Rumen Ammonia Concentration as Influenced by Storage Time, Freezing and Thawing, Acid Preservative, and Method of Ammonia Determination

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ABSTRACT

Twenty-five milliliters of rumen fluid from five cows on a variety of diets were added to either 25 ml .1 N HCl, 5 ml 25% metaphosphoric acid, 5 ml 20% trichloroacetic acid, 5 ml 3 N H_2SO_4, or no preservative. Replicate samples were stored at −20°C until analyzed. One replicate set was analyzed at 7 d, refrozen and analyzed at 35 d, refrozen, thawed, and reanalyzed at 65 d. Another set was thawed and analyzed at 35 d and yet another set kept frozen until analyzed at 65 d. Fresh and frozen samples were quantitated for ammonia by Conway microdiffusion and phenol-hypochlorite reaction.

Ammonia recoveries were 93.8 and 94.1% for the Conway and phenol-hypochlorite methods. Storage time and refreezing and thawing had a variable effect on ammonia concentration. Samples with no preservative yielded higher ammonia than those with preservative; ammonia was accentuated by refreezing and thawing up to 65 d. Hydrochloric acid samples had lower ammonia than other acids, especially for the phenol-hypochlorite method. Overall, the Conway method resulted in higher ammonia for frozen samples than the phenol-hypochlorite method (10.4 vs. 9.0 mg/dl); however, this varied depending on acid.

When rumen fluid samples are to be stored with an acid preservative, .1 N HCl is not recommended. Repeated freezing and thawing of rumen samples used for ammonia analysis should be avoided because of variable results.

INTRODUCTION

Ruminal ammonia concentrations are frequently quantitated as an indicator of rumen nitrogen metabolism with particular reference to ruminal protein degradation. In studies that involve quantitation of a large number of samples for ruminal ammonia, questions often arise as to sample storage time, freezing and thawing, as well as preservative effects on ammonia analyses.

The microdiffusion method (5) of ammonia analysis has been the most used technique for rumen ammonia analysis until recent years. The need for convenience, speed, and simplicity has brought colorimetry into focus as a viable alternative. The phenol-hypochlorite color reaction is used manually (2) or automated (1, 9). The reaction is pH sensitive, and the use of acidified rumen fluid may depress ammonia (2).

It is often desirable to store rumen fluid for later analysis to reduce labor requirements. Rumen fluid has been stored for varying lengths of time, with intermittent thawing and refreezing, with little research conducted to verify storage effects on ammonia analysis. Preservatives (acidification) must be added to rumen fluid when stored to reduce volatilization of ammonia and halt microbial activity. Samples have been prepared by adding H_2SO_4 (8), trichloroacetic acid (3), HCl (4), and H_3PO_4 (6). However, little is known regarding these preservatives and their interaction with storage time and methodology of ammonia analysis.

The objectives of this research were to determine the influence of two commonly used methods of rumen ammonia analysis, rumen fluid storage time and commonly used preservatives, on ruminal fluid ammonia concentration.

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MATERIALS AND METHODS

Rumen fluid was collected from five fistulated lactating Holstein cows 3 h postfeeding. Cows were receiving rations listed in Table 1. These rations were partially designed to produce differences in rumen ammonia concentration. Rumen fluid samples were collected from four different locations in the rumen and strained through four layers of cheesecloth. Samples were magnetically stirred while 25-ml aliquots were pipetted into replicated screw cap, 50-ml centrifuge tubes containing preservatives shown in Table 2.

Ammonia concentration was determined on fresh unpreserved rumen fluid by the phenol hypochlorite (PHC) color reaction (2) and by a modified Conway (CON) microdiffusion (5) in which dishes were incubated 1 h at 37°C and ammonia corrected for recovery. Analysis on these samples commenced within 1 h of collection. Other sets of samples were frozen (−20°C) for 7 d, thawed and analyzed, refrozen, thawed and analyzed at 35 d, refrozen, and thawed at 65 d for analysis. Other sets of rumen fluid samples were frozen, thawed, and analyzed at either 35 or 65 d. All samples were thawed gradually by placement into a refrigerator (4°C) from the freezer (approximately 3 h) then submersed into a room temperature water bath until thawed. All ammonia concentrations were determined in duplicate by CON and PHC methods, and standard curves were determined during each analysis for the PHC method utilizing 0, 5, 10, 15, 20, and 25 mg/dl ammonium sulfate. A standard was included for every 10 analyses to correct for recovery on the CON method. Ammonia concentrations were corrected for differences in dilution associated with preservative addition.

