

# Predicting the Effect of Proteolysis on Ruminant Crude Protein Degradation of Legume and Grass Silages Using Near-Infrared Reflectance Spectroscopy<sup>1</sup>

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## ABSTRACT

Two studies were conducted to assess whether routine applications of near infrared reflectance spectroscopy could predict the effects of silage proteolysis on ruminal crude protein (CP) degradation of legume and grass silages. A preliminary study was conducted to assess the effect of laboratory drying method on ruminal CP degradation of silages. Thirty legume and grass silages were freeze-, oven-, or microwave-dried and incubated in situ in the ventral rumen of three ruminally cannulated cows for 24 h. Freeze-drying was considered least likely to alter ruminal CP degradation of the silages; therefore, oven- and microwave-drying were compared using first-order regression with freeze-drying. Oven-drying for 48 h at 55°C compared favorably ( $R^2 = 0.84$ ) with freeze-drying. Microwave-drying resulted in a large bias (2.84 g/10<sup>-1</sup> kg of CP) and was poorly related ( $R^2 = 0.48$ ) to freeze-drying.

In a second study, alfalfa and timothy were cut at three maturities and allowed to wilt for 0, 10, 24, 32, 48, and 54 h. Forages were ensiled in triplicate cylindrical mini silos and allowed to ferment for 120 d. After fermentation, silages were oven-dried, ground, and scanned on a near-infrared reflectance spectrophotometer. Duplicate, dried, 2-mm ground silage samples were incubated in the ventral rumen of three ruminally cannulated cows for 24 h. Forage species, maturity, and wilting time significantly affected 24-h ruminal CP degradation of the silages. Near-infrared reflectance spectroscopy accurately predicted ( $R^2 = 0.91$ ) 24-h ruminal CP degradation of silages. Data suggest near-infrared reflectance spectroscopy can accurately assess the effects of forage species,

maturity, and wilting time (proteolysis) on 24-h ruminal CP degradation of legume and grass silages. (**Key words:** silage, proteolysis, near infrared, in situ)

**Abbreviation key:** CPR = CP remaining, NE = not ensiled, NIRS = near-infrared reflectance spectroscopy.

## INTRODUCTION

Near-infrared reflectance spectroscopy (NIRS) is commonly used in commercial forage testing because it is efficacious and economical. In the past two decades, major improvements have been made in NIRS hardware and calibration techniques to improve prediction accuracy of the nutrient composition of forages (19). The primary application of NIRS in forage testing is to predict nutrients such as CP, ADF, and NDF. In a review of NIRS technology, Sherk and Westerhaus (19) suggested that the greatest potential of NIRS was direct prediction of animal response or biological nutrients such as DM digestibility or CP degradability. To date, there have been few investigations exploring the possibilities of the use of NIRS to predict animal response or biological nutrients. The potential for NIRS to predict ruminal CP degradation of forages is of particular interest because common molecular bonds N-H, C-H, C=O, and C-N of protein have NIRS absorption bands (19). The relationship between NIRS and CP content of forages is high ( $R^2 > 0.96$ ) because the relationship of these bonds to the total N content of forages is high. Because differences in protein molecular structure define ruminal CP degradation (12), prediction of ruminal CP degradation of forages by NIRS is theoretically feasible. Halgerson et al. (7) observed that NIRS accurately predicted in situ and ficin CP degradability of forages on a DM basis. Although promising, the prediction of CP degradation of forages by NIRS on a DM basis (7) may be confounded by the simple positive relationship between CP content and CP degradability (8). A

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better approach to evaluating the potential of NIRS to predict CP degradation of forages may be to evaluate forages with similar CP contents that differ in CP degradability. Proteolysis created by ensiling forages can result in changes in molecular protein size and structure, which changes CP degradability (11, 15) even when forage CP contents are similar. Messman et al. (12) demonstrated that only 9% of macro-protein molecules remained intact after fermentation (proteolysis). Those data (11, 12) indicate that proteolysis creates major changes in molecular structure of forage proteins, and, because NIRS can detect changes in molecular structure, NIRS should theoretically be able to predict differences in ruminal CP degradation that result from proteolysis. This study was implemented to explore this hypothesis.

