ABSTRACT

Validation of the Combs -Goeser *in vitro* procedure to test if it improves repeatability and precision of neutral detergent fiber (NDF) digestibility measurements to allow improved near-infrared reflectance spectroscopy (NIRS) calibration.

More precise and accurate estimates of NDF digestibility (NDFD) are needed to better predict forage energy utilization by dairy cattle. Currently, NDFD measurement accuracy and repeatability are weak due to two facets, lab-to-lab variability and run-to-run variability, making it a poor tool for feed formulation. The objective of this research was to validate the Combs - Goeser *in vitro* fiber digestion procedure that was developed and the concurrent Near-Infrared Reflectance Spectroscopy (NIRS) calibration that is based upon the wet data from this procedure. I hypothesize that the new *in vitro* method will significantly reduce variation in measurement of NDFD, and this will allow improved NIRS calibration. This new procedure and concurrent NIRS calibration was validated against the *in vitro* procedures and calibrated NIRS procedures currently used by major commercial forage testing laboratories. A more precise and repeatable NDFD procedure would have great impact on the dairy industry in Wisconsin and the Midwest by allowing nutritionists and producers to more accurately predict forage digestion and ultimately milk production from forage crops.

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TITLE:

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Abstract:

More precise and accurate estimates of NDF digestibility (NDFD) are needed to better predict forage energy utilization by dairy cattle. Currently, NDFD measurement accuracy and repeatability are weak due to two facets, lab-to-lab variability and run-to-run variability, making it a poor tool for feed formulation. The objective of this research was to validate the Combs - Goeser *in vitro* fiber digestion procedure that was developed and the concurrent Near-Infrared Reflectance Spectroscopy (NIRS) calibration that is based upon the wet data from this procedure. I hypothesize that the new *in vitro* method will significantly reduce variation in measurement of NDFD, and this will allow improved NIRS calibration. This new procedure and concurrent NIRS calibration was validated against the *in vitro* procedures and calibrated NIRS procedures currently used by major commercial forage testing laboratories. A more precise and repeatable NDFD procedure would have great impact on the dairy industry in Wisconsin and the Midwest by allowing nutritionists and producers to more accurately predict forage digestion and ultimately milk production from forage crops.

Introduction:

The neutral detergent fiber (NDF) fraction of feedstuffs is an important source of digestible energy for dairy cattle, and the digestibility of NDF is directly related to the amount of energy available to the animal (Hall and Mertens, 2008; Kitessa et al., 1999). NDF is digested by fiber–digesting bacteria that grow in the rumen of dairy cattle; the major bacteria species involved include: *Bacteroides succinogenes, Ruminococcus flavefaciens, Ruminococcus albus, Butyrivibrio fibrisolvens, Bacteroides rumincola,* and other *Ruminococcus spp.* (Van Soest, 1994). NDF is the collective term for the components of the plant cell wall, which is mainly composed of cellulose, hemicellulose, and lignin. None of the aforementioned cell wall components are easily digestible by

polysaccharide composed of unbranched D-glucose molecules linked by ß 1→4 glycosidic bonds, which are difficult to break down and are slowly hydrolyzed by special microorganisms in the rumen that contain the cellulase enzyme. Cellulose digestion is limited by the amount of time the feed is retained in the rumen. Hemicellulose is a heterogeneous polymer of pentoses, hexoses, and sugar acids; the plethora of different glycosidic bonds make the polysaccharide more difficult to hydrolyze than starch, yet the random structure gives the molecule little tensile strength making it more digestible than cellulose (Badal, 2003). Lignin is indigestible in the rumen because there are no microorganisms present with the ability to break down the molecule. Thus, of these components hemicellulose is the most digestible, cellulose is less digestible, and lignin is indigestible. Furthermore, there are interactions between the three cell wall components; for example, lignin has the ability to crosslink with cellulose and further lower the digestibility of cellulose. Thus, depending on the proportions of hemicellulose, cellulose, and lignin in the cell wall and the level of interactions between them, the overall digestibility of NDF can vary.

It is important to note that the animal cannot derive energy from ingested fiber (NDF) unless it is first digested by the microbes that live in the animals' rumen, thus the digestibility of NDF is related to the amount of energy available from a feed. As previously mentioned, the digestibility of NDF varies amongst different feeds, and thus testing of NDF digestibility (NDFD) has become commonplace to estimate the energy content of feedstuffs. Accurate NDFD measurements are essential because NDF can account for as much as 30% of the energy value of a forage (Oba and Allen, 1999). Commercial laboratories in the Midwest routinely analyze thousands of forage samples each year to predict the energy content of forages from their energy yielding components

used: Dairyland labs and Rock River labs, two of the largest forage testing labs in the Midwest report that 28% and 34%, respectively, of forage samples submitted are analyzed for NDFD (Dr. David Combs, 2008).