Data were initially analyzed by split-split plot analysis of variance techniques using method of ammonia analysis, method of sample preservation, and storage time as main effects. Because of anticipated differences in ammonia concentrations among cows, cows were used as blocking effects in the analyses, and no attempt was made to characterize interactions with cow effects. Due to a significant interaction between method of sample preservation and storage time, data were pooled by method of ammonia analysis and further analyzed by separate factorial analysis (7) for the presence of storage time effects by individual method of preservation and for method of preservation by each storage time. Finally, differences between methods of ammonia analyses were evaluated by separate single way analysis of variance for each storage time and method of preservation. Duncan's multiple range test was used to determine mean treatment differences when the overall F statistic was significant ($P<.05$).

TABLE 1. Ingredient and chemical composition of experimental diets fed to fistulated cows.

Ingredient	Cow no. 982	994	1046	922	1008
	(% dry matter basis)				
Soybean meal	8.8	13.3	...	15.3	...
Corn gluten meal	...	...	12.9	...	14.3
Soybean hulls	...	27.2	27.2	...	...
Corn	9.7	32.2	32.4	38.0	38.9
Cottonseed hulls	...	9.6	9.6	16.1	16.1
Corn silage	76.0	...	...	...	...
Corn stover	...	...	...	22.0	22.0
Molasses	.5	10.3	10.4	2.6	2.6
Mineral-vitamin mix[1]	5.0	.5	2.6	2.7	2.7
Chemical composition					
Crude protein	14.8	15.1	16.2	14.5	14.1
Acid detergent fiber	21.0	22.2	22.5	25.2	27.2

[1] Added to meet National Research Council Requirements.

TABLE 2. Methods used to preserve rumen fluid.[1]

Volume and concentration of ruminal preservative	Approximate final normality of rumen fluid	pH of stored rumen fluid
No preservative	...	5.5
25 ml .10 N hydrochloric acid	.05	4.3
5 ml 25% wt/vol metaphosphoric acid	.6	2.7
5 ml 3.0 N sulfuric acid	.5	1.3
5 ml 20% wt/vol trichloroacetic acid	.2	1.6

[1] Each tube received 25 ml of rumen fluid in addition to the designated preservative.

RESULTS AND DISCUSSION

Figure 1 illustrates standard curves of the PHC method conducted for different sample groups. Regression coefficients for individual regressions were linear ($P<.001$) through the standard concentrations utilized. The pooled coefficient of determination was .96 ($P<.001$). Recoveries ranged from 89.5 to 98.6% (mean 94.1%, CV 3.4%). Percent recoveries for the CON procedure ranged from 90.5 to 96.0% (mean 93.8%, CV 1.4%). Mean ruminal ammonia concentrations for cows numbered 982, 994, 1046, 922, and 1008 for the CON method were 8.6, 6.9, 15.4, 9.3, and 11.9 mg/dl and for the PHC method were 6.2, 4.7, 15.1, 7.6, and 10.9 mg/dl, respectively.