## MATERIALS AND METHODS

### Trial 1

Because most NIRS applications require samples to be dried and ground before scanning, a preliminary study was conducted to assess the effects of drying method on CP degradability of silages. Thirty legume or legume and grass silage samples were obtained from samples (Marshfield Soil and Forage Testing Analysis Laboratory, Marshfield, WI) submitted for routine forage analysis. Forage samples were divided into three subsamples weighing approximately 200 g per subsample. Subsamples were freeze-dried, oven-dried at 55°C for 48 h, or partially microwave-dried according to the procedures of Undersander et al. (22). The partial microwave-drying procedures of Undersander et al. (22) involve microwaving at various power settings until the silage samples are approximately 90 to 95% DM and sufficiently dry for grinding. The residual moisture content is then predicted ( $R^2 > 0.98$ ) by NIRS during scanning (19). These procedures were chosen because they are commonly used by commercial forage testing laboratories (D. J. Undersander, 1997, personal communication), and procedures used in this trial were designed to mimic commercial NIRS applications. After drying, freeze-, oven-, and microwave-dried subsamples were ground through a 2-mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA).

In situ evaluation of each subsample representing each drying procedure was conducted in three 24-h periods. Ten forage samples with all three drying methods represented were evaluated during each period. Dacron bags (20 × 10 cm; 52- $\mu$ m pore size) were filled with a 5-g sample of dried forage. Dacron

bags were placed in a mesh net secured to the ruminal cannula via a nylon cord. Dacron bags for each drying method subsample were incubated in the ventral rumen of three midlactation ruminally cannulated cows for 24 h. Cows were fed diets containing (DM basis) 58% alfalfa silage, 32% high moisture shelled corn, 7% CP supplement, and 3% vitamins and minerals.

In addition, duplicate bags containing 5 g of a standard forage (bud stage alfalfa) were incubated in the rumen of each cow during each period and evaluated for DM and CP disappearance to assess period effects. The DM or CP disappearance of the standard forage did not vary more than 5% for any period; therefore, no periods were repeated.

Upon removal of bags from the cow, bags were immersed in ice water to stop microbial activity and were washed according to the procedures of Cherney et al. (6). Bags were dried at 55°C for 48 h and weighed, remaining DM was determined, and duplicates were composited. Microbial CP contamination was assumed to be constant across main forage samples and not influenced by drying method (subsample), and, as a result, in situ residues were not evaluated for microbial CP. Residue CP was determined using AOAC procedures (2) and the percentage of CP remaining (**CPR**) after 24 h was calculated. Data were evaluated using regression procedures of SAS (17). Freeze-drying was assumed to be the procedure least likely to alter CP degradation (14), and oven- and microwave-drying data were regressed against freeze-drying data with the strength of the regression coefficient, standard error, and bias of a first-order equation serving as evaluation criteria.

### Trial 2

Approximately 25 kg of alfalfa (*Medicago sativa* L.) were harvested at growth stage 2 as described by Kalu and Fick (9). Harvested alfalfa was chopped to a 1-cm theoretical length of cut then spread to a uniform depth of 7.5 cm on a wooden table and allowed to wilt at 22°C for 0, 10, 24, 32, 48, and 54 h. After wilting, forage was inoculated with an inoculant containing *Lactobacillus plantarum* and *Streptococcus faecium* at 100,000 cfu/g and immediately ensiled in triplicate 10-cm × 40-cm cylindrical mini silos fitted with gas relief valves. Silages were packed to a uniform density, stored at 20°C, and allowed to ferment for 120 d. A nonensiled (**NE**) sample of stage 2 alfalfa was also saved and served as a control sample to validate the extent of proteolysis created by the experimental silage protocol. The NE sample was harvested, chopped to a 1-cm theoretical length of cut,

and immediately dried in an oven at 55°C for 48 h. Procedures were repeated for alfalfa harvested at growth stages 4 and 6 as described by Kalu and Fick (9) and for timothy (*Phleum pratense* L.) harvested at growth stages 32, 45, and 58 as described by Simon and Park (20). An NE sample was processed for all forage species and maturities. General descriptive maturity classifications at harvest for alfalfa were late vegetative, late bud, and midbloom. For timothy, descriptive maturity classifications at harvest were late vegetative, boot, and anthesis.

