Comparative evaluation of methods for ammonia (NH_3) determination in rumen fermentation of legume and concentrate samples

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Abstract. Ammonia (NH_3) is main product of ruminal protein degradation and serves as an indicator of nitrogen availability for microbial protein synthesis. Several analytical methods exist to quantify NH_3 concentration, but their reliability varies with the analytical approach and experimental setup. This study aimed to compare four NH_3 determination methods: Conway microdiffusion, Ion Selective Electrode (ISE), Indophenol, and Nessler, for evaluating *in vitro* fermentation of legumes and concentrate samples. Supernatants from legumes (n=7) and concentrates (n=7) after 4 h incubation, with four replicates each, were analyzed using these methods. Data were subjected to a 2x4 factorial randomized block design followed by Duncan test. Correlation and regression analyses were conducted between the Conway method and other methods across legume, concentrate, and mixed samples. The ISE method produced the highest NH_3 concentration (93.63 mM), while the Indophenol and Nessler methods yielded values closer to Conway. Significant correlations ($P<0.05$) were observed between Conway and other methods in mixed samples, with coefficients ranging from 0.5327 to 0.6663, but depended on substrate type. In conclusion, Indophenol and Nessler methods are comparable to Conway method in concentrate samples, but are inconsistent in legumes, while the ISE method requires further validation for NH_3 determination in rumen fermentation studies.

1 Introduction

Accurate quantification of ammonia (NH_3) is essential in rumen fermentation studies, as NH_3 represents the primary end-product of ruminal protein degradation and the main nitrogen source for microbial protein synthesis (MPS). Its concentration reflects the dynamic balance between protein breakdown and microbial utilization, making NH_3 a critical indicator of nitrogen efficiency in ruminant nutrition systems. Different feed ingredients generate different NH_3 patterns. Legume forages often contain secondary metabolites such as tannins

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that suppress ruminal degradability [1], whereas concentrate feeds generally produce higher NH_3 due to their greater rumen-degradable protein (RDP) content [2]. These differences highlight the importance of selecting reliable analytical methods for NH_3 determination in rumen fermentation studies.

The Conway microdiffusion technique has long been considered the reference technique due to its simplicity and robust accuracy [3]. This method relies on the passive diffusion of volatile NH_3 into an acid trap within a sealed chamber, followed by titrimetric quantification [4]. However, the lengthy incubation period and limited throughput make it impractical for experiments requiring rapid processing or large numbers of samples. Several alternative analytical approaches have been developed, including colorimetric assays (Nessler and Indophenol methods) and Ion Selective Electrode (ISE) techniques [4, 5]. The Nessler method involves the reaction of NH_4^+ with a mercuric iodide reagent, which forms a yellow-brown complex [6]. Despite good sensitivity, the use of mercury restricts its practicality. The Indophenol method is based on the reaction of phenol and hypochlorite with ammonium to form a blue indophenol chromophore, offering high sensitivity but requiring careful control of pH and reagent stability [7]. Meanwhile, ISE techniques measure the activity of NH_4^+ directly in solution through a selective membrane that responds to changes in ionic activity; the resulting potentiometric signal is proportional to NH_4^+ concentration [8]. Although rapid and reagent-free, ISE measurements are highly dependent on accurate calibration and stable ionic strength, both of which may vary substantially in samples.

Although these colorimetric and electrochemical techniques are well established in water quality monitoring, their performance for NH_3 measurement in rumen fermentation studies remains uncertain due to the complex matrix and different pH conditions, which pose additional analytical challenges. Colorimetric reactions may be inhibited by plant secondary metabolites or by turbidity, while ISE performance can deteriorate due to fluctuations in ionic strength or the presence of competing cations. These challenges justify comparative evaluations to identify methods that remain robust under rumen fermentation conditions and across different feed types. Therefore, this study aimed to compare four NH_3 determination methods: Conway microdiffusion, ISE, Indophenol, and Nessler, for evaluating *in vitro* fermentation of legumes and concentrate samples.