Table 3 shows the influence of storage time and method of ammonia analysis on ruminal ammonia concentration within the various preservation methods. Ammonia concentration for fresh rumen fluid was higher ($P<.05$) when analyzed by PHC than by CON. Relative differences between methods of analysis were similar to fresh rumen fluid when samples with no preservatives were frozen, thawed, and analyzed d 7, 35, and 65, although the 35-d analyses were not different ($P>.05$). Analyses by CON method did show increased ($P<.05$) ammonia due to refreezing, storage, and thawing. For the PHC method, there was no effect ($P>.05$) of thawing and refreezing from d 7 to 35. Additional refreezing and thawing to 65 d elevated ammonia considerably for both methods of analyses. Ruminal samples stored with no preservative for 35 d had a higher ($P<.001$) ammonia concentration when determined by CON than the PHC procedure. For the CON method, 35 d of storage had higher ($P<.05$) ammonia concentrations than 65 or 7 d; conversely, with the PHC procedure, ammonia was lower ($P<.05$) at 35 than 65 d with no difference ($P>.05$) between d 65 and 7.

With HCl as the preservative, methods of analysis differed at each storage time, except for the 65 d frozen sample, with the CON procedure yielding higher ammonia than PHC. For the CON method, ammonia at d 35 was higher than when refrozen and thawed at 65 d.

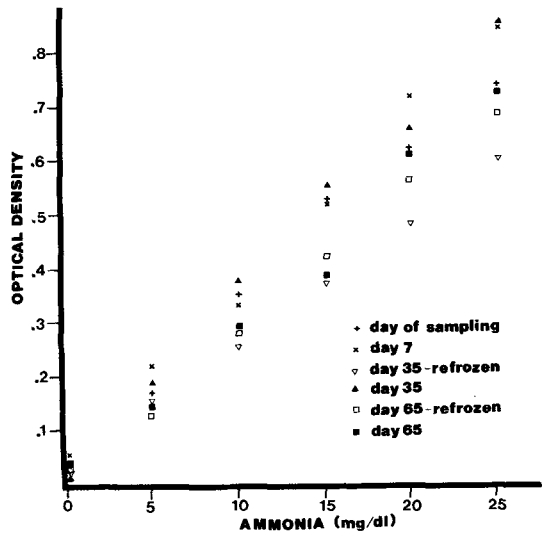

Figure 1. Standard curves conducted on each day of rumen fluid ammonia analyses for the phenol-hydrochlorite colorimetric procedure (2). Standard curve determinations for d 35 and for d 35 refrozen were on the same day; however, a separate standard curve was conducted for each set of samples (same for d 65 and for 65 d refrozen samples).

TABLE 3. The effect of freezing, thawing, storage time, and analytical method on rumen fluid ammonia concentration treated with different acid preservatives.

Preservative[1]	Analytical method, and significance[2]	Fresh[3]	Refrozen[4] 7 d	Refrozen[4] 35 d	Refrozen[4] 65 d	Frozen[5] 35 d	Frozen[5] 65 d	SEM[6]
			mg/dl					
NO PRES	CON	11.8	11.2[c]	12.7[b]	19.4[a]	13.2[b]	11.0[d]	.74
	PHC	13.8	13.2[b]	13.2[b]	23.7[a]	9.9[c]	12.8[b]	1.00
	$P<$[7]	.05	.001	NS[8]	.001	.001	NS	
HCl	CON	...	9.3[ab]	9.7[a]	8.8[b]	8.9[ab]	5.7[c]	.52
	PHC	...	10.7[a]	3.6[d]	4.6[c]	3.2[e]	6.2[b]	.55
	$P<$		.002	.001	.001	.001	NS	
MPA	CON	...	9.7[b]	11.4[a]	9.6[b]	10.3[b]	7.0[c]	.46
	PHC	...	10.9[a]	5.1[e]	7.9[b]	7.3[c]	6.4[d]	.55
	$P<$		NS	.001	NS	.001	NS	
SA	CON	...	10.6[ab]	10.9[a]	10.7[ab]	10.2[b]	7.1[c]	.50
	PHC	...	10.9[a]	6.3[e]	10.2[b]	8.2[c]	7.1[d]	.57
	$P<$		NS	.001	NS	.003	NS	
TCA	CON	...	9.9[b]	11.0[a]	11.3[a]	11.4[a]	8.9[c]	.44
	PHC	...	10.8[a]	6.4[c]	8.7[b]	6.4[c]	6.6[c]	.56
	$P<$		NS	.001	.004	.001	.04	

[a,b,c,d,e]Means in the same row with different superscripts differ ($P<.05$).

