A comparison of methods to assess unavailable nitrogen in ruminant feeds

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Abstract

Intestinal digestibility of cattle diets is not routinely analyzed as part of diet formulation. The unavailable nitrogen (uN) assay of Ross (uN_{Ross}) was developed to predict the uN of non-forage feeds, and questions have been raised about the applicability to forages. The rumen residency time of forages is about 30 h, whereas the current uN assay uses 16 h to represent the residency time of non-forage feeds. The objective was to evaluate the ability of the uN_{Ross} assay to measure the uN of forages when adopting a longer *in vitro* digestion period. A total of 12 feeds were analyzed and for all feeds both 16 h and 30 h *in vitro* fermentations were conducted prior to the uN analysis for comparison. All samples were then evaluated using both acid detergent insoluble nitrogen (ADIN) assay and uN_{Ross} assay to determine uN. Comparisons were made among the results from ADIN assay, uN-16h assay and uN-30h assay. Most feeds had a lower or equal uN after 30 h of incubation with rumen fluid compared to the standard 16 h. The 30 h of fermentation with rumen fluid apparently did not allow for adequate carbohydrate degradation to provide adequate surface area to digest the proteins integrated in the fiber and this might be related to some enzymes present in cattle that are not currently used in the uN assay. For non-forage feeds 16 h of *in vitro* ruminal exposure was adequate to allow the intestinal enzymes to function appropriately. Future research should focus on the addition of other enzymes to evaluate the ability to digest the fiber matrix to predict protein digestibility.

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List of Abbreviations

AD	Acid Detergent
ADF	Acid Detergent Fiber
ADIN	Acid Detergent Insoluble Nitrogen
CNCPS	Cornell Net Carbohydrate and Protein System
CP	Crude Protein
DM	Dry matter
DMI	Dry Matter Intake
IDEA	Immobilized Digestive Enzyme Assay
MCP	Microbial Crude Protein
ME	Metabolizable Energy
MML	Mixed Mostly Legume
MP	Metabolizable Protein
mTSP	Modified Three-step Procedure
N	Nitrogen
ND	Neutral Detergent
NDF	Neutral Detergent Fiber
RDP	Rumen Degradable Protein
RUP	Rumen Undegradable Protein
TCA	Trichloroacetic Acid
TN	Total Nitrogen
TSP	Three-step Procedure
uN	Unavailable N

uN_{Ross}	The unavailable nitrogen assay of Ross

Literature Review

In terms of milk production and environmental impact, N in feed plays an important role, especially when N is typically overfed in most dairy cattle diets for several reasons. The efficiency of N utilization has been one of the main concerns in the field for years as there is more pressure on dairy producers to reduce the environmental impact of milk production and also to reduce feed costs to improve profitability. Protein consumed by ruminants is either degraded in the rumen (rumen degradable protein, RDP), or escapes microbial digestion in the rumen and passes out of the rumen intact (rumen, undegradable protein, RUP), and is digested in the intestine. In the rumen, protein is utilized by the microbes to form microbial crude protein (MCP). In contrast, RUP escapes the utilization of rumen microbes and is digested and absorbed in the intestine or excreted in the feces if unavailable to digestion. Both MCP and RUP forms what is known as metabolizable protein (MP), which is highly correlated with milk yield. Ideally, about 45 to 50% of MP should come from MCP and the balance of it, 50 to 55% should come from RUP. Besides, in order to make better use of inexpensive soluble feed protein and reduce environmental impact, 50% or greater of dry matter intake (DMI) is recommended to be forage to ensure adequate carbohydrate fermentation in the rumen to produce MCP. Also, a balance of metabolizable energy (ME) and MP guarantees that nutrients are used most efficiently so that one is not limiting. When ME is not first limiting, the availability of MP or N becomes the predominant factor in limiting milk yield. For a long period of time, studies in the dairy cattle nutrition field have been focused on crude protein (CP), however, discussion on the level of CP does not consider the digestibility of protein in the intestine. Therefore, developing methods to determine intestinal unavailable N is of great significance to reduce protein feeding and ensure more accurate diet formulation.