After fermentation, silages (n = 108) were removed from the cylindrical silos, and 200 g were dried at 55°C for 48 h. Absolute DM of silage and NE samples was determined by AOAC procedures (2). Samples were ground through a 2-mm screen in a Wiley mill, and an in situ evaluation was conducted on each silage replicate and NE sample by the procedures described for trial 1 with the following exceptions. Silages were evaluated in three 24-h periods with all forage species and maturities and NE samples represented in each period. In situ residues were evaluated for microbial CP contamination using an NIRS calibration (3) that estimates ( $R^2 = 0.81$ ) milligrams of RNA CP per gram of DM. Base chemistries for the NIRS calibration (3) were the procedures of Zinn and Owens (24) with modifications by Aharoni and Tagari (1). Dried, 2-mm ground silage samples were reground through 1-mm screen via a Udy mill (UDY Corp., Boulder, CO) and scanned on a spinning cup near infrared reflectance spectrophotometer (model 6500; NIR Systems, Perstop Analytical, Silver Spring, MD). Spectra were saved, and a calibration (n = 41) subset was selected using the center and select procedures of Infrasoft International Software (NIR Systems) (18). Samples not selected (n = 67) for the calibration set served as a validation set. The NE samples were not considered for the calibration or validation sets. Calibration and validation procedures were conducted on the parameter, CPR after 24 h of ruminal incubation expressed as grams per  $10^{-1}$  kg of CP. All values were adjusted for microbial CP contamination by contamination using previously described procedures. Statistical inferences of the calibration and validation procedures were determined using Infrasoft International Software (18). Only first-order derivatives were considered in the calibration procedures to avoid overfitting the data.

The effect of forage species, maturity, and wilting time on CPR after 24 h of ruminal incubation of the silages was evaluated by the GLM procedure of SAS (17) using the following model:

$$Y = \mu + F_i + M_j + W_k + P_l + E_{ijkl}$$

where

- $\mu$  = overall mean of the population,
- $F_i$  = mean effect of forage species i,
- $M_j$  = mean effect of maturity j,
- $W_k$  = mean effect of wilting time k,
- $P_l$  = mean effect of period l, and
- $E_{ijkl}$  = unexplained residual error, assumed to be normally and independently distributed.

There was no effect ( $P > 0.10$ ) of period on silage CPR after 24 h of ruminal incubation, and a second model was run without period. In addition, ruminal DM and CP degradation of the standard alfalfa sample did not differ more than five percentage units among periods. No interactions were considered in the model because the sole objective of the statistical evaluation was to determine whether main effects (forage species, maturity, or wilting time) in the experimental protocol produced significant changes in silage CP degradation for subsequent NIRS prediction.

## RESULTS AND DISCUSSION

### Trial 1

The effect of drying method on CPR after 24 h of ruminal incubation in experimental silages is presented in Table 1. The CP content of silages ranged from 12.3 to 22.3% CP with a mean CP content of 19.4%. The CPR after 24 h of ruminal incubation of freeze-dried silage samples ranged from 9.7 to 21.2  $g/10^{-1}$  kg of CP.