[1] NO PRES = No preservative, HCl = .1 N hydrochloric acid, MPA = 25% (wt/vol) metaphosphoric acid, SA = 3 N sulfuric acid, TCA = 20% (wt/vol) trichloroacetic acid.

[2] CON = Conway microdiffusion method (5), PHC = phenol hypochlorite colorimetric method (2).

[3] Samples were not preserved with acid or frozen prior to analyses.

[4] Rumen fluid samples were frozen (−20°C), thawed, and analyzed on d 7, refrozen, thawed, analyzed on d 35, refrozen, thawed, and analyzed on d 65.

[5] Separate sets of samples were frozen (−20°C), thawed on each d 35 and 65, and analyzed.

[6] SEM = Standard error of the mean.

[7] Probability of difference between analytical methods.

[8] NS = Nonsignificant ($P>.05$).

Refreezing and thawing considerably reduced ($P<.05$) ammonia from d 7 to 35 for the PHC procedure. Additional refreezing and thawing increased ($P<.05$) ammonia concentration slightly for d 35 (4.6 mg/dl). Additional storage time to d 65 reduced ammonia concentration ($P<.05$) compared with 7 with the CON method. Storage to d 35 or 65 did reduce ($P<.05$) ammonia concentration compared with d 7 for the PHC method.

Method of ammonia analysis was different ($P<.001$) at the 35 d refrozen and 35 d storage times; the CON method was higher than PHC for rumen samples preserved with metaphosphoric acid (MPA). Refreezing and thawing increased ($P<.05$) ammonia concentration to d 35 with the CON method and reduced ($P<.05$) concentration from d 7 with the PHC procedure. Additional refreezing and thawing to d 65 reduced ($P<.05$) ammonia concentration for the CON method compared with the 35 d analyses but not when compared with d 7 analyses. As with HCl, the PHC procedure had an opposite effect on ammonia concentration than did the CON method in relation to refreezing and thawing. However, the reduction in ammonia with MPA was not as severe as with HCl. Storage to 65 d reduced ($P<.05$) ammonia concentration when the CON method was used. A linear decline in ammonia concentration was observed with storage time with the PHC method.

Methods to determine ammonia concentration were different for the refrozen 35 d ($P<.001$) and the 35 d storage ($P<.003$) when sulfuric acid (SA) was used as the rumen fluid preservation, as occurred with MPA. There was no effect of refreezing and thawing on ammonia concentration for the CON method. Ammonia concentrations for ruminal fluid samples were similar at 7 and 35 d, but the 65-d storage length depressed ($P<.05$) ammonia concentration compared with 7 or 35 d. Except for the refrozen and thawed value at 35 d, the PHC method of ammonia analysis followed a trend similar to the CON method in effect of refreezing and thawing and storage time on ammonia concentration.

When TCA was used as the rumen fluid preservative, all but the 7 d samples were different between method of analyses; CON was higher than the PHC method. Ammonia concentration increased as samples were refrozen and thawed from d 7 to 65 with the CON method. Ammonia concentration increased as storage time increased from d 7 to 35; however, ammonia concentration decreased with 65 d of storage. For the PHC procedure, ammonia concentration decreased with refreezing and thawing from d 7 to 35 but increased with subsequent refreezing and thawing to d 65. No differences were detected between storage times of 35 and 65 d; however, both were lower ($P<.05$) in ammonia concentration than d 7.

Fresh rumen fluid analyzed for rumen ammonia concentration immediately after sampling may be considered the closest to the "true" ammonia concentration in this experiment. Assuming this fact, it would appear that by numerical comparison, ammonia concentrations for samples with no preservative (except for 65 d refrozen) remained relatively close to the "true" value compared with many of the other samplings, which included a preservative.