2 Materials and methods

2.1 Sample collection and *in vitro fermentation*

Samples consisted of legume (n=7) and concentrate feed (n=7). The legumes included *Leucaena leucocephala*, *Calliandra calothyrsus*, *Indigofera zollingeriana*, *Gliricidia sepium*, *Moringa oleifera*, *Acacia mangium*, and *Albizia chinensis*. Legume samples were oven-dried at 60 °C and ground to pass a 1 mm sieve. The concentrate feed comprised soybean meal, palm kernel meal, coconut meal, corn gluten meal, corn gluten feed, tofu dregs, and pollard. *In vitro* rumen fermentation was carried out following the method of Tilley and Terry [9]. Approximately 0.5 g of each sample was weighed into incubation tubes, followed by the addition of 10 mL rumen fluid and 40 mL McDougall's buffer. The mixture was flushed with CO_2 for 15 sec and sealed with a rubber stopper. Tubes were incubated in a shaking water bath at 39 °C for 4 h. After incubation, the samples were centrifuged at 3500 rpm for 15 min to obtain the supernatant, which was stored at –20 °C for subsequent NH_3 analysis.

2.2 NH_3 measurement

2.2.1 Conway method

Conway microdiffusion [3] was performed by coating the edges of the Conway dish with petroleum jelly to ensure an airtight seal. 1 mL of the sample was placed in one compartment, and 1 mL of Na_2CO_3 solution was added to the other compartment. The central well contained 1 mL of boric acid solution with an indicator. The dish was tightly closed and incubated for 24 h at room temperature (25 °C) to allow NH_3 to diffuse and react with the boric acid. After incubation, the boric acid solution was titrated with 0.005 N H_2SO_4 solution until the color changed from blue to red, indicating the titration endpoint.

2.2.2 Ion-selective electrode (ISE) method

The ISE method [5] used a Thermo Scientific Orion Star A214 (made in Singapore) electrode, which was rinsed with distilled water and dried prior to measurement. The electrode wasthen immersed in 30 mL of each standard solution (100, 200, and 400 ppm) and the sample supernatant until a stable potential was obtained. Voltages recorded from the standards were used to generate a regression equation, and NH_3 concentrations in the samples were calculated from the corresponding calibration curve.

2.2.3 Indophenol method

For the Indophenol method, NH_3 was quantified through its reaction with phenol and sodium hypochlorite, forming a blue indophenol complex [10]. For each determination, 1 mL of the supernatantwas combined with phenol reagent, hypochlorite reagent, and sodium nitroprusside, then brought to a final volume of 10 mL. The mixture was incubated for 30 min at 40 °C, and absorbance was subsequently measured at 630 nm using a UV–Vis spectrophotometer (LW Scientific UV-200-RS, USA). Ammonia concentration was determined from a calibration curve constructed using NH_3 standard solutions of 50, 100, 150, 200, and 300 ppm.

2.2.4 Nessler method

Samples (1 mL) from the supernatant of *in vitro* rumen fermentation were mixed with 0.1 mL of Nessler reagent and diluted to 10 mL with distilled water in test tubes. The mixture was homogenized using a vortex, and absorbance was measured at 425 nm with a UV–Vis spectrophotometer [7]. The NH_3 concentration was quantified from a calibration curve generated with NH_3 standards ranging from 0 to 7 ppm prepared from a 100 ppm NH_4Cl stock solution.

2.3 Data analysis

Data were analyzed using a 2x4 factorial randomized block design with 4 replications, followed by Duncan's multiple-range test. Correlation and regression analyses were conducted to evaluate relationships between the Conway method with the alternative techniques across legume, concentrate, and mixed samples.

3 Results and discussion

Table 1 summarizes the NH_3 concentration obtained using four analytical methods across legume and concentrate substrates. The Conway, Indophenol, and Nessler methods produced NH_3 values within a comparable range for both feed types. On legum samples, these methods yielded concentrations between 4.59 and 9.52 mM, while concentrates produced slightly higher values ranging from 7.49 to 11.85 mM. These concentrations exceed the minimum threshold (>3.6 mM) required to support MPS in the rumenand are consistent with values typically observed under normal rumen conditions [11]. The higher NH_3 release in concentrates aligns with their RDP content compared to legumes [2].