Currently the most common methods used to test NDFD are modified versions of the Goering and Van Soest *in vitro* digestion procedure (1970). Briefly, the original procedure involved incubating feed in rumen fluid and an artificial buffer solution, then measuring how much dry matter disappeared after 96 hours of fermentation. The Goering and Van Soest procedure was not created to be used for short term fiber digestibility analyses; rather it was created to estimate digestion of total dry matter within the entire intestinal tract over a long period of time (Goering and Van Soest, 1970). The modified procedures that are currently used in the industry attempt to mimic the microbial digestion of the rumen, where most of the fiber digestion occurs. The procedure has been modified by incubating the feed for much shorter periods of time (24, 30, and 48 hours) to simulate the time that forages would normally be retained in the rumen. Furthermore, the procedure was modified to measure NDF disappearance rather than dry matter disappearance (Grant and Mertens, 1992; Goering and Van Soest, 1970).

The modified procedures are much more variable than the original procedure. It is well-documented that there is substantial run-to-run variability associated with the modified procedures i.e. the variance values (standard deviation and standard error) are high between trials and within trials (Iantcheva et al., 1999; Grant and Mertens, 1992a; Madsen et al.,1997; Nelson et al., 1972). To further complicate matters, different labs have further modified the short-term fermentation procedures by altering their respective fermentation methods, which has lead to high lab-to-lab variability. It is well-documented

at 24, 30, and 48 hours.

More specifically, different buffers, vessel type, method of carbon dioxide gassing, and the use of reducing solution and/or additives can lead to different NDFD values.

First, changes in the pH of the inoculum due to different buffers employed result in significantly different NDFD values ($p<0.05$). Lower pH (5.8) of the buffer results in significantly decreased NDFD values relative to a buffer pH of 6.8. With respect to the NDF kinetics, a decreased pH significantly lowers NDF rate of digestion and increases lag time (Grant and Mertens, 1992; Grant and Weidner, 1992).

Second, different vessel types used during incubation result in significantly different NDFD values ($p<0.05$), with the use of Erlenmeyer flasks resulting in the most effective NDF digestibility (Hall and Mertens, 2008; Grant and Mertens, 1992).

Third, the method of carbon dioxide gassing also results in significantly different NDFD values ($p<0.05$), with constant carbon dioxide gassing throughout preparation of the inoculum and incubation resulting in superior NDF digestion. Relative to NDF kinetics, constant gassing results in significantly decreased lag time ($p<0.05$), and increased rate of NDF disappearance relative to purging with carbon dioxide solely at inoculation (Grant and Mertens, 1992; Hall and Mertens, 2008).

Lastly, use of reducing solution and nutrient additives in the inoculum can result in significantly different NDFD values. Superior NDF digestion occurs when both are used because the lag time is significantly reduced ($p<0.05$) (Grant and Mertens, 1992).

To summarize, currently NDFD measurement accuracy and repeatability are poor due to two facets, lab-to-lab variability and run-to-run variability. It is essential that research and commercial labs adopt one common method and remove lab-to-lab

in vitro procedure could be adopted by labs, there would be significantly improved estimates of forage quality.

Run-to-run variability is mostly due to natural differences in the inoculum, which is the medium of rumen fluid and artificial buffer solution that is used in fiber-digestibility procedures (Nelson et al., 1972). However, it has been demonstrated that the inoculum can be standardized by priming it with substrates before inoculation. Nelson et al., demonstrated that adding nutrients to the inoculum resulted in a decreased standard deviation between trials, from 2.81 with no nutrients added to 1.74 with nutrients added. Furthermore, in a previous study, I examined the effect of priming the inoculum with four different levels (control, 0.015g, 0.030g, and 0.045g) of the disaccharide, cellobiose, before inoculation. I calculated the standard deviations from mean gas production (which is an indicator of feed breakdown by bacteria) between different trials to determine if priming the solution lowered run-to run variability between the trials. A lower standard deviation indicated a decrease in run-to-run variability. The results showed that priming the inoculum made the rumen fluid fermentative activity more consistent by allowing bacteria to regenerate, and thus erasing the tremendous variability of bacteria number and type. There is high variability of the bacteria in the rumen fluid due to the shock the bacteria ensue when being transported from the rumen to the lab and the natural discrepancy of rumen bacteria attributed to diet and time of feeding (Nelson et al., 1972). Allowing the bacteria to regenerate resulted in decreased run-to-run variability. The mean standard deviation from the 0.030g group was significantly lower than the control standard deviation, with a p-value less than 0.05 (Goldschmidt, unpublished data). This research supports our labs' hypothesis that run-to run variability can be reduced by altering the inoculum in the *in vitro* procedure.

redeveloped the *in vitro* procedure (Combs – Goeser *in vitro* procedure) so that it specifically addresses fiber digestibility at shorter time frames and minimizes variability. We hypothesize that by adding a step to standardize rumen fluid inoculum and modifying the *in vitro* assay to reduce gravimetric errors associated with improper weighing and rinsing of feed residues after incubation, we can improve repeatability compared to the modified Goering and Van Soest (1970) procedures most widely used in research and commercial labs. The first objective of this research is to validate that the Combs – Goeser *in vitro* procedure improves repeatability and precision relative to the current procedures used in the major commercial laboratories.

