# Near infrared spectroscopic analysis of intact grass silage and fresh grass for dry matter, crude protein and acid detergent fiber

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## Introduction and objective

The analysis of intact fresh grass and grass silage by near infrared (NIR) spectroscopy to quickly determine the chemical composition and nutritional value for dairy cattle is an attractive prospect due to the increased demand for rapid analysis results. Farmers no longer want to wait for samples to be dried and analyzed by conventional methods. The demand for same day analysis is increasing.

The objective of this study was to evaluate the efficiency or error of NIR spectroscopy calibrations for the prediction of dry matter, crude protein and acid detergent fiber (ADF) on intact fresh grass and grass silages.

## Materials and methods

Fresh grass and ensiled grass samples were collected from 138 different dairy farms located in the Southwestern corner of British Columbia in Southwestern Canada. The samples were brought to the lab where they were mixed and presented in quadruplicate in a natural products cell in their intact (undried, unground) form to an NIRSystems 6500 instrument (Perstorp Analytical, Silver Spring, MD) equipped with a transport device. The samples were not cut or changed in any way once they were received in the lab so moisture losses were minimized. This meant the samples were coarse, uneven in size, shape and composition. Some of the samples were a mixture of leaves, stems and species. Spectral information was collected from 400 to 2500 nm at 2 nm intervals in the reflectance mode and the four spectra were averaged for each sample.

The samples were chosen to include as much variation as possible in the growing area and the variation included:

1. Two crop years—1993 and 1994. The 1993 crop year was characterized by a warm, dry growing season while the 1994 crop year was cool and wet. Four samples from the 1992 crop year were included in the sample set as well and these samples had been ensiled for more than a one year period.

2. Different harvests or cuts from each crop year were also included. These samples covered a range of quality and maturities and included high quality as well as poor quality samples. Poor quality grass silages included poorly fermented samples, samples high in acid detergent insoluble nitrogen and fiber and samples low in protein and dry matter. The sample set included 39 fresh grass samples and 244 ensiled samples to give a total of 283 samples.

Table 1 demonstrates the breakdown of samples collected over the crop years and the number of samples collected from each harvest. Due to weather conditions, the majority of the first cut harvest in the area is ensiled so the majority of the grass silage is a first cut forage. After the first cutting, more of the harvest is stored as hay resulting in less later-cut silages available as indicated by their numbers in the table.

3. Different chop lengths were represented. The chop length of the fresh grass was up to 20 cm long while the ensiled samples were 1.5–2.5 cm long.

4. Different grass species and mixtures were sampled and are listed in Table 2. Orchardgrass and perennial ryegrass combinations are common in the area so the majority of the samples reflected this factor. Orchardgrass/ryegrass combinations in different ratios totalled 201 out of the 283 samples.

Pure varieties such as orchardgrass, ryegrass and tall fescue were sampled as well. Also, seventeen ryegrass varieties from a research variety trial were included and were scanned in the fresh, unwilted form, ensiled in research silos for 34 days and scanned again after ensiling.

5. Different additives were used on the farms to aid the ensiling process and the samples included different additives as indicated in Table 3. Approximately 35% of the samples were ensiled with a microbial inoculant and/or enzyme, 6% with an acid such as propionic and 59% were ensiled without the aid of an ensiling additive or were sampled fresh before ensiling.

6. Storage facilities for the grass silages were varied (Table 4) with the majority of farmers utilizing bunker silos. Samples were obtained from other types of storage facilities as well, such as ag bags, oxygen limiting silos, tower silos, research silos and round bales.

After spectra were collected, the samples were sent to the lab for analysis. The samples were split and a portion was dried at 60°C in a forced-air oven for subsequent grinding and chemical analysis and another portion was dried for 24 hours at 105°C for dry matter determination. Crude protein was determined by the Kjeldahl method and ADF was determined by the method of Goering and van Soest.[1] All samples were analyzed in duplicate and the results converted to a dry matter basis.