Table 1. Ammonia measurements using various methods in the fermentation of legume and concentrate

Methods	Type of feed		Average±SD
	Legumes	Concentrate	
Conway	9.52±1.85^{c}	11.85±1.74^{c}	10.68±2.08^{b}
ISE	55.88±13.97^{b}	131.38±2.26^{a}	93.63±41.41^{a}
Indophenol	5.59±0.50^{c}	10.87±1.34^{c}	8.23±2.97^{b}
Nessler	4.59±0.43^{c}	7.49±1.03^{c}	6.04±1.71^{b}
Average±SD	18.89±23.02^{b}	40.39±54.30^{a}	

Note: ISE= ion selective electrode; SD= standard deviation

In contrast, the ISE method produced markedly higher NH_3 values, reading 55.88 mM in legumes and 131.38 mM in concentrates, far above the physiological ranges. This discrepancy results from the electrohchemical nature of ISE, which detects the total ionic ammonium activity (NH_4^+), not the free NH_3fraction relevant to rumen metabolism [12]. Because electrode response is strongly influenced by pH, matrix ionic strength, and species distribution, converting raw potential to biologically meaningful NH_3 concentrations requires strict calibration procedures and continuous verification [8]. Without these adjustments, ISE-derived values cannot be directly compared to those generated by colorimetric or diffusion-based assays.

Table 2. Coefficient correlation between Conway methods and other NH_3 measurement methods

Methods	Legumes	Concentrate	Mixed
ISE	-0.4282^{ns}	0.0832^{ns}	0.5327*
Indophenol	0.1142*	0.5000*	0.6663*
Nessler	-0.5608^{ns}	0.5083*	0.5434*

Note: ns=not significant; *significant at $P<0.05$

As the Conway microdiffusion method has been considered a long-standing reliability technique in rumen NH_3 analysis [3], it was used as the reference for evaluating correspondence with the other methods. Table 2 shows the correlation between Conway methods and other NH_3 measurement methods with legume, concentrate, and combined datasets (mixed samples). For legumes, only the Indophenol method exhibited a weak but significant positive correlation with the Conway method ($r=0.1142$; $P<0.05$), whereas the ISE and Nessler methods showed nonsignificant negative correlations. However, the coefficient of determination for Indophenol in legume samples ($R^2=0.0131$) indicates that only about 1% of the variance was explained, reflecting negligible predictive strength despite statistical significance. These inconsistent relationships may be attributed to the chemical complexity of legumes, particularly the presence of tannins and other phenolics [13], which

can reduce protein solubility and interfere with color development in spectrophotometric assays, thereby weakening their alignment with the Conway method.

In concentrate samples, all methods demonstrated positive correlations with the Conway method, with both Indophenol ($r=0.5000$; $P<0.05$) and Nessler ($r=0.5083$; $P<0.05$) showing moderate and significant relationships. The simpler chemical matrix and high proportion of RDP in concentrates [2] likely reduce interference, enabling colorimetric techniques to more closely track NH_3. Across the mixed samples, correlation strength improved, with the Indophenol method exhibiting the highest overall correlation with the Conway method ($r=0.6663$; $P<0.05$), followed by Nessler ($r=0.5434$; $P<0.05$). These findings reinforce previous reports that the colorimetric method can reliably quantify ruminal NH_3 when applied under appropriate conditions and properly standardized [10]. For example, the Indophenol reaction offers high linearity and sensitivity at low concentrations [14], although the reaction may be inhibited by compounds such as amines, sulfides, and thiols [15]. Conversely, the Nessler reaction can be affected by metal ions (e.g., Fe^{2+}, In^{3+}, Ru^{3+}, Ni^{2+}), which interfere with the formation of the yellow complex measured at 420–425 nm [14].