In association with validating the new *in vitro* procedure, I will also be validating the Near-Infrared Reflectance Spectroscopy (NIRS) procedure that is coupled with the wet (*in vitro)* data. NIRS is a quick and inexpensive method capable of analyzing feed composition, and it is frequently used in the industry for this reason (Lawler et al., 2006; Foley et al., 1998). NIRS relies on establishing a statistical relationship between the spectrum of near-infrared light reflected by a sample and the spectrum of light known to be reflected by a set component of interest.

It is important to note that for NIRS to be effective it requires a calibration equation, which is set by reference data from an *in vitro* procedure The accuracy of the calibration equation relies on the accuracy and repeatability of the base data (Foley et al., 1998; Kitessa et al., 1999). Poor precision of the *in vitro* NDFD assay has limited the use of NIRS for accurate NDFD measurement. Currently, NIRS has a very poor calibration coefficient of determination (R^2) for NDFD of approximately 0.6 (Mentick et al., 2006; Redshaw et al., 1986 as cited in Kitessa et al., 1999). Even though NIRS produces variable NDFD measurements, it is still used in the industry for NDFD assays.

Soest (1970) procedure, and widely use this method to predict nutrient components, such as NDF and NDFD (Foley et al., 1998). Thus, the second objective of my research is validating that the calibration equation for NIRS is improved when using reference data from the improved Combs – Goeser *in vitro* procedure versus the currently used protocols.

Furthermore, the data gathered by this research will be used to view the overall variance that is present in the current procedures. Numerous studies exist that observe the effect of slightly different lab procedures, or that document run-to-run variability within a single procedure. Yet, no data exists that quantifies the overall variability in the current procedures used, or that locates the main source of variability. The plethora of data collected from commercial labs in this project will be used to observe the overall trends of variance associated with NDFD measurements.

This research is important because forage analysis results are used as the basis of feed formulation in dairy cows to maximize milk production. Currently in the industry there is a gap between predicted value of a forage and actual observed production values (Kitessa et al., 1999; Iantcheva et al., 1999; Adesogan et al., 1998). High NDF digestibility has two expected *in vivo* responses in a ruminant: higher milk production due to more available energy, and higher feed intake. Voluntary feed intake is mandated by the amount of fill in the rumen. Because fibrous material is slowly digested, the amount of fiber in the rumen is the main determinant for fill, thus when NDF is digested more rapidly there is decreased physical fill and the animal will have an increased voluntary feed intake (Oba and Allen, 1999). Currently, *in vitro* and NIRS methods poorly predict milk production and feed intake levels. I hypothesize that decreased variation in NDFD measurements will allow for accurate prediction of forage digestion,

industry in Wisconsin and the Midwest.

Methods:

To validate that the Combs - Goeser *in vitro* procedure decreases run-to-run variability and decreases variability in the concurrent NIRS procedure, the following study was conducted. A standard alfalfa silage feed was sent anonymously over the course of three weeks (once per week) to four major commercial labs: UW Soil and Plant Analysis lab (Marshfield, Wisconsin), Dairy One (730 Warren Road, Ithaca, New York 14850), Cumberland Valley Analytical Services, Inc. (PO Box 669, Maugansville, MD 21767), and Dairyland Labs (217 E. Main St., Arcadia, WI 54612). All of the labs provide wet chemistry analysis of forage NDF and NDFD, and three of the labs provide NIRS analysis for NDF and NDFD. All commercial laboratories use their own modified procedure of the original Goering and Van Soest (1970) procedure. The three laboratories which run NIRS use their respective *in vitro* data to set the NIRS calibration equation. The silage was also run in our lab, which uses the Combs - Goeser *in vitro* procedure and concurrent NIRS calibration equation.

The silage was dried (60° C for 48 h), ground through a 2mm screen in a Wiley mill, and then approximately 20 grams of the test feed was packaged in separate sample bags. Each bag was uniquely labeled so that it was not readily apparent that each bag contained feed from a single source. Each week, three separate sample bags of the test feed was sent to each lab for wet chemistry analysis, and three separate sample bags were sent for NIRS analysis. This allowed for observation of both within run variance and run-to-run variance of the respective labs *in vitro* procedures and calibrated NIRS procedures.

The method used by our lab is as follows:

gram forage samples are first divided into 0.5 sub-samples and are placed into an Ankom F57 fiber bag and heat sealed. The day prior to inoculation, the sealed bags are placed in 125 ml Erlenmeyer flasks. Thirty millilters of pre-media solution is added to each flask, as well as 40 ml of a combination of pre-media solution and buffer. The combination of pre-media solution and buffer is made in bulk and contains 1,500 ml pre-media solution and 500 ml of buffer solution. The chemical components of each solution are presented in table 1 in the appendix. The flasks are sealed with a rubber stopper, which is connected to a carbon dioxide manifold to allow for constant CO_2 gassing. The flasks are then placed in a 39°C shaking water bath for the night so that the contents can be thoroughly purged with CO_2 and warmed.