Development of in vitro and in situ assays

It is widely understood that *in vivo* methods to determine N digestibility of feeds are costly, time consuming and labor intensive. Therefore, animal scientists started working on the development of *in vitro* and *in situ* assays to determine the N digestibility of forages. In 1963, a two-step method containing sequential processes of rumination and acid pepsin digestion was developed (Tilley & Terry, 1963). In 1985, a nylon bag technique was introduced into the estimation procedure (Hvelplund, 1985) that allowed for feeds to be placed in the bag and then within the rumen of a cow. Later on, Casamiglia and Stern (1995) developed a three-step procedure (TSP) to estimate protein digestion in ruminants, which included an *in situ* bag process followed by acid pepsin and pancreatic digestion. However, because the unavailable protein was determined by the addition of trichloroacetic acid (TCA) solution to precipitate the undigested proteins, this procedure didn't describe the digestibility of the individual amino acids (AA). A decade later, Gargallo et al. (2006) modified the TSP (mTSP) by removing TCA from the procedure and utilizing a batch incubator (Daisy^{II,} Ankom Technologies, Macedon, NY), which further reduced the labor and financial cost. A good correlation of the AA digestibility of RUP was shown in comparison with the *in vivo* results (Gargallo et al., 2006). Nevertheless, concerns about the use of the bag remained unsolved. There are two main issues with the use of a bag. One is that it reduces the exposure of feed to the rumen microbes by creating a barrier to entry which increases the lag time while reducing the flow of fluids through the bag. Also, any portion of the feed that solubilizes or is of very small particle size will be lost during the procedure, resulting in the underestimation of intestinal protein digestion. In 2009, Boucher et al. compared the mTSP method with an immobilized digestive enzyme assay (IDEA). Although the comparison revealed that both mTSP method using the batch incubator and the IDEA method worked well for the sample feeds

(Boucher et al., 2009), the enzymes used in the IDEA method were from chicken, which suggests that the enzyme profile might influence the digestibility results as well.

Acid detergent insoluble nitrogen (ADIN) assay

The ADIN analysis is based on the acid detergent insoluble protein or ADIN fraction (Higgs et al., 2015). The ADIN is the N fraction remaining in the acid detergent fiber (ADF) residue and ADF by assay and definition is the cellulose and lignin in the plant cell wall (Goering & Van Soest, 1970). ADIN usually refers to the protein that is associated with those fibers. The N content in ADF is measured by combustion or Kjeldahl test (Higgs et al., 2015).

The protein and N associated with ADF (ADIN) is typically a result of excessive exposure to heat during processing or storage and cannot be utilized by the animal. As such, it has been used as a determinant for indigestible N (Pichard & Van Soest, 1977) for all feed ingredients in diet formulation for over 40 years. However, recent work on digestibility suggests this fraction might not represent the indigestible protein of all feeds, especially non-forage feeds (Ross et al., 2013). The committee that developed the 2001 Dairy NRC publication (NRC, 2001) suggested that at least 5% of the protein in ADIN could be digested, thus they assigned a 5% digestibility to the ADIN fraction which was a step towards moving to something more dynamic, however a similar digestibility for all feeds does not seem realistic.

uN_{Ross} assay and cow study

Stern et al. (1997) pointed out that in order to precisely predict protein digestion, it is important to use enzymes with similar activity and specificity as those found in the digestive tract of animals. They further argued that non-ruminant enzymes might have different behaviors in comparison to ruminant enzymes (Stern et al., 1997). In comparison to the mTSP method, in the uN_{Ross} assay, rather than placing a bag of feed into the rumen of a cow, feed is incubated with collected rumen

fluid *in vitro*, and the enzyme profile is optimized in sequential steps to simulate the digestive environment of ruminant animals. In the uN_{Ross} assay, fermentation typical of the environment of the rumen takes place in an Erlenmeyer flask containing buffer and fresh rumen fluid maintained at 39 ^{o}C in a warm water bath. Also, in terms of intestinal enzyme profile, proteolytic enzymes (trypsin, chymotrypsin), lipolytic enzymes (lipase, bile) and carbohydrate hydrolyzing enzymes (amylase) are included in this assay as suggested in a previous study (Ben-Ghedalia et al., 1974).

A comparison of ADIN assay and uN_{Ross} assay was conducted on many feed samples including high quality blood meal and the same blood meal subjected to very high temperatures (>130 degree C) to create heat damage and decrease the digestibility (Ross et al., 2013). Due to the Maillard reaction, a condensation reaction between carbohydrates and AA, primarily lysine, heat damaged blood meal contains less digestible N. Results showed that using ADIN the percentage of unavailable N (uN) in two feeds was not different; while using the uN_{Ross} assay, the percentage of unavailable N in heat damaged blood meal was much higher than that of untreated blood meal, which is a more logical result. This study indicated that the uN_{Ross} assay might be a better method for determining uN for animal-sourced byproduct feeds.

