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Research article

Comparison of two in situ reference methods to estimate indigestible NDF by near infrared reflectance spectroscopy in alfalfa

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ABSTRACT

Undigested forage neutral detergent fiber (uNDF) from long-term ruminal in situ incubations are used to estimate indigestible neutral detergent fiber (iNDF). Measurement of iNDF is important in forage evaluation because it defines the potentially digestible pool of neutral detergent fiber (NDF). Near-infrared reflectance spectroscopy (NIRS) can be calibrated to in situ reference sets to rapidly predict uNDF. Our objective was to compare uNDF estimates after 240 h of incubation when two types of bags were used in the in situ reference method. The bags compared were 4 cm × 5 cm Ankom F57 bags (25 micron pore size), and 5 cm × 10 cm Ankom in situ bags (50 micron pore size). Alfalfa samples from Pennsylvania and Wisconsin ($n = 144$) of different varieties and harvest intervals were used. One-half or two gram samples, respectively, were weighed into the small and large bags in triplicate. Mass to surface area was 0.05 and 0.02 g/cm^2 for the small and large bags, respectively. The iNDF content after 240 h incubation was evaluated by two types bags in three rumen-cannulated Holstein cows. Each dried and ground forage was also scanned to determine the visible–near-infrared-reflectance spectra with a FOSS 6500 spectrophotometer. Prediction equations were developed for each bag type using modified partial least square regressions. The estimated iNDF fraction from small and large bags were 13.75% and 9.97%, respectively (SED = 0.39, $P < 0.001$). The coefficient of determination for calibration (R^2), cross-validation (1 - VR), calibration standard deviation (SEC), and interactive authentication standard deviation (SECV) was 0.94, 0.92, 0.85 and 0.98 for values determined with the small bag and 0.88, 0.85, 1.12 and 1.27 for iNDF for values determined with the large bag, respectively. Results indicate that iNDF varies among alfalfa cultivars and NIRS can be used to quickly and quantitatively estimate iNDF content in alfalfa. Bag type influences 240h NDF residues. NIRS predictions of iNDF from the small bag calibration set had higher R^2 and lower SEC and SECV than the large bag calibrations.

1. Introduction

Alfalfa hay (*Medicago sativa* L.), a perennial legume with a unique anatomy comprising relatively digestible fibers, is one of the most utilized forages for dairy rations in the world. Maturity and environmental differences influence lignification of stems, which, in turn, alters fiber digestibility in alfalfa (Palmonari et al., 2014). Indigestible NDF (iNDF) represents a uniform feed fraction with zero true digestibility (Lucas, 1964). Thus, iNDF is also a reliable intrinsic marker that has been successfully used to predict the In vivo nutrient digestibility (Lee and Hristov, 2013), which is directly related to TDN for ruminants and animal performance (Van Soest, 1994). Moreover, the iNDF fraction of forages affect passage rates, physical effectiveness, and gut fill (Van Amburgh et al., 2015).

The iNDF was used as an important measure to regulate feed intake and net energy in ruminants by Cornell net carbohydrate and protein system (CNCPS) (Fox et al., 2004). The accuracy and precision of iNDF estimates depend on the evaluation techniques used. At present, the methods for evaluating forage iNDF content mainly include in situ or in vitro techniques (Bender et al., 2016). The in situ techniques attain the degradability of forages by incubating samples in nylon bags which are

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placed in the rumen. Bags are then extracted at 244–288h (Harper and McNeill, 2015). Rumen fluid can provide a large number of enzymes that can effectively digest the diet, however, changes in bacterial activity, and the degradation products could not be recovered, the digestibility of NDF was significantly different from the determination results of iNDF content. But can be reduced with the adoption of a common methodology such as the standardisation of bag porosity, forage grinding length, and sample weight to bag size (Harper and McNeill, 2015). Thus, the objective of this study was to determine the differences between two different sizes Ankom bag types used in the in situ studies, to identify a standardisation of bag.

Near infrared reflectance spectroscopy (NIRS) can be used as a valuable tool for accurately determining the chemical composition, energy values, and digestibility of feedstuffs, and also used for in situ studies (Berzaghi et al., 1997). The principle of NIRS is to measure reflectance of infrared light with matter, giving information on the chemical makeup of feed nutrient fractions (Wang et al., 2010). NIRS allows a reduction of in situ animal studies and laboratory analyses that are time consuming, costly, and laborious, especially for iNDF determination (Mentink et al., 2006). Many previous studies use NIRS to determine the macronutrients such as CP, NDF, starch, NFC, and fat in feedstuffs (Mentink et al., 2006; Nie et al., 2008). However, for the determination of feed iNDF, previous studies still need the in situ residues for NIRS scanning (Berzaghi et al., 1997). Recently, Brogna et al. (2018) attempt using NIRS to predict fecal iNDF for dairy cows, which suggests the possibility of using NIRS in estimating forage iNDF. However, to the best of our knowledge, estimating alfalfa iNDF on commercial dairies to improve TDN estimations for alfalfa inclusive in the diet formulation by NIRS has not been fully tested or standardized. Thus, the objective of this study was to build NIRS calibrations and equations for alfalfa iNDF by a large number of alfalfa samples, using an in situ approach, and then to evaluated the feasibility of using NIRS to predict concentrations of iNDF in alfalfa forages.