The next major step of the *in vitro* procedure occurs the following morning, and it involves preparing the inoculum. First, 0.3215 g of primer, 250 ml of buffer solution, and 50 ml of reducing solution (table 1) is added to each 1000 ml Erlenmeyer flask. The primer consists of 40% cellulose, 20% urea, 20% starch, and 20% cellobiose; this combination is based off the ratio fed to dairy cattle. The flasks are then held under constant CO_2 gassing until the rumen fluid is added.

Rumen fluid is collected into pre-warmed thermoses from cannulated-lactating cows. The rumen fluid is strained through four layers of cheesecloth in a warm room (39°C). Strained rumen fluid (250 ml) is added to each of the pre-prepared 1000 ml Erlenmeyer flasks. The flasks, which now contain rumen fluid, buffer solution, reducing solution, and primer are then placed within a shaking incubator (39°C). The flasks remain sealed in the shaking incubator until they reach a pressure of 20 inches of water, which indicates that the microbial population is breaking down feed and releasing gas. Once the

within the water bath.

Samples are removed from inoculation at 0, 4, 24, 48, 54, and 72 hours after the start of inoculation. Fermentation is halted by rinsing the bags with cold, distilled water. The amount of NDF in the bag is then determined by using a neutral detergent solution containing ∝ amylase and sodium sulfite in an Ankom200 Fiber Analyzer. NDF residue remaining after incubation for 24, 30, and 48 hours is determined by the following equation:

NDF residue (% of DM) = [((bag wt. + residue)-(bag wt. X bag correction factor))/((bag wt. + sample)-(bag wt.)] X 100.

NDF digestion is calculated by comparing the ratio of NDF residue remaining after incubation to the amount of NDF of the feed originally weighed into the bag. The formula for this calculation is:

NDF digestibility (% of NDF) = 100 – (NDF residue (expressed as a % of DM) /(NDF of the original feed sample (expressed as a % of DM)

The calibration equation for the NIRS procedure is then set using data from the Combs - Goeser *in vitro* procedure explained above.

The dry matter, NDF, NDFD – 30 hour, and NDFD – 48 hour values collected from the different procedures were analyzed using the SAS computer program. Dry matter was analyzed because it is a standardized procedure, which the forage testing labs in this study are certified by the National Forage Testing Association. Dry matter should have a low variance compared to the other measurements; in essence it was used as a pseudo control allowing observation of variance produced by the different labs of an easily measured compound (rather than NDFD, which is difficult to analyze). The data was first analyzed using the Nested analysis of variance procedure, allowing separation

measurements produced by *in vitro* and NIRS were observed using the difference in least square means. Lastly, the respective repeatability of each lab's *in vitro* and calibrated NIRS procedure was evaluated. *In vitro* and NIRS procedures were evaluated separately. Total variance for a set component (NDF, NDFD – 30, or NDFD – 48) refers to the standard deviation of all measurements produced from all runs. Within run variance was calculated by averaging the standard deviation associated with each run, i.e between the three samples that were sent per run. Run – to run variance was determined by averaging the respective component values from a run, and then calculating the standard deviation between the three mean run values. It is important to note that not all commercial labs run both NDFD – 30 and NDFD – 48, and that all labs are coded in the results section so that it is not apparent within this paper which lab produced which measurements.

Results and discussion:

Source of variability in DM, NDF, NDFD – 30 hour, and NDFD – 48 hour measurements

The greatest contributor to variance in DM, NDF, NDFD – 30, and NDFD - 48 measurements is from lab – to – lab variability. Figure 1 shows the source of variation between all measurements of a component (*in vitro* and NIRS). Within this model, percent variance due to the method used (*in vitro* vs. NIRS) is low indicating that the wet data and NIRS are well coupled and produce similar measurements. For DM, percent variation due to run - to - run and within run variability are also low, which is expected since analysis of DM was used as a pseudo control. For NDF, both variance due to run - to – run variability and within run variability did not substantially contribute to overall variance, this is also expected because like DM, NDF analysis is a certified procedure for all laboratories. On the contrary, within run variance is a large source of overall variance

procedures are much less repeatable. However, the percent variance due to run – to - run variability is not substantially higher in digestibility values than in DM and NDF. But, this is due to the digestibility procedures biasing the results rather than the procedures being as repeatable as the DM and NDF analyses. Laboratories will add a known standard sample to each run of unknown samples when testing for NDFD, and will then adjust the unknown values according to the standard. In other words, if the standard sample results in a higher than normal NDFD value, all samples (including the unknowns) will be reduced accordingly to decrease the standard to its' expected value. Thus, laboratories attempt to reduce the run-to-run variability by adjusting the results of the unknown sample to a feed standard used within the individual lab. It should be pointed out that labs assume that there is no interaction between feed composition and run - to - run variability. This assumption is probably erroneous because fiber digestion is affected by the amount of starch, protein and lignin contained in the feed.