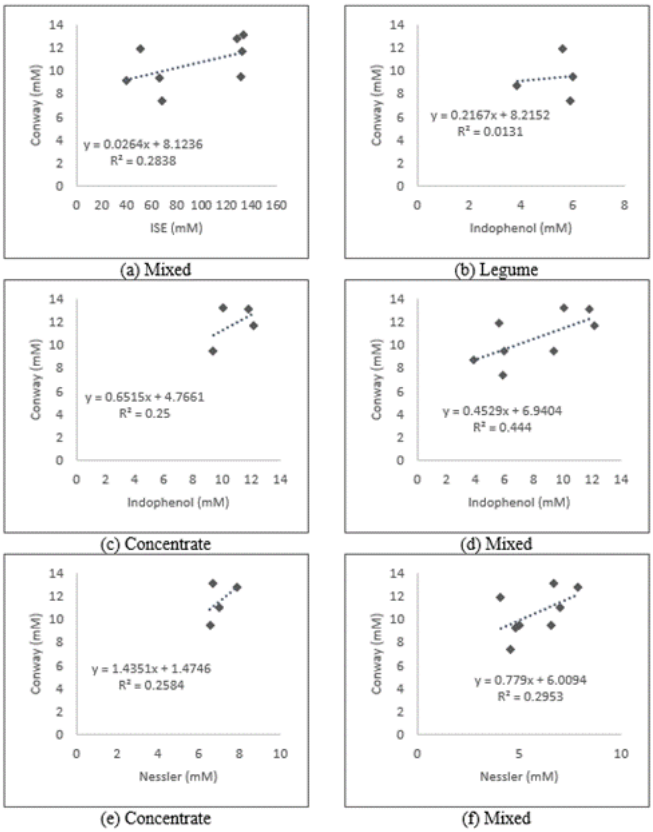

Fig. 1. Relationship between the Conway method (*x*) and other methods (*y*), including Ion Selective (ISE), Indophenol, and Nessler, for legume, concentrate, and mixed samples

In contrast, the ISE method showed a significant correlation in the mixed dataset ($r=0.5327$; $P<0.05$), but the coefficient of determination ($R2=0.2838$) indicates weak predictive performance, explaining less than 30% of the variance relative to Conway. The ISE method also remained nonsignificant within individual feed types. The relatively weak agreement might be due to the fundamental constraints of ammonia-selective electrodes,

which perform optimally at high pH (around 11) to shift NH_4^+ to NH_3[7]. Because rumen fermentation supernatants generally fall within pH 6–7 [10], substantial pH adjustment and protocol standardization are required for accurate ISE use.Without the stringent control of installation, calibration, maintenance, and data validation procedures [8], ISE measurements may not accurately reflect ruminal NH_3 dynamics.

The regression plots highlight varying levels of agreement between each method and the Conway technique across substrates (Figure 1). In mixed samples, Indophenol showed moderate coefficients of determination (R^2=0.444), while Nessler and ISE exhibited only a weak fit (R^2=0.2953 and 0.2838, respectively). In concentrate samples, both Indophenol and Nessler produced steeper and more consistent slopes. To strengthen the robustness of these predictive models, future work should incorporate a larger, more diverse dataset, along with matrix correction, improved calibration procedures, or adaptive non-linear modeling to better capture variability across feed ingredients.

4 Conclusion

These findings show that the Indophenol and Nessler methods provide NH_3 estimates comparable to those obtained with the Conway method in concentrate samples but exhibit inconsistent performance in legumes. Although the ISE technique offers the fastest measurement option, it is limited in predictive strength across feed types, suggesting it requires further calibration with a larger sample set to strengthen its correlation and reliability.