2. Materials and methods

2.1. Plant material

From August 2018 to July 2019, 72 and 72 involved alfalfa hay samples were obtained from 39 commercial Midwest dairy farms located in Wisconsin and Pennsylvania, respectively. These samples were with different varieties and harvest intervals.

2.2. Sample preparations

Dry matter content of the alfalfa samples was determined after drying in a forced-air drying cabinet at 60 °C for 48 h. Samples for chemical analysis were dried and ground through a 2-mm screen in a Wiley mill. The experimental procedures of the animals involved in this experiment were approved by the University of Wisconsin Animal Care and Use Committee. Two types bags were used for the in situ studies. One is a small bag, 4 cm × 5 cm Ankom F57 bag (25-μm pore size), and another is a large bag, 5 cm × 10 cm Ankom nylon bag (50-μm pore size). 1.5 and 2 g samples (n = 3) were weighed into the small and large bags, respectively. Mass to surface area was 0.05 and 0.02 g/cm^2 for the small and large bags, respectively.

2.3. In vivo digestibility and rumen incubations

All *in situ* incubations followed the protocol of Lee and Hristov (2013). Three rumen-cannulated Holstein cows (BW approximately 500 kg) were used for rumen incubations. Cows were fed at a high forage TMR diet (44.5% alfalfa silage, 26.8% corn silage, 10.7% alfalfa hay, 6.5% straw, 11.5% concentrate mix; DM basis) with a measured nutrient composition of 14.7% CP, 46.1% NDF, 10.6% starch, and 3.4% ether extract. All bags were incubated at the same time of the day (0700 h) before the morning feeding and taken out after 240 h incubation. After removal from rumen, bags were rinsed with cold tap water thoroughly until the wash water ran clear. Subsequently, bags were dried in a forced-air drying cabinet at 60 °C for 48 h to determine the residue mass and NDF concentration. DM was determined by drying forage samples under 105 °C for 4 h. The NDF concentration was determined by the method of Goering and Van Soest (1970), with sodium sulfite, α-amylase, and the Ankom Fiber Analyzer (Ankom Technology Corp., Fairport, NY).

2.4. NIRS scanning

For spectra acquisition, each alfalfa sample was packed into cylindrical sample holders equipped with a quartz window and scanned (wavelength between 400 and 2.498 nm) in duplicate according to the procedures of Marten et al. (1983) on a near-infrared reflectance spectrophotometer (model 6500; FOSS-NIR System, Silver Spring, MD) fit with a spinning cup holder. Near infrared spectra (log 1/reflectance) was recorded for each 2-nm interval. The CEMNTER algorithm was used to evaluate the population characterization based on the spectral variability of samples (Shenk and Westerhaus, 1991). This procedure was conducted using the WinISI III software package (version 1.61, Infrasoft International, Port Matilda, PA), with a maximum standardized Mahalanobis distance from the average spectrum of 3.0. A modified partial least squares regression method was used to develop calibration equations with the full spectrum for iNDF (Brogna et al., 2018).

2.5. Statistical methods

Prediction equations were developed for each bag type using modified partial least square (**MPLS**) regressions. The coefficient of determination for calibration ($\mathbf{R^2}$) and cross-validation (**1-VR**, where **VR** = variance ratio), standard error of calibration (**SEC**), standard error of laboratory (**SEL**), and cross validation (**SECV**) were used to evaluate calibration and validation results. 14 spectral pretreatments were tested (WinISI III software, version 1.61, Infrasoft International, Port Matilda, PA) to improve the calibration models. Two criteria were used to select the best spectral pretreatment parameters: simultaneous low standard errors of cross-validation and high coefficients of determination in cross-validation. Four cross-validation groups were selected when developing the NIRS equations so as to choose the optimal number of terms and avoid overfitting (Shenk and Westerhaus, 1991). All data were analyzed using the one-way analysis of variance (**ANOVA**) procedure of the SAS software system. $P < 0.05$ was used to define statistical significance.