			Percent variance due to:			
Assay	**Mean**	**SE**	**Method**	**Lab**	**Run**	**W/in run**
DM	94.6	0.042	< 1%	96.9%	2.2%	0.9%
NDF	45.7	0.758	< 1%	86.7%	6.3%	7.0%
NDFD - 30	43.5	1.22	< 1%	73.2%	7.1%	19.7%
NDFD - 48	48.1	1.31	9.7%	74.8%	2.2%	13.3%

Figure 1: Sources of variation between all measurements (in vitro and NIRS)

The source of variation in measurements produced only by the *in vitro* procedures is shown in Figure 2. The same general trend is seen as in figure 1: the largest percent of variance is due to lab – to - lab variability, and the digestibility measurements have a

DM measurements.

			Percent variance due to:		
Assay	**Mean**	**SE**	**Lab**	**Run**	**W/in run**
DM	94.7	1.1	97.6%	2.2%	0.3%
NDF	45.0	1.4	92.2%	3.0%	4.8%
NDFD - 30	44.8	3.3	83.6 %	5.5 %	10.9%
NDFD - 48	49.4	1.7	87.1%	3.5%	17.4%

Figure 2: Sources of variation between in vitro measurements

The source of variance in DM, NDF, NDFD – 30, and NDFD – 48 values measured only by the NIRS procedures are shown in Figure 3. Lab – to – lab variability is the highest contributor to the overall variance in DM, NDF, and NDFD - 48. However, percent variance due to lab – to – lab variability is low for NDFD – 30 values. This is most likely due to the low number of data points available for this measurement, rather than repeatability within different NIRS calibration equations.

			Percent variance due to:		
Assay	**Mean**	**SE**	**Lab**	**Run**	**W/in run**
DM	94.6	1.3	96.5 %	2.2 %	0.1 %
NDF	46.5	0.8	74%	13.8%	12.1%
NDFD - 30	42.3	0.4	<1%	18.3%	79.3%
NDFD - 48	46.8%	1.4	78.6%	1.4%	19.9%

Figure 3: Sources of variation between NIRS measurements

Effect of lab procedure on NDF, NDFD – 30, and NDFD – 48 measurements

values, as well as the overall mean (horizontal line) of generated values is documented below.

NDF

Different *in vitro* laboratory procedures produce significantly ($p < 0.05$) different NDF values. The only comparisons that were not statistically significant were between the NDF values generated by the Combs – Goeser and lab 4 procedures, and between the NDF values generated from lab 1 and lab 3 procedures. Conversely, only one NIRS calibration produced a significantly ($p<0.05$) different NDF value compared to the other laboratories.

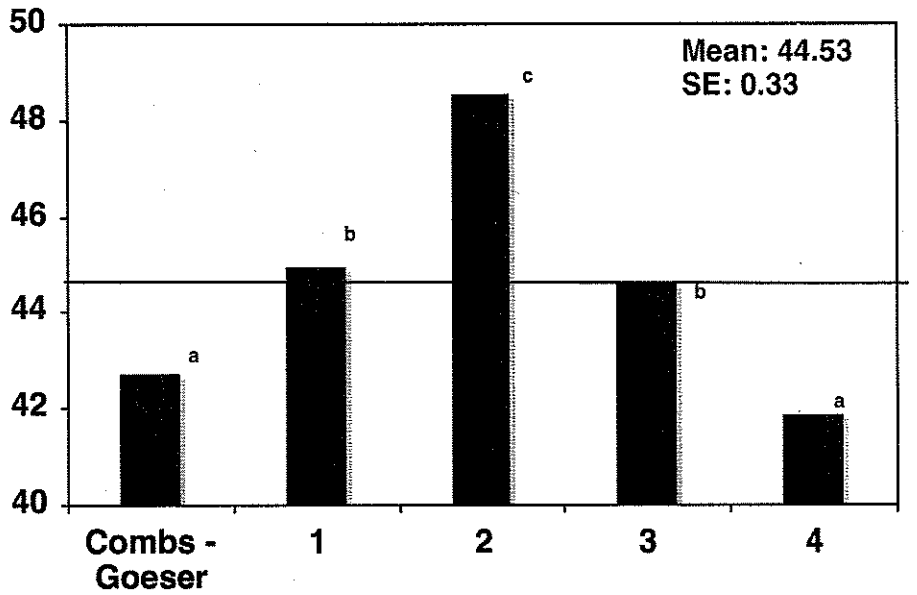


Figure 4a: Mean NDF values from different laboratory in vitro procedures, laboratories with different superscripts have significantly (P < 0.05) different mean NDF values