Because previous research (10, 16) has demonstrated that freeze-drying results in negligible changes in chemical composition of forages, freeze-drying was considered to be least likely to alter ruminal CP degradation characteristics of silages in this experiment. As a result, regression analyses were conducted on the relationships between oven-drying and freeze-drying and microwave-drying and freeze-drying on CPR after 24 h of ruminal incubation. These relationships are presented in Figures 1 and 2. Oven-drying (Figure 1) resulted in a small (0.64  $g/10^{-1}$  kg of CP) bias, and the relationship between oven- and freeze-drying was relatively strong ( $R^2 = 0.84$ ). Data suggest that oven-drying at 55°C for 48 h results in small changes in the CPR after 24 h of ruminal incubation of silages. The relationship between microwave- and freeze-drying (Figure 2) on silage CPR is presented in Figure 2. Microwave-drying resulted in a large bias (2.84  $g/10^{-1}$  kg of CP),

and the relationship between microwave- and freeze-drying was poor ( $R^2 = 0.48$ ). Because heating of forages is known (23) to decrease ruminal CP degradation and because microwave-drying can result in extensive heating in a relatively short period (<10 m), it is logical that microwave-drying would increase the CPR in silages. Our data are similar to the observations of Karn (10) and Reed and Belyea (16). Karn (10) measured the effects of freeze-, oven-, and microwave-drying on N, ADF, and N content in ADF of forages. Oven-drying at 50°C resulted in a small increase in N, ADF, and the N content in ADF of forages as compared with freeze-drying. Microwave-drying resulted in larger increases in N, ADF, and N content in ADF of forages when compared with freeze-drying. Reed and Belyea (16) observed similar results. Oven-drying at 55°C did not alter 24-h rumi-

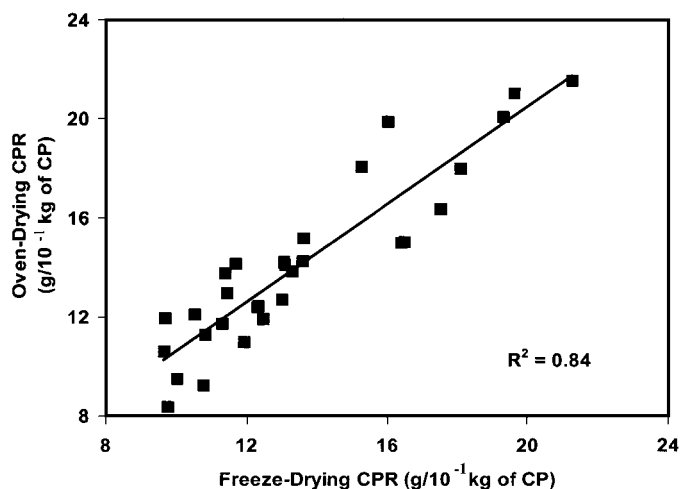


Figure 1. Relationship between freeze-drying and oven-drying on the CP remaining (CPR) in trial 1 silages after 24 h of ruminal incubation.

TABLE 1. The effect of drying method on the amount of CP remaining (CPR) after 24 h of ruminal incubation of the experimental silages (Trial 1).

Sample	CP (% of DM)	CPR <sup>1</sup> (g/10 <sup>-1</sup> kg of CP)		
		Microwave	Oven	Freeze
1	12.3	22.1	21.5	21.2
2	13.7	15.5	16.4	17.5
3	16.7	23.4	20.1	19.3
4	16.7	15.5	14.1	13.1
5	16.9	13.7	12.7	13.0
6	17.1	17.0	13.9	13.3
7	18.1	22.6	15.0	16.4
8	18.4	16.2	19.9	16.0
9	18.6	22.6	21.1	19.6
10	18.7	15.2	18.0	18.1
11	19.4	16.7	13.8	11.4
12	19.4	12.2	11.9	12.5
13	19.5	15.4	11.3	10.8
14	19.6	13.3	13.0	11.4
15	19.8	16.0	14.2	11.7
16	19.9	12.6	14.2	13.0
17	20.1	12.5	12.1	10.5
18	20.2	14.5	14.3	13.6
19	20.2	10.2	9.3	10.8
20	20.6	12.7	11.7	11.3
21	20.7	16.4	18.1	15.3
22	20.9	13.5	15.2	13.6
23	21.4	20.7	9.5	10.0
24	21.5	11.9	12.4	12.3
25	21.5	9.4	10.6	9.7
26	21.7	14.8	15.0	16.5
27	21.7	11.3	12.0	9.7
28	21.9	15.1	11.0	11.9
29	22.2	13.6	12.5	12.3
30	22.3	11.8	8.4	9.7
$\bar{X}$	19.4	15.3	14.1	13.5
SD	2.4	3.7	3.4	3.1

<sup>1</sup>Subsamples were freeze-dried, oven-dried at 55°C for 48 h, or partially microwave-dried according to the procedures of Under-sander et al. (22).

nal CP degradation of grass ingesta as compared with ingesta frozen using dry ice.