3. Results

The iNDF composition from the two types bags of alfalfa samples data are reported in Table 1. The alfalfa samples used for calibration varied in their iNDF composition, which ranged from 8.60 to 21.96% of DM from small bags, and from 4.99 to 18.24% of DM in large bags. Most of the iNDF concentration of Pennsylvania samples were greater than that of Wisconsin samples (Figure 1). Thus, both of the iNDF concentration of two types bags showed a similar range of variability, demonstrating a good result of the selection process. The iNDF concentration of small bag showed a clear higher value than large bag for both of the Wisconsin and Pennsylvania samples (Table 1, Figure 1). Our results from the comparison between small and large bags were consistent with the previous findings, confirming that the small bag with 25 μm pore size was more accurate to estimate the iNDF concentration. As shown in Figure 1, both of the Wisconsin and Pennsylvania samples showed a liner correlation between in situ and NIR method. It was found that a clear absorption was observed in the regions 1,200 to 1,620 nm and 2,200 to 2,392 nm for the prediction of alfalfa iNDF. It was reported that ratio of performance deviation (**RPD**) value was 3.3 and range error ratio (**RER**) 10.5.

The calibration and cross-validation statistics for near infrared spectroscopy analysis of alfalfa iNDF concentration are listed in Table 2. The R^2,

Table 1. Summary statistics of indigestible neutral detergent fiber (iNDF) concentration for the alfalfa samples used in the calibration data set.

iNDF (% of DM)	n	Minimum	Maximum	Range 1	Mean*	SD
Small bag	144	8.60	21.96	13.36	13.79	3.50
Large bag	144	4.99	18.24	13.25	9.94	3.29

1 Range = maximum − minimum.
* $P < 0.05$, significant different between small bag and large bag.

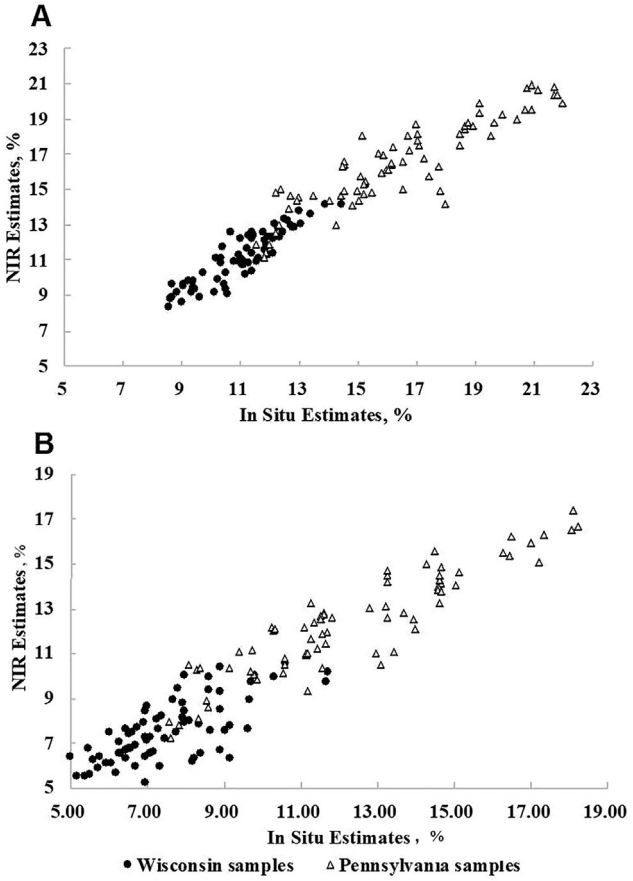

Figure 1. Comparison of iNDF contents of alfalfa in situ to NIR estimates, small bags (A), large bags (B).

1-VR, SEC, SEL and SECV was 0.94, 0.92, 0.85, 1.37 and 0.98 for the small bag, respectively. And the values of R^2, 1-VR, SEC, and SECV were 0.88, 0.85, 1.12, 1.53 and 1.27 for the large bag, respectively. The fraction of variance accounted for MPLS calibration was relatively high for small bags ($R^2 = 0.94$) but not for large bags ($R^2 = 0.88$). After cross-validation, the coefficient of determination (1 − VR) for iNDF from small bags and large bags were both lower than the R^2 of the calibration. As the accuracy indicators shown in Table 2, the RER values were both >10, and the RPD values were >3 for small bags (3.60) but not for large bags (2.58), suggesting a proper calibration for small bags.

4. Discussion

The iNDF concentration of feedstuff indicates the availability of NDF in the rumen and thereby the energy availability for the dairy cow (Huhtanen et al., 2006). Moreover, iNDF could be an excellent marker to predict the apparent nutrients digestibility because it can be traced from the diet to the feces (Lee and Hristov, 2013). Thus, the variation of iNDF content of alfalfa samples might further indicate their feeding value for the dairy cow.