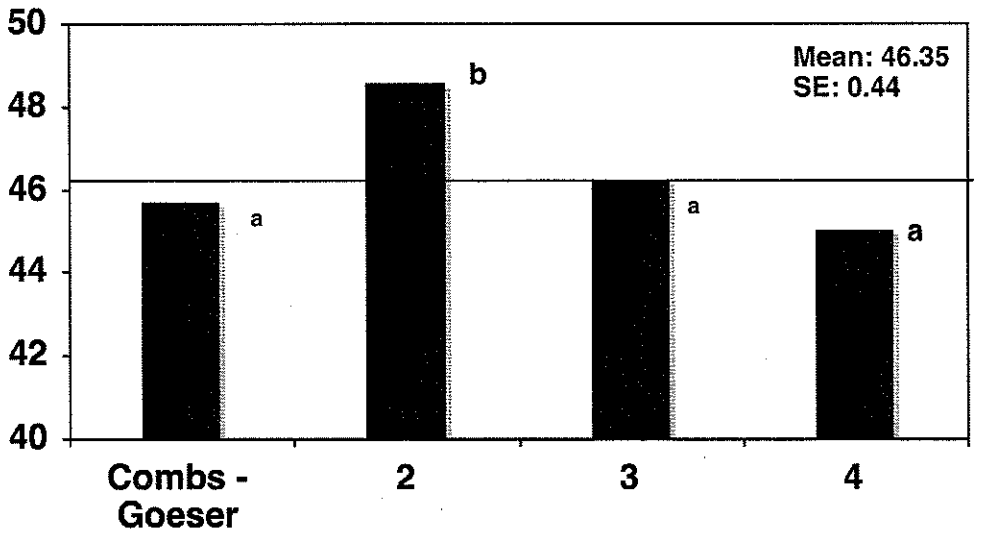


Figure 4b: Mean NDF values from different NIRS calibrations, laboratories with different superscripts have significantly (P < 0.05) different mean NDF values

NDFD – 30 hour

Different *in vitro* procedures utilized by the different labs produced significantly ($p < 0.05$) different NDFD – 30 values. None of the NDFD -30 values generated by the different NIRS calibrations are statistically different.

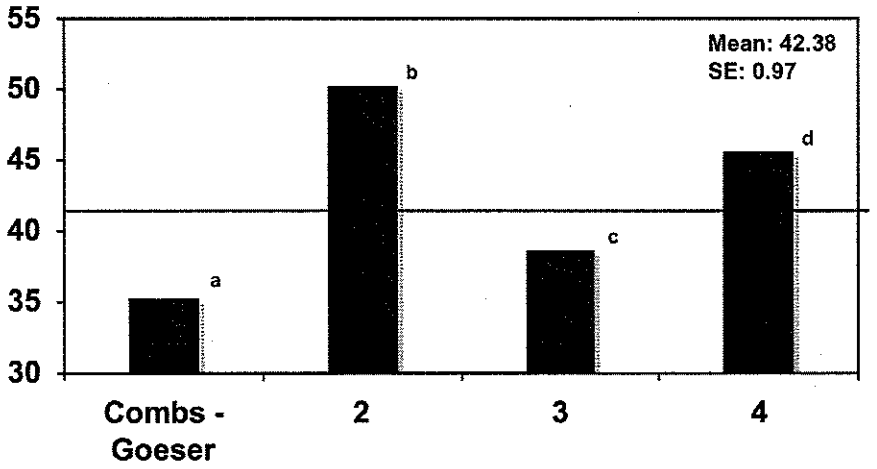


Figure 5a: Mean NDFD – 30 values from different in vitro procedures, laboratories with different superscripts have significantly (P < 0.05) different mean NDFD - 30 values

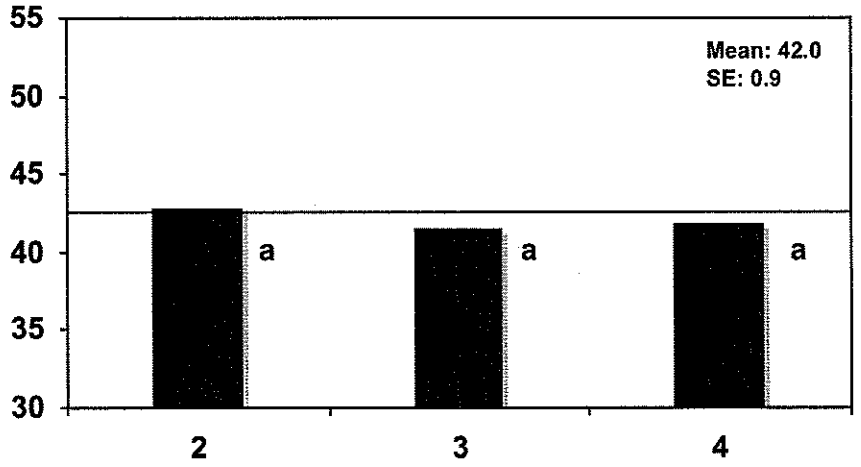
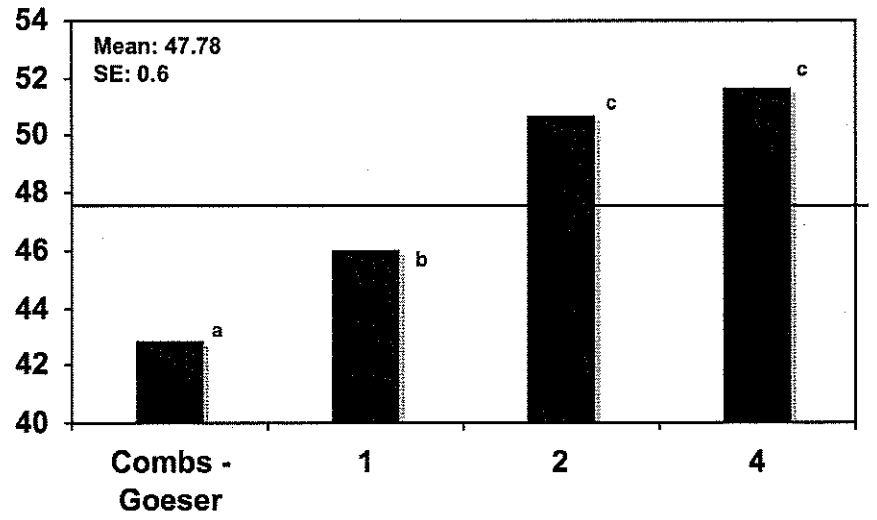