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References

1. R. Pazla, M. Zain, Despal, U.H. Tanuwiria, E.M. Putri, M. Makmur, R. Zahera, L.A. Sari, I.M. Afnan, A. Rosmalia, Y.I. Yulianti, S.D. Putri, A. Mushawwir, R.A. Apriliana, Evaluation of rumen degradable protein values from various tropical foliages using *in vitro* and *in situ* methods. Int. J. Vet. Sci. **12**, 6, 860–868 (2023).
2. I.G. Permana, A. Rosmalia, S.F.I. Rahmat, Despal, R. Zahera, Characterization of protein degradation in tropical dairy feedstuff using the *in sacco* method. J. Ilmu Ternak dan Vet. **29**, 4, 181–192 (2024).
3. B. Masterson, Conway's microdiffusion analysis: eight years on and still counting!. Biochem. (Lond). **36**, 1, 34–39 (2014).
4. H. Mayulu, T.P. Daru, I. Tricahyadinata, *In vitro* evaluation of ruminal digestibility and fermentation characteristics of local feedstuff-based beef cattle ration. *F100Research*. **11**, 843–858 (2025).
5. L. Kure, V. Pedersen, G. Sven, S. Gjedde, Validation and comparison of ammonia measurement techniques used to determine ammonia absorbed by passive absorption samplers. Can. J. Soil Sci. **100**, 2, 175–178 (2020).
6. V. Kudrna, M. Marounek, Influence of feeding whole sunflower seed and extruded linseed on production of dairy cows, rumen and plasma constituents, and fatty acid composition of milk. Arch. Anim. Nutr. **62**, 1, 60–69 (2008).
7. H. Jeong, J. Park, H. Kim, Determination of NH_4^+ in environmental water with interfering substances using the modified nessler method. J. Chem. **359217**, 1–9 (2013).
8. S. Papias, M. Masson, S. Pelletant, S. Prost-boucle, C. Boutin, *In situ* continuous monitoring of nitrogen with ion-selective electrodes in a constructed wetland receiving

treated wastewater : an operating protocol to obtain reliable data.Water Sci. Technol. **77**, 6, 1706–1713 (2018).
9. J.M.A. Tilley, R. A. Terry, A two-stage technique for the *in vitro* digestion of forage crops. Grass Forage Sci. **18**, 2, 104–111 (1963).
10. N.K.P. Souza, E. Detmann, S.C.V. Filho, V.A.C. Costa, D.S. Pina, D.I Gomes, A.C. Queiroz, H.C. Mantovani, Accuracy of the estimates of ammonia concentration in rumen fluid using different analytical methods. Arq. Bras. Med. Vet. Zootec. **65**, 6, 1752–1758 (2013).
11. P. McDonald, R.A. Edwards, J.F.D. Greenhalgh, C.A. Morgan, L.A. Sinclair and R.G. Wilkinson, Animal Nutrition 7th ed, (Pearson, London, 2010).
12. Y. Wen, Y. Mao, Z. Kang, Q. Luo, Application of an ammonium ion-selective electrode for the real-time measurement of ammonia nitrogen based on pH and temperature compensation. Measurement. **137**, 98–101 (2019).
13. Despal, Y.I Yulianti, R. Zahera, I. Agustiyani, A. Rosmalia, I.M. Afnan, M. Zain, U.H. Tanuwiria, Comparison of chemical composition, *in vitro* digestibility, and near infrared reflectance spectroscopy in estimating *in situ* rumen degradable protein of tropical foliage.Trop. Anim. Sci. J. **46**, 2, 211–220 (2023).
14. Y. Zhao, R. Shi, X. Bian, C. Zhou, Y. Zhao, S. Zhang, F. Wu, G.I.N, Waterhouse, L. Wu, C. Tung, T. Zhang. Ammonia detection methods in photocatalyticand electrocatalytic experiments: how to improve the reliability of NH_3 production rates?Adv. Sci. **6**, 8, 1802109 (2019).
15. Y.B. Cho, S.H. Jeong, H. Chun, Y.S. Kim, Selective colorimetric detection of dissolved ammonia in water via modified Berthelot's reaction on porous paper. Sensors Actuators B. Chem. **256**, 167–175 (2017).