Gutierrez-Botero et al. (2014) conducted a cattle study to evaluate the predictions of the uN_{Ross} assay. In many cases, assays have been developed to test N digestibility or indigestibility, but in vivo evaluations conducted with cattle have not been done to evaluate the specificity and precision of the assays. Thus, an experiment was conducted on high producing dairy cattle to evaluate the precision and accuracy of the assay. Cattle were divided into two groups with one group fed a low uN diet and another group fed a high uN diet. The diet was formulated with identical ingredients in the same proportions except for the N digestibility of the two blood meals utilized in the study. One of the blood meals had a high uN of 34% whereas the other blood meal had a low uN value

at 9%, thus there was a 23-unit difference in predicted intestinal digestibility between the two ingredients. The total protein content was also the same in the two diets and dry matter intake was not significantly different so total N intake averaged about 670 g/d for both treatments. In the end, a 2.0 kg decrease in milk yield and energy corrected milk was observed in the group of cows fed the high uN diet. The result that cows fed high uN diet produced less milk compared to those fed low uN diet was convincing. Furthermore, this result showed that the uN_{Ross} assay was a more accurate and specific method to determine the uN and this feed characteristic was integrated into the CNCPS system to more precisely predict the intestinal digestibility of protein (Gutierrez-Botero et al., 2014).

Previously, for most of the assays described the rumen incubation time used was 16 hours (Calsamiglia & Stern, 1995; Gargallo et al., 2006; Ross et al., 2013). However, based on the current understanding of rumen residency time of forages compared to concentrate feeds, forage samples might require a longer incubation time to allow for adequate ruminal digestion prior to lower tract digestion. Studies have described an average of about 30-hour of rumen retention time for 65% to 70% of a forage to either disappear or pass out of the rumen with selective retention of the digestible fraction of forages (Colucci et al., 1982; Allen & Mertens, 1988). Therefore, in experiments reported in this thesis, the influence of time of incubation with rumen fluid was evaluated on a group of feeds to determine if 30h *in vitro* rumen digestion created differences in the uN_{Ross} assay results compared to the normal 16h *in vitro* digestion and if the results of the longer fermentation time correlated to the ADIN content of forages, as that value has been used as the unavailable N in forages for over 40 years.

Introduction

Due to the cost of feeding dairy cattle, proper chemical characterization of feeds is important to improve diet formulation to improve income over feed costs and reduce the environmental impact of milk production. Dairy cattle are ruminants, which are fed on a diet containing both forages and non-forages. Forages include corn silage, alfalfa hay, grasses and many other plants while non-forage ingredients can include soybean meal, canola meal, blood meal and soybean meal, etc. The feed consumed by dairy cows will sequentially pass through the rumen, abomasum and intestine, being digested, and absorbed, and the portion of indigestible materials will be excreted. Protein in the feed that escapes rumen fermentation will be absorbed in the form of AA or short peptides and these AA will be further utilized or metabolized by the cow for milk synthesis, maintenance, tissue growth and repair and pregnancy. The indigestible fraction of protein will be excreted in feces which can later convert to ammonia or nitrous oxide gas and eventually cause environmental concerns. Therefore, it is of great importance to study nitrogen (N) utilization efficiency of dairy cows considering its impact on both production and environment.

Nitrogen efficiency can be calculated as the ratio of productive N (milk, tissue) to feed N. Studies have revealed that theoretically this value could reach 40% to 45% in lactating dairy cows (Van Vuuren & Meijs, 1987; Madsen et al., 1995). However, with the management level of most farms in the United States, the current value they could arrive at is about 20% to 32%. Nevertheless, there are cows within groups approaching the theoretical limits of N efficiency. Case studies showed that cows producing 76 kg/d of milk had a N efficiency of 41% (Van Amburgh personal communication). This suggests that with better diet formulation and feeding management, farms could achieve a higher N efficiency and eventually make more profits and reduce environmental pollution. For the purpose of better diet formulation, scientists have been working on the

refinement of formulation models for years and with this, improved methods to chemically analyze feeds might be required to improve the ability to balance for rumen N and post-rumen amino acids.