Figure 5b: Mean NDFD – 30 values from different NIRS calibrations, laboratories with different superscripts have significantly (P < 0.05) different mean NDFD - 30 values

NDFD – 48 hour

Once again, different *in vitro* procedures utilized produce significantly ($p < 0.05$) different values. With the exception of lab 2 and lab 4, which produced NDFD – 48 values that were not significantly different from each other. Only the NIRS calibration utilized by lab 2 produced a significantly different measurement than the other laboratories.



different superscripts have significantly (P < 0.05) different mean NDFD - 48 values

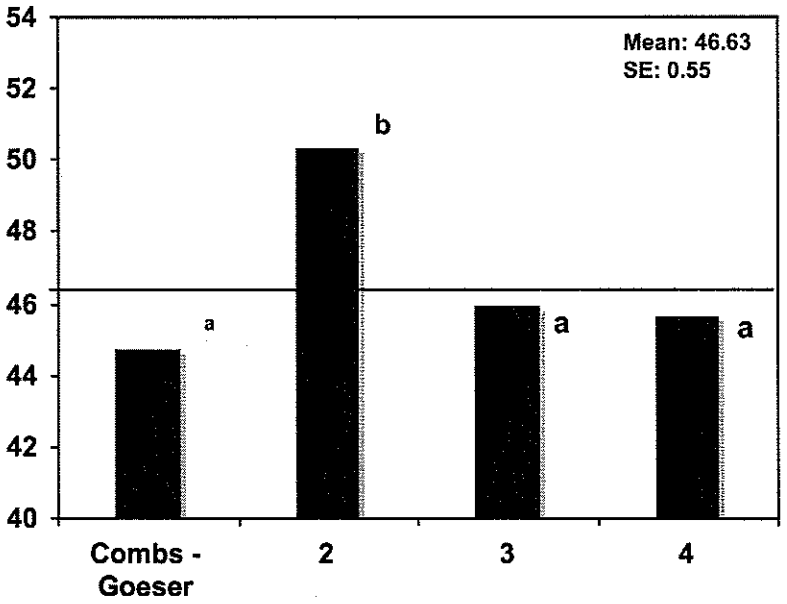


Figure 6b: Mean NDFD – 48 values from different NIRS calibrations, laboratories with different superscripts have significantly (P < 0.05) different mean NDFD - 48 values

Variability of Combs – Goeser in vitro procedure versus commercial laboratories

The Combs – Goeser *in vitro* procedure variability in the measurement of NDF, NDFD – 30, and NDFD – 48 relative to commercial laboratories is shown below.

Concerning NDF analysis (figure 7), the Combs – Goeser procedure has low total variability and run – to - run variability compared to the other labs. Yet, the within run variance is moderate compared to other labs, and all lab procedures have fairly low variability associated with the measurement of NDF.

The Combs – Goeser procedure has the lowest run – to – run variance associated with the measurement of NDFD 30 (Figure 8). Yet, total variance and within run variance were both extremely high. This data implies that the Combs – Goeser procedure may

on the procedures' ability to increase repeatability of the measurement of NDFD – 30 cannot be made.

Lastly, the Combs – Goeser procedure has the highest amount of variance associated with the NDFD – 48 measurement (figure 9). Thus, it can be concluded that the Combs – Goeser procedure does not lower variability in NDFD – 48 analysis compared to the commercial *in vitro* procedures.

Overall, thirty hour NDFD measurements had more variance than 48 hour NDFD estimates. For the NDFD - 30 and NDFD - 48 *in vitro* measurements, lab 3 had consistently lower total variance in their lab assay than any of the other lab methods, including the Combs - Goeser method.

Furthermore, lab 3 consistently had the lowest within run variances. This could be due the number of replicates they use for each *in vitro* analysis, or it could be because this lab's *in vitro* method and lab techniques are superior to the other labs. However, it is unknown if they run duplicate or triplicate samples of each unknown, if they run three or more samples, if they throw out an apparent outlier, or if they report the average of all samples. Within run variance can be greatly minimized if the average of triplicate samples is reported versus duplicates. Combs - Goeser values are based on duplicate samples within runs, and no outliers are thrown out.