Figures 2A through E illustrate the influence of method of ammonia analysis and preservative on ruminal ammonia concentration within a given storage time. A readily visible finding is higher ammonia for samples with no preservative regardless of method of analysis or storage time. Differences ($P<.001$) in ammonia concentration between methods of analyses for the d 7 sample (Figure 2A) were exhibited for samples with no preservatives ($P<.001$) and HCl. No differences ($P>.05$) were observed among added preservatives for the PHC method. For the CON method, samples preserved with SA had highest ($P<.05$) and those with HCl lowest ammonia. Samples preserved with TCA were higher in ammonia than HCl, but lower than SA, with MPA not being different from HCl or TCA.

Refreezing and thawing after 35 d once again showed significant differences in ammonia concentrations between methods for all preservatives (Figure 2B). The general influence of preservative on ammonia concentration was similar to d 7, except ammonia for the PHC procedure was considerably lower than ammonia for the CON method. After a subsequent refreezing and thawing sequence, ammonia at d 65 (Figure 2C) was similar between methods of analyses for MPA and SA and different between HCl and TCA. For the CON method, HCl was lowest and TCA highest in ammonia concentration, whereas for the PHC method, HCl was lowest and SA highest in ammonia concentration. Trends for ammonia concentration in relation to method of analyses and preservative for 35 and 65 d storage were quite similar to those previously described for refreezing and thawing at 35 and 65 d (Figure 2D and E).

The reason for differences between analytical procedures to measure ammonia concentration is not readily known. Coefficients of determination and recoveries for both procedures were comparable. Broderick and Kang (3) indicated that CV for ammonia recoveries from ruminal fluid by the automated PHC procedure were about half those for the CON microdiffusion technique. In general, for samples stored beyond 35 d with a preservative, the CON procedure yielded higher ruminal ammonia values than the PHC procedure. Inhibitory substances in the rumen fluid may be interfering with the formation of chloramine or indophenol chromagen complexes with the PHC procedure, therefore reducing ammonia recovery (3). In addition, the difficulty of accurately pipetting 50 μl of rumen fluid, due to its viscous nature, may be contributing to the greater error associated with this procedure.

Time of storage, as well as freezing and thawing, had a definite but variable effect on ammonia concentration, depending on the