**Table 1. Number of fresh grass and grass silage samples by crop year and cut number.**

| Crop year | Cut #1 | Cut #2 | Cut #3 | Cut #4 | Cut #5 | Cut #6 | Total |
|---|---|---|---|---|---|---|---|
| 1992 | 2 | 1 | 1 | 0 | 0 | 0 | 4 |
| 1993 | 50 | 38 | 22 | 13 | 13 | 0 | 136 |
| 1994 | 81 | 27 | 9 | 5 | 18 | 3 | 143 |
| Total | 133 | 66 | 32 | 18 | 31 | 3 | 283 |

**Table 2. Grass species mix and combinations in the sample population.**

| Species | # of samples |
|---|---|
| Orchardgrass/perennial ryegrass mix | 201 |
| Perennial ryegrass | 34 |
| Orchardgrass | 33 |
| Tall fescue | 3 |
| Orchardgrass/tall fescue grass mix | 6 |
| Orchardgrass/clover mix | 4 |
| Miscellaneous | 2 |
| Total | 283 |

**Table 3. Ensiling additives.**

| | # of samples | % of samples |
|---|---|---|
| None | 166 | 59 |
| Enzymes and/or microbial inoculants | 100 | 35 |
| Acid (propionic) | 17 | 6 |
| Total | 283 | 100 |

## Results and discussion

When the population was viewed in Symmetry, within the ISI Software (InfraSoft International, Port Matilda, PA), there appeared to be a second population that included the fresh, unwilted rye grass variety trial samples and their associated research silo samples. They were all low in dry matter with values ranging from 14 to 18%. Some of the samples that appeared between the two populations were fresh, wilted grass samples. The remainder of the fresh samples overlapped the main population of fermented grass.

The sample set of 283 samples was characterized by the statistics listed in Table 5. The large dry matter range from 13.9 to 72.3% resulted in a standard deviation of 13.04%. The range for crude protein corrected to a dry matter basis was from 9.5 to 27.8% and the range for ADF was from 22.6 to 46.6%, which provided reasonably large standard deviations of 3.5 and 3.9%.

There are many different approaches to the analysis of data and for this paper the results were analyzed using ISI software and all 283 samples were treated as one, broad based, multi-species population. The sample set was ordered by global “H” and every 5th sample was selected for a validation set.

Prediction equations were developed on 226 samples utilizing modified partial least squares (PLS) regression and using both no scatter correction and SNV and detrend along with different

**Table 4. Storage facilities.**

| | # of samples |
|---|---|
| Fresh grass | 39 |
| Bunker silos | 166 |
| Ag bags | 25 |
| Oxygen limiting silos | 18 |
| Tower silos | 14 |
| Research silos (34 days) | 13 |
| Round bales (plastic wrapped) | 7 |
| Stack with plastic covering | 1 |
| Total | 283 |

math treatments, wavelength segments and outlier elimination. Elimination passes were used due to the difficulty of obtaining good agreement between the spectral information from the intact sample and the lab reference values.

These equations were then used to predict the validation set and the best equation was chosen based on the lowest standard error of cross validation (*SECV*) with consideration given to the number of terms used along with a low standard error of prediction (*SEP*) on the validation set.

Table 6 summarizes the calibration equation statistics that were obtained. The dry matter equation developed with all 226 samples included produced a $R^2$ of 0.99 and a *SECV* of 1.36 and after the elimination of t-outliers, the $R^2$ increased to 1.00 and the *SECV* decreased to 1.04. This was utilizing a 2 5 5 1 math treatment with scatter correction from 800 to 2500 nm. The crude protein equation also improved after the elimination of six outliers which then increased the $R^2$ from 0.90 to 0.93 along with a decrease in *SECV* from 1.24 to 1.09. This was utilizing a 0 4 4 1 math treatment and no scatter correction.

The ADF equation started with a $R^2$ of 0.94 and a *SECV* of 1.28 and after the elimination of seven outliers, the $R^2$ increased to 0.95 and the *SECV* decreased to 1.03. These results were achieved using a 2 5 5 1 math treatment and no scatter correction. The outliers that were eliminated did not consistently contain any one type of variation.

