table 1 and Figure 1). In addition, the 1:10 ratio was evaluated because this ratio was recommended in the original method based on steam distillation (Fenner, 1965).

In spite of the results obtained with the standard solutions (Table 2), the N-NH₃ in the ruminal samples was not detected using the 1:10 ratio of KOH solution to rumen fluid, which seems to indicate deficiency with regard to acid neutralization.

Table 2. Average contents of ammonia nitrogen (N-NH₃, mg/dL) in the standard solutions obtained using the two analytical methods

Method	Standard solution (mg N-NH ₃ /dL) ⁴				
	0	3	6	12	24
KD ^{1 2}					
10:1	2.60±0.09	5.46±0.05	8.09±0.06	13.80±0.07	24.97±0.08
7.5:1	2.69±0.05	5.63±0.06	8.09±0.04	13.45±0.09	26.34±1.24
5:1	2.25±0.03	5.13±0.03	8.07±0.25	14.41±0.79	25.38±1.09
2.5:1	2.09±0.03	4.80±0.04	7.57±0.05	12.92±0.08	23.82±0.05
1:1	1.62±0.05	4.23±0.07	6.94±0.03	12.27±0.06	23.60±0.19
1:2.5	1.13±0.03	3.76±0.06	6.31±0.06	11.58±0.03	22.45±0.14
1:5	0.82±0.04	3.48±0.03	6.15±0.08	11.39±0.09	22.14±0.13
1:7.5	0.77±0.06	3.35±0.03	6.04±0.06	11.28±0.07	22.16±0.18
1:10	0.47±0.03	3.29±0.04	5.93±0.05	11.43±0.09	21.90±0.40
CICR ³	0.79±0.03	3.96±0.34	6.84±0.50	11.41±0.65	24.98±0.88

¹Kjeldahl distillation. ² Using different ratios of the KOH solution to the standard solutions (mL/mL). ³ Catalyzed indophenol colorimetric reaction. ⁴ Mean ± standard error.

The KD method (5:1 ratio) estimated the N-NH₃ concentrations with higher precision than the CICR method; however, those values were, on average, higher than the values obtained using the CICR method (Table 3).

The intercept for the relationship between the KD (5:1) and CICR methods (Figure 2) for the rumen fluid samples was similar to that obtained when standard solutions were evaluated by the KD method (5:1 ratio; Table 2), which emphasizes the occurrence of deamination of true protein. However, unlike the observations with the standard solutions, the KD did not allow the complete recovery of N-NH₃ in the rumen fluid samples (P<0.05; Figure 2). Such a pattern seems to be plausible because rumen fluid

samples are more complex than the standard solutions; the former comprises varied concentrations and proportions of VFA and presents compounds that were not included in the standard solutions (e.g., lactic acid and branched-chain volatile fatty acids).

Despite the differences, N-NH₃ concentrations estimated by the two methods were strongly correlated (r = 0.9701; Figure 2). Considering that there are situations where only the KD method could be used, N-NH₃ concentrations estimated by this method could be converted into values that are equivalent to those obtained by the CICR method (Figure 2). Such a conversion could be performed as follows:

$$N - NH_3(a) = \frac{[N - NH_3(K)] - [N - NH_3(d)]}{RR} = \frac{[N - NH_3(K)] - 2.11}{0.83} \quad (3),$$

where N-NH₃(a) = the adjusted N-NH₃ concentration in rumen fluid (mg/dL); N-NH₃(K) = the N-NH₃ concentration in rumen fluid estimated by the KD method using a 5:1 ratio of KOH solution to the rumen fluid sample (mg/dL); N-NH₃(d) = the N-NH₃ arising from the deamination of true protein (mg/dL); and RR = the recovery rate of N-NH₃ in the medium.

Table 3. Descriptive statistics for the ammonia nitrogen concentration (mg/dL) in the rumen fluid samples estimated by the catalyzed indophenol colorimetric reaction (CICR) and Kjeldahl distillation (KD) methods

Statistic	Method	
	CICR	KD ¹
Mean	9.31	9.82
Median	7.61	6.03
Maximum	33.88	31.70
Minimum	0.17	1.75
Standard deviation	7.07	6.03
Relative standard deviation (%)	75.9	61.4
n	93	

¹ Using a 5:1 ratio of the KOH solution to the rumen fluid sample.

CONCLUSIONS

The method based on a catalyzed indophenol colorimetric reaction produces accurate estimates of ammonia concentrations in rumen fluid. Conversely, the method based on the Kjeldahl distillation produces biased estimates due to the deamination of true protein and the incomplete recovery of ammonia nitrogen. However, these methods were observed to be strongly correlated. Therefore, the estimates obtained by the Kjeldahl distillation using a 5:1 ratio of potassium hydroxide solution (2mol/L) to the rumen fluid sample could be adjusted to avoid such biases. A model to adjust the ammonia concentration obtained by the Kjeldahl distillation is presented in this work.

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