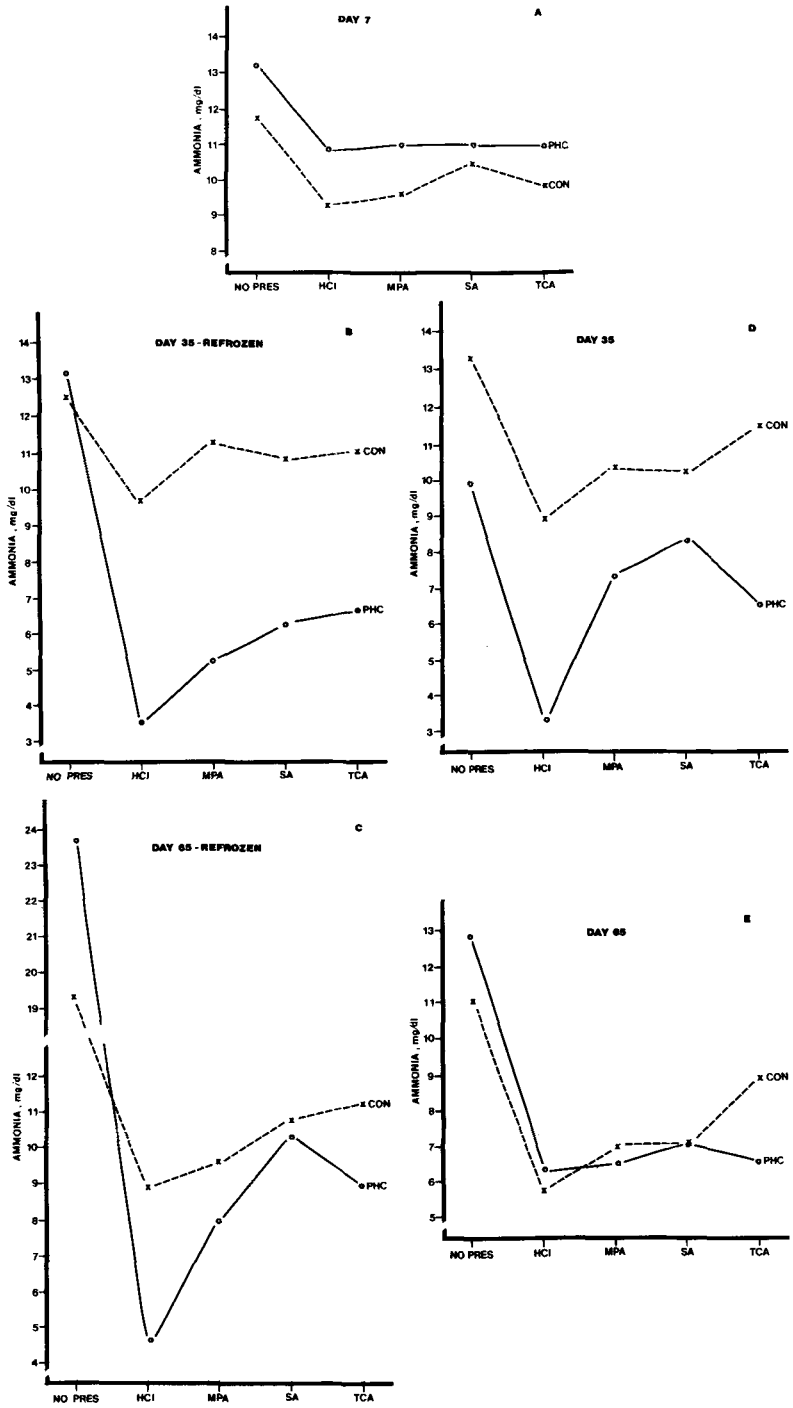

Figure 2. Ammonia concentration of rumen fluid samples determined by the Conway microdiffusion procedure (5) or phenol-hypochlorite procedures (2). Different acid preservatives (NO PRES = no preservation, HCl = hydrochloric acid, MPA = metaphosphoric acid, SA = sulfuric acid and TCA = trichloroacetic acid) were evaluated within various storage times: sample collected and frozen for 7 d (A), same sample refrozen and thawed at 35 d (B), and sample refrozen and thawed at 65 d (C). Separate samples were collected and frozen for either 35 d (D) or 65 d (E) then analyzed for ammonia.

preservative used. Ruminal ammonia values from samples with no preservatives were generally double those values with samples containing preservative. This was especially apparent after samples were refrozen and thawed twice (65 d). This elevation would most likely be associated with microbial proteolysis of the ruminal fluid protein constituents; however, one might also suspect this ammonia to be volatilized, since these samples were not acidified. Baetz et al. (1) observed that ammonia content of frozen rumen fluid was stable for 3 wk.

Among samples treated with an acid, HCl-treated rumen fluid generally yielded the lowest ammonia concentration with other preservatives being similar. These results correspond to HCl having the lowest normality compared with the other acid treated samples (HCl; .05 N, MPS; .6 N, SA; .5 N and TCA; .2 N). Degree of normality did not appear associated with ammonia concentration above .2 N. Rumen fluid pH (Table 2), however, did appear associated with ammonia loss in preserved samples (i.e., lower ammonia concentration with higher pH).

This trial illustrates a variety of scenarios that one may normally follow in the handling of ruminal fluid for ammonia analyses. No general conclusions can be drawn with regard to storage time or freezing and thawing on ammonia concentration of rumen fluid, since many superimposed factors appeared to influence the results and must be evaluated under given sets of conditions. However, rumen fluid samples with no preservation (acidification) resulted in higher ammonia concentrations than those that were treated, regardless of analytical method to quantitate ammonia. In addition, except for a few storage times, ammonia concentrations for samples containing no preservative were relatively close to rumen fluid that was analyzed for ammonia immediately after sampling. When a preservative is needed, .1 N HCl is not recommended because this level and type of acid was not effective in reducing rumen fluid pH, and ammonia loss was higher than with other acids.

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