As far as run – to - run variance, all labs use a standard feed to adjust values and reduce run – to – run variance, excluding the Combs - Goeser procedure. However, it is important to note that adjusting values does not result in similar measurements between labs - it was shown that the average measurements vary widely between the labs.

Lab	Total variance	Within run variance	Run - to- run variance
1	0.788032007	0.263240249	0.839585436
2	0.938527215	0.716381267	0.608200138
3	0.462180821	0.288080473	0.422075998
4	1.071052183	0.406338659	1.050742242
Combs - Goeser	0.555895174	0.457811262	0.440887153

Figure 7: Variability of NDF measurement

Lab	Total variance	Within run variance	Run - to- run variance
2	3.527668415	1.912425411	2.268953095
3	0.888037787	0.313798314	0.971444133
4	1.490165912	0.681353796	1.135215757
Combs - Goeser	2.39042767	2.617247441	0.810331437

Figure 8: Variability of NDFD – 30 measurement

Lab	Total variance	Within run variance	Run - to- run variance
1	1.099751234	0.694024924	0.995167955
2	1.936491673	0.681353796	0.881917104
3	0.650128192	0.650813477	0.141421356
4	1.263087487	0.312191847	0.935737974
Combs - Goeser	2.270042829	1.900998321	1.563913657

Figure 9: Variability of NDFD – 48 measurement

Variability of Combs – Goeser NIRS calibration equation versus commercial laboratories

The Combs – Goeser NIRS calibration equation is moderately variable in measuring NDF and NDFD – 30 compared to the other laboratories' calibration equations (Figure 10, Figure 11). However, the Combs – Goeser calibration equation is much less variable in measuring NDFD – 48. This data implies that Combs – Goeser calibration equation for NDFD – 48 is much more precise than the other commercial calibration equations. Yet, it is not prudent to assume that this procedure is more accurate than other

which is variable.

Lab	Total variance	Within run variance	Run - to- run variance
2	0.324465372	0.295751297	0.153960072
3	1.0252371	0.225509125	1.14519932
4	0.846632217	0.874707942	0.199786924
Combs - Goeser	0.615726761	0.592198258	0.315899656

Figure 10: Variability of NDF measurement

Lab	Total variance	Within run variance	Run - to- run variance
2	3.333333333	2.571816242	2.268953095
3	0.670820393	0.389615168	0.669161997
4	2.409997118	0.450141662	2.226510307

Figure 11: Variability of NDFD – 30 measurement

Lab	Total variance	Within run variance	Run - to- run variance
2	1.5	1.271238936	1
4	1.10449209	0.563216684	0.992171207
Combs - Goeser	0.49911032	0.461552788	0.272892599

Figure 12: Variability of NDFD – 48 measurement

Conclusion:

The Combs – Goeser *in vitro* procedure does not significantly lower variability relative to the commercial procedures. The NIRS calibration does appear to be more repeatable, yet the accuracy of this data is questionable.

The bottom line of this study is that it is one of the first attempts to compare labs in their analysis of NDFD. Laboratories clearly struggle with NDFD measurements, and the final reported number varies considerably from lab to lab. However, labs are more effective in "controlling" between run variation than was initially suspected. This is probably because they use a standard feed and adjust the results of the tested feeds to this unknown. The Combs – Goeser method did not stand out as much as was anticipated, but

variation, and we did not throw out or re-run any samples. Our approach to comparing our assay to commercial labs fell short because we did not account for how these labs may try to control within and between run variance.

The overall conclusion that should be taken away from this data is that lab – to – lab variability is the problem that must be addressed immediately. Lab – to – lab variability was the largest contributor to overall variance in both *in vitro* and NIRS procedures. Furthermore, it was shown that different *in vitro* procedures produce significantly different measurements for the same silage. Thus, it is essential that one common *in vitro* method is adopted by all commercial laboratories to erase this tremendous source of variability. It is essential that the NDFD variability be lowered by accepting one common *in vitro* procedure, to better predict forage energy utilization by dairy cattle

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Appendix :

Table 1: *chemical components of solutions used within the in vitro procedure*

Pre-media solution (for 150 samples)

- 3000 ml distilled H_2O
- 1500 ml Solution A
- 12.0 g trypticase peptone
- 0.6 ml Solution B
- 6.0 ml resazurin indicator

Solution A:
- 18.0 L distilled H_2O
- 102.6 g Na_2HPO_4
- 111.6 g KH_2PO_4
- 10.5 g $MgSO_4 \cdot 7H_2O$

Solution B:
- 13.2 g $CaCl_2 \cdot 2H_2O$

- 1.0 g $CoCl_2 \cdot 6H_2O$
- 8.0 g $FeCl_3 \cdot 6H_2O$
- Brought to 100ml with distilled H_2O

Buffer solution
- 18.0 L distilled H_2O
- 630 g $NaHCO_3$
- 72.0 g NH_3HCO_3

Reducing Solution
- 1.875 g Cysteine HCl
- 1.875 g $Na_2S.9H_2O$
- 290 ml distilled H_2O
- 12 ml 1M NaOH