Currently there are multiple methods to determine uN in the gastrointestinal (GI) tract. The oldest of these and the one currently used for all feeds, forages and concentrates is the Acid Detergent Insoluble Nitrogen (ADIN) assay (Pichard & Van Soest, 1977). The ADIN fraction of protein is associated with the cell wall of plants and considered insoluble and unavailable to dairy cows by intestinal digestion. By measuring the ADIN values, nutritionists can predict the N digestibility which will later be used in the ration formulation models, such as Cornell Net Carbohydrate and Protein System (CNCPS) (Tylutki et al., 2008).

A recently developed bioassay (uN_{Ross} assay, Ross et al., 2013), has been used in place of the ADIN assay for non-forage feeds for use in the CNCPS for its precision of predicting uN. This is an *in vitro* assay, which has overcome a lot of obstacles in previous assays to more accurately determine the amount of uN in common feed ingredients. In detail, feeds are inoculated with rumen fluid and subjected to a 16 hour *in vitro* rumen fermentation, acidified and then incubated with a pepsin HCl cocktail to mimic the abomasum in a shaking water bath for 1 hour, and finally incubated with an enzymatic cocktail to mimic intestinal environment in a shaking water bath for 24 hours. After these three steps, the remaining N recovered on a 1.5 µm pore size glass filter paper is determined using the Kjeldahl method and considered the uN to the animal. Results from this uN_{Ross} assay have proved to be more accurate in determining the amount of uN in feeds, primarily concentrates like blood meal, feather meal and soybean meal; however, further evaluations of the assay are required. One such evaluation is to determine if this assay might be useful for forages. To evaluate forages, a longer *in vitro* rumen fermentation step is necessary to more accurately depict the resident time of forages in the rumen (Colucci et al., 1982) in order to

understand if the assay can hydrolyze the protein bound to the cell walls once a larger portion of the cell wall is digested by microbes. The objective of this study is to evaluate differences in uN by subjecting forages to either a 16 hour or 30 hour *in vitro* rumen fermentation and then conducting the same intestinal digestion steps used in the uN_{Ross} assay, and comparing these results to ADIN. The hypothesis is that the use of a 30 hour *in vitro* rumen fermentation should more accurately represent the actual indigestible N of forages compared to the 16 hour fermentation when then further evaluated against ADIN by allowing for greater digestion of the carbohydrate matrix that envelopes the proteins of the plant more likely to be digested in the intestine.

Materials and Methods

A total of twelve samples were used in this project, including five forages and seven non-forage feeds (Table 1). Both the ADIN assay and uN assay at the two time points, 16h and 30h were performed on them. Duplicates were used for all the sample analyses in this project. Dry matter was measured after drying overnight in the forced-air oven at 105 °C. All N was measured by Kjeldahl (AOAC Official method 2001.11; Foss, 2003; Tecator Digestor 20 and Kjeltec 2300 Analyzer, Foss Analytical AB, Höganäs, Sweden; AOAC 2001.11).

Table 1. List of feeds analyzed.

Forage Feeds	**Non-forage Feeds**
Timothy Hay	Corn Gluten Feed
Grass Hay	Corn Germ
Corn Silage I	Soybean Meal
Corn silage II	Wheat Distiller
MML Silage*	Citrus Pulp
	Peanut Hulls
	Cottonseed

*MML Silage - Mixed Mostly Legume.

ADIN assay

The samples were analyzed using the ADIN assay (Pichard & Van Soest, 1977) using the following procedure:

1. Weigh the sample and record the weight.
2. A 1g sample was boiled for 1 hour in 100 mL of acid detergent (AD) solution, in 600 ml flasks under a cold water refluxing apparatus.
3. Samples were filtered through Whatman 541 filter paper under vacuum and rinsed with boiling water.
4. The samples were rinsed with acetone to remove fat, water and any remaining detergent residues.
5. Nitrogen in the residue was measured using Kjeldahl.

uN_{Ross} assay

Portions of feed samples were analyzed using the uN_{Ross} assay according to the published procedure but with the comparison of 16 h and 30 h incubation time (Ross et al., 2013). Briefly, 0.5g of sample was placed into a 125ml Erlenmeyer flask and 40ml of rumen buffer and 10ml of rumen fluid were added to each flask. Rumen fluid was gathered from two healthy lactating cows at the Cornell University Ruminant Center (CURC). For each cow, the rumen fluid was taken from at least five different positions in the rumen. It was then brought to the lab and filtered. The fermentation buffer was made using trypticase, distilled water, micromineral solution, sodium bicarbonate, macromineral solution, resazurine, cysteine, and reducing solutions (Van Soest, 2015).