**Table 5. Fresh grass and grass silage sample reference method statistics.**

| Variable | *n* | Mean | *SD* | Range (%) |
|---|---|---|---|---|
| Dry matter | 283 | 34.7 | 13.04 | 13.9–72.3 |
| Crude protein (DM) | 283 | 17.1 | 3.51 | 9.5–27.8 |
| ADF (DM) | 283 | 34.3 | 3.90 | 22.6–46.6 |

**Table 6. Calibration equation statistics before and after outlier removal.**

| | All samples | | | After outlier removal | | |
|---|---|---|---|---|---|---|
| Variable | *n* | $R^2$ | *SECV*[1] | *n* | $R^2$ | *SECV*[1] |
| Dry matter | 226 | 0.99 | 1.36 | 215 | 1.00 | 1.04 |
| Crude protein (DM) | 226 | 0.90 | 1.24 | 220 | 0.93 | 1.09 |
| ADF (DM) | 226 | 0.94 | 1.28 | 219 | 0.95 | 1.03 |

[1]Standard error of cross-validation.

Since the equations were developed with outlier elimination, the t-statistic outliers from the validation set were also eliminated. These included a cross sample of variations with no one variation consistently present.

The validation set reference method results and the NIR spectroscopy predicted results for all three constituents produced means and standard deviations very similar to each other.

After prediction of the validation set, the *SEP* for dry matter was 1.00% with a $r^2$ of 0.99, a bias of –0.13 and a slope of 1.00 as indicated in Table 7. The correlation statistics for crude protein resulted in a *SEP* of 1.06%, a bias of –0.05, a slope of 1.04 and a $r^2$ of 0.92. The correlation plot for ADF produced a *SEP* of 1.03, a bias of –0.14, a slope of 1.03 and a $r^2$ of 0.92.

This is an example of the accuracy of NIR spectroscopic calibration equations that can be obtained on fresh pasture grass and grass silage using this type of sampling procedure, reference methods, scanning method, data treatment and regression analysis on the dry matter corrected results. There was no spectral repeatability file used on these calibrations.

Equations were then developed from the "as received" results for crude protein and ADF. The crude protein equation had a $R^2$ of 0.99 and a *SECV* of 0.33 after two elimination passes as shown in Table 8. The validation set produced a $r^2$ of 0.98 with a *SEP* of 0.32.

The ADF (as received) equation resulted in a $R^2$ of 0.99 and a *SECV* of 0.53. Upon validation, a $r^2$ of 0.98 and a *SEP* of 0.55 was achieved.

An equation is considered to be acceptable if the *RPD* (standard deviation divided by the *SEP*) is greater than three.[2] This comparison is referred to as the ratio of the standard error of performance to the standard deviation and all five equations meet this criterion, as shown in Table 9. The dry matter equation has a *RPD* of 12.3. The "as received" equations show improvement in accuracy over the dry matter corrected equations. The *RPD* of the crude protein equation corrected for dry matter is 3.55 and rises to 7.53 when the equation is developed on "as received" lab values.

**Table 7. Performance statistics for prediction of dry matter, crude protein (DM) and ADF (DM).**

| Variable | $r^2$ | *SEP* | Bias | Slope |
|---|---|---|---|---|
| Dry matter | 0.99 | 1.00 | –0.13 | 1.00 |
| Crude protein (DM) | 0.92 | 1.06 | –0.05 | 1.04 |
| ADF (DM) | 0.92 | 1.03 | –0.14 | 1.03 |

**Table 8. "As received" equation and validation set statistics for intact grass silage and fresh grass samples.**

| | Equation set | | | Validation set | | |
|---|---|---|---|---|---|---|
| | *n* | $R^2$ | *SECV*[a] | *n* | $r^2$ | *SEP*[b] |
| Crude protein (as rec.) | 218 | 0.99 | 0.33 | 53 | 0.98 | 0.32 |
| ADF (as rec.) | 213 | 0.99 | 0.53 | 48 | 0.98 | 0.55 |

[a]Standard error of cross-validation.
[b]Standard error of prediction.