4.1. Two different sizes Ankom bag types used in the in situ studies

The iNDF concentration of small bag showed a clear higher value than large bag for both of the Wisconsin and Pennsylvania samples (Table 1, Figure 1). It was reported that the use of larger porosity bags would magnify the loss of small feed particles during rumen incubation that will overestimate digestion compared to the smaller porosity bags (Udén, 2006). Besides, the pore size with a range from 6 to 17 μm was determined as the best compromise to minimize particle inflow and outflow (Nousiainen et al., 2004). Our results from the comparison between small and large bags were consistent with the previous findings, confirming that the small bag with 25 μm pore size was more accurate to estimate the iNDF concentration. For large bags, the low correlation and the lower iNDF concentration might be related to the fact that samples were larger pore size which might easily result the disappearance of iNDF. It was reported that more samples are required to strengthen the calibration when difference between R^2 and 1 - VR is wide (Brogna et al., 2018). In most of the previous iNDF studies, the 1- VR were less than 0.90. The calibration for iNDF from small bags, thus, looked promising and was more robust than the large bags, despite the limited number of samples.

In addition, most of the iNDF concentration of Pennsylvania samples were greater than that of Wisconsin samples. The reason for the different iNDF content of the samples from 2 different areas is unclear. It was reported that maturity influences fiber fractions and digestibility of alfalfa hay, as the indigestible fraction of fiber increasing with maturity (Palmonari et al., 2014). Thus, different iNDF concentration between Wisconsin and Pennsylvania samples might be due to the various maturity of alfalfa from the different places as well as unmeasured environmental differences.

4.2. NIRS the prediction of alfalfa iNDF

The prediction of ME, microbial protein, and milk production of dairy cow relies on dietary TDN (Schalla et al., 2012), and dietary iNDF is directly related to TDN for ruminants and animal performance (Van Soest,

Table 2. Calibration and cross-validation statistics for near infrared spectroscopy analysis of indigestible neutral detergent fiber from small bags and medium bags.

	Calibration statistics 1							Cross validation statistics 2			
Item	n	MPLS terms	Mean	SD	SEC	SEL	R^2	SECV	1-VR	RPD	RER
Small bag	135	9	13.75	3.52	0.85	1.37	0.94	0.98	0.92	3.60	13.68
Large bag	139	9	9.97	3.29	1.12	1.53	0.88	1.27	0.85	2.58	10.39

1MPLS terms = number of factors in modified partial least square equation; SEC = standard error of calibration; SEL = Standard error of laboratory.
2SECV = standard error of cross validation; 1 − VR = coefficient of determination of cross-validation, where VR = variance ratio; RPD = ratio of performance deviation (SD/SECV), RER = range error ratio (range/SECV).

1994). Although several studies have been conducted, no experiments have shown the correlation between NIRS spectra and alfalfa iNDF of dairy cows. As shown in Figure 1, both of the Wisconsin and Pennsylvania samples showed a liner correlation between in situ and NIR method.

Furthermore, the available calibrations for alfalfa feed iNDF are still lack, the NIRS analysis from this study make up for the deficiency of the prediction of the digestibility and TDN of alfalfa.

On the other hand, it was found that a clear absorption was observed in the region 1,900 to 2,000 nm, at 2,450 nm, and in the regions 1,150 to 1,650 nm and 2,000 to 2,350 nm for the prediction of fecal iNDF240 (Brogna et al., 2018), which was similar to the results of alfalfa iNDF determination in the current study. It was reported that ratio of performance deviation (**RPD**) value should be at least 3 and range error ratio (**RER**) at least 10 (Williams and Sobering, 1996). The previous study found that the fecal iNDF can be well predicted by NIRS (Brogna et al., 2018). The good correlation among peaks based on the small bag between spectra of this study suggests that the NIRS technique can be used successfully for the prediction of alfalfa iNDF in the current study.

5. Conclusions

NIRS accounted for the majority of variance ($R^2 = 0.94$ for small bag; $R^2 = 0.88$ for large bag) in the experimental alfalfa after 240 h ruminal incubation, indicating that NIRS calibration developed from this experiment can be used to quickly and quantitatively estimate iNDF content in alfalfa for future commercial application. The results indicated the NIRS predictive equations from small bag had a better predictive value than large bag.

Declarations

Author contribution statement

G.J. Zhang: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Y. Wang: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Y. H. Yan; M.H. Hall: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

D. J. Undersander: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

D. K.Combs: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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