Flasks were incubated in a water bath at 39°C for 16 h or 30 h under continuous CO2. Samples were then acidified with 3M HCL to lower the pH to 2. Samples were then incubated in a shaking bath for one hour after the addition of 2ml of pepsin and pH 2 HCl and then neutralized with 2ml of 2M NaOH to stop the pepsin reaction. An enzyme mix containing trypsin, chymotrypsin, lipase, amylase and bile salts were added to the flask and incubated for 24 h in the shaking bath at 39°C. Samples were then filtered through a 1.5 μm glass filter (Whatman 934AH) and boiling water. The N content of the residue was determined by Kjeldahl and expressed as a % of total N in the sample. The definitions of enzyme activity are described in Table 2.

Statistical Methods

The comparison between uN-16hr assay and uN-30h assay was conducted using a two-tailed paired T test, while the comparison between uN-16h assay and ADIN assay was conducted using a two-tailed T test.

Table 2. Enzyme activity definitions

Pepsin	ΔA_{280nm} of 0.001 per min at pH 2.0,37°C measured as TCA-soluble products using hemoglobin.
Trypsin	ΔA_{253nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-arginine ethyl ester (BAEE).
Chymotrypsin	ΔA_{256nm} of 0.001 per min at pH 7.6, 25°C equals one unit using Benzoyl-tyrosine ethyl ester (BTEE).
Amylase	One unit will liberate 1.0 mg maltose in 3 min at pH 6.9, 37°C.
Lipase	One unit releases 1 uEq of acid from olive oil per min.

Results and Discussion

Neutral Detergent (ND) Digestibility

The data of ND digestibility of the corn silage used as a control was 31.0% for the 16 h and 71.8% for the 30 h fermentation demonstrating that the fermentation worked and digestibility continued as expected.

ADIN assay, uN-16h and uN-30h assay

The data of residual N of the samples using ADIN assay, uN-16h assay and uN-30h assay is presented in separate tables for forage feeds (Table 3) and non-forage feeds (Table 4).

For the majority of the feeds analyzed, the uN value after 30 h rumen fermentation was lower than that of 16 h rumen fermentation but was not significantly different for any of the feeds analyzed. Extending the fermentation time for the forages (Table 3) lowered the uN value of the timothy and grass hay and also corn silage I, but not enough to be significant and not uniformly as expected. The uN for the corn silage II and the MML silage were not different for the two time

points of fermentation and that was surprising as we know the NDF digestibility was shown to be different (Ross et al., 2013). The protein in the forage fiber is integrated with the carbohydrate and in this case the hemicellulose, cellulose and lignin matrix which makes it difficult for the digestion enzymes to gain access to the proteins and hydrolyze them from the plant materials. The implication of this is that the rumen incubation would have to be longer than 30 h to potentially digest the fiber adequately to be able to achieve proper comparisons to intestinal digestion.

To evaluate the differences between the ADIN and the uN values for a couple of the forages evaluated in this study with a cattle trial dataset using the CNCPS, the ADIN and uN values from the corn silage II and MML silage (Table 3) were entered in the CNCPS along with the feeding trial data of Hoff (Hoff, 2018). In the Hoff dataset, cattle were fed diets with animal proteins that differed in uN and their productivity was evaluated. Actual milk yields of the cattle were 44.5 and 43.1 kg/d for the high digestibility and low digestibility protein diets, respectively. Using the uN assay evaluations for the animal proteins provided MP allowable milk yields of 45.8 and 43 kg/d for the high and low protein digestibility diets, respectively using uN data for the animal proteins and ADIN for the forages. The forage chemistry was updated with the uN data from the current study for the two forages (corn silage II and MML silage) used in the study by Hoff and the MP allowable milk yields were predicted to be 36.6 and 34.8 kg/d for the high and low digestibility diets, respectively. Thus, the use of the uN values for forages generated in this study demonstrated an obvious over prediction of uN given the higher actual milk yield compared to the MP allowable milk production predicted. This strongly suggests that the uN_{Ross} assay as designed even with the extended fermentation time of 30 h does not effectively predict the indigestible N in forages most likely because the enzymes cannot gain access to the proteins in the plant carbohydrate matrix.