**Table 9. Ratio of the standard error of performance to the standard deviation (RPD).**

| | *SEP* | *SD* | *RPD* |
|---|---|---|---|
| Dry matter | 1.00 | 12.32 | 12.32 |
| Crude protein (DM) | 1.06 | 3.77 | 3.55 |
| Crude protein (as rec.) | 0.32 | 2.41 | 7.53 |
| ADF (DM) | 1.03 | 3.65 | 3.54 |
| ADF (as rec.) | 0.55 | 3.94 | 7.16 |

The dry matter corrected ADF equation results in a *RPD* of 3.54 and increases to 7.16 for the "as received" equation.

The error on the dry matter equation may be high due to spectral alterations caused by the presence of water. Studies by Reeves[3–5] have shown shifts in the absorption bands depending on the compounds in solution and the amount of moisture in the sample and 88% of these samples had moisture contents greater than 50%. The error may possibly be lowered for the dry matter equation by using the Karl Fischer method for true moisture determination as the reference method so the volatile acids and alcohols are not lost as in oven drying.[6–8] However, the accuracy exhibited by this equation is accurate enough for some limited applications as it stands.

The crude protein and ADF calibration equations show promise and one way to improve the error may be to reduce the chop length of the fresh hay samples. Some of the samples had visible air pockets when packed in the natural products cell which indicated variations in packing densities. What the instrument saw may not have been what was analyzed in the lab.

Other directions that could be taken with this sample set could include separation of the different species or separation of the ensiled samples from the fresh grass. Also, a repeatability file could be added to see if there is an improvement in prediction accuracy. Another approach could be to investigate the calibration equations developed utilizing only the 800 to 1850 nm range to avoid non-linear regions of the spectra.

## Conclusions

- The error associated with NIR spectroscopy prediction equations developed from intact fresh grass and grass silages for dry matter, crude protein and ADF determinations is higher than for calibration equations developed from dried ground samples when samples are scanned and analyzed using this study's particular methods.
- Equations developed on "as received" reference values may be more accurate than equations developed on dry matter corrected values for the prediction of intact fresh grass and grass silages.
- Due to the higher error associated with equations developed from intact samples, the equations have limited use as they stand, but, they are acceptable and show promise.

## References

1. H.K. Goering and P.J. van Soest, *Forage Fiber Analysis*. USDA Agricultural Handbook No. 379, USDA, Washington, DC (1970).
2. P.C. Williams, "Variables Affecting Near Infrared Reflectance Spectroscopic Analysis", in *Near Infrared Technology in the Agricultural and Food Industries*, Ed by P.C. Williams and K. Norris. American Assoc. of Cereal Chemists, St Paul, MN, pp. 143–167 (1987).
3. J.B. Reeves III, *J. of AOAC International*, **76(4),** 1993 (1993).
4. J.B. Reeves, III, and T.H. Blosser, *J. Dairy Sci.* **72,** 79 (1989).
5. J.B. Reeves, III, and T.H. Blosser, *J. Dairy Sci.* **74,** 882 (1991).
6. J.S. Shenk, *Analysis of Undried, Unground Forage with a Visible-Near-Infrared Monochrometer*. Proc. 17th Int. Grassland Cong., pp. 592–593 (1993).
7. J.S. Shenk and M.O. Westerhaus, *Analysis of Agriculture and Food Products by Near Infrared Reflectance Spectroscopy*. NIRSystems Inc., Silver Spring, MD (1993).
8. J.S. Shenk and M.O. Westerhaus, "The Application of Near Infrared Reflectance Spectroscopy to Forage Analysis", in *Forage Quality, Evaluation and Utilization*, Ed by G.C. Fahey. ASA Inc., Madison, WI, pp. 406–449 (1994).