Further work will be necessary to understand the discrepancies between how a cow digests and processes a feed *in situ* versus what occurs in an assay *in vitro*. It is possible there are some other enzymes in the small intestine of the cow that are not present in the assay and this affects the efficiency of degradation and extraction of the fiber bound proteins. For example, an enzyme that might be important for this type of digestion is carboxypeptidase A, which is currently not included in the assay due to cost and the lack of differences in digestibility of many other substrates previously investigated (Ross et al., 2013). Carboxypeptidase A catalyzes the hydrolysis of the carboxyl-terminal peptide bond in peptides and proteins. It is primarily specific to aromatic and hydrophobic side chains such as phenylalanine, tryptophan or leucine and it also exhibits esterase activity. The esterase activity could be important as ester and ether linkages are involved in the bonds between hemicellulose and lignin and anything that could cleave those linkages and help linearize the molecule would potentially enhance the digestibility of the fiber, and improve the digestibility of the associated proteins (Raffrenato et al., 2017).

In comparison, the non-forage feeds again showed no significant difference between the uN assay conducted on samples fermented for 16 or 30 h demonstrating that 16 h exposure to rumen fluid *in vitro* was adequate to provide access of proteolytic enzymes for digestion. This finding is identical to the data generated during the development of the assay (Ross et al., 2013). The values obtained in the current study are similar to values previously observed and reinforce that for non-forage feeds a 16 h *in vitro* fermentation is adequate to provide a viable estimation of the intestinal digestibility in bovines for use in diet formulation.

Summary

In summary, our data show that longer *in vitro* digestibility time results in a lower uN value for forages but not the non-forage feeds. This was not unexpected for the non-forage feeds, but the lack of improvement in predicting intestinal digestibility in forages was unexpected as the longer fermentation time should have provided greater surface area and exposure for intestinal enzymes to digest the associated proteins. Further work is necessary to evaluate the addition of other enzymes to the *in vitro* uN_{Ross} assay to determine if they can improve the efficiency of digestion and provide more accurate and precise predictions of intestinal digestibility of forages.

Table 3. Comparison of unavailable N of forage feeds using uN-16h, uN-30h and ADIN assays

Feed	**Total N, % DM**	**ADIN, % TN**	**uN-16h, % TN**	**uN-30h, % TN**	**uN-16h vs. uN 30h P-value**	**uN-30h vs. ADIN P-value**
Timothy Hay	1.79	12.78 ± 0.05	42.10 ± 13.19	32.69 ± 0.81	0.52	0.02
Grass Hay	1.05	13.30 ± 0.53	62.03 ± 6.53	36.55 ± 1.39	0.14	0.01
Corn Silage I	1.10	6.36 ± 0.69	45.99 ± 1.78	31.86 ± 3.48	0.06	0.05
Corn Silage II	1.31	6.99 ± 0.49	27.09 ± 11.47	25.26 ± 0.16	0.86	0.009
MML Silage	3.73	8.93 ± 0.77	28.19 ± 1.51	29.71 ± 0.30	0.45	0.007

Table 4. Comparison of unavailable N of non-forage feeds using uN-16h, uN-30h and ADIN assays

Feed	**Total N, % DM**	**ADIN, % TN**	**uN-16h, %TN**	**uN-30h, % TN**	**uN-16h vs. uN-30h**	**uN-16h vs. ADIN**
					P-value	**P-value**
Corn Gluten Feed	3.21	8.65 ± 0.65	28.05 ± 5.88	21.60 ± 1.03	0.31	0.13
Corn Germ	4.43	6.52 ± 1.34	21.67 ± 2.46	22.98 ± 4.96	0.85	0.03
Soybean Meal	8.85	3.77 ± 0.29	9.20 ± 2.41	5.97 ± 1.56	0.46	0.19
Wheat Distiller	5.73	17.25 ± 0.34	27.47 ± 5.22	30.97 ± 6.56	0.16	0.22
Citrus Pulp	1.09	13.55 ± 1.17	38.09 ± 8.03	58.30 ± 2.08	0.13	0.14
Peanut Hulls	1.21	40.39 ± 0.99	61.51 ± 3.72	69.36 ± 4.09	0.39	0.06
Cottonseed	3.19	11.08 ± 0.04	29.11 ± 6.26	24.84 ± 1.30	0.44	0.15

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