We concluded that the extensive heat associated with rapid microwave-drying decreases ruminal CP degradation of silages and that microwave-drying is unsuitable as a preparatory method for in situ or NIRS analysis of ruminal CP degradability. Oven-drying of silages at 55°C for 48 h does result in small decreases in ruminal CP degradation but is an acceptable preparatory method for in situ or NIRS analysis of silages to determine CP degradation characteristics.

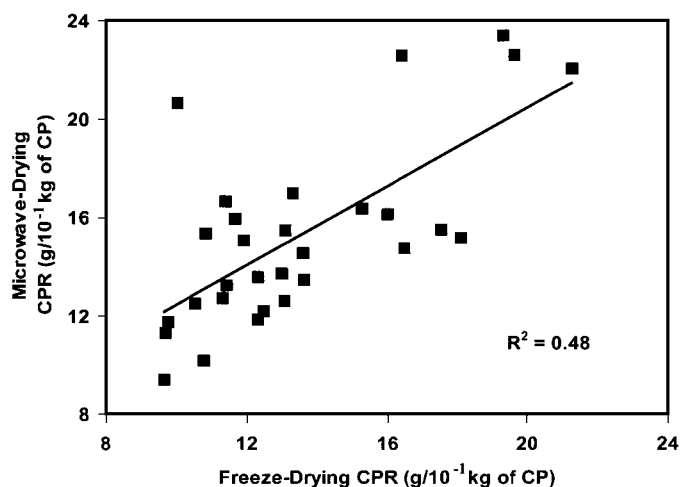


Figure 2. Relationship between freeze-drying and microwave-drying on the CP remaining (CPR) in trial 1 silages after 24 h of ruminal incubation.

TABLE 2. Dry matter and CP contents of experimental silages (Trial 2).

Wilting time (h)	Alfalfa stage			Timothy stage		
	Vegetative	Late Bud	Midbloom	Vegetative	Boot	Anthesis
	(% of DM)					
0	14.7	11.0	16.5	11.4	16.5	13.1
10	14.8	12.4	16.8	12.5	19.2	14.4
24	17.9	15.8	19.7	24.8	24.7	17.0
32	20.4	25.1	22.5	29.9	24.2	19.1
48	23.3	26.7	26.1	31.0	27.2	21.8
54	36.1	42.5	33.2	41.5	33.4	26.5
	(% of CP)					
0	27.3	19.0	16.1	20.8	13.5	10.5
10	26.7	18.1	15.6	20.2	12.4	11.2
24	27.0	18.7	16.7	19.8	12.7	11.9
32	27.2	21.6	16.1	19.3	12.7	11.8
48	27.9	21.7	16.9	20.6	13.2	11.8
54	28.7	21.3	15.7	20.0	13.7	12.3

### Trial 2

The DM content of the experimental silages upon removal from the cylindrical silos is presented in Table 2. In general, all silages contained less DM than applied recommendations for optimum fermentation (35 to 45%) (13). Silage DM contents were kept low in this experiment for two reasons: first, to ensure extensive proteolysis in the silages, because proteolysis is known to be correlated inversely with DM content of the silage (13), and second, to prevent possible heat damage in silages created when forages are ensiled at high DM contents (13). Changes in silage CP degradation caused by fermentation temperature are important, but this issue was beyond the scope of this study. Fermentation characteristics of the experimental silages were not evaluated, but secondary fermentation characteristics (13) were empirically observed in alfalfa and timothy when ensiled without wilting (0 h). Some secondary fermentation characteristics were also empirically observed in timothy silages wilted for 10 h.

The CP content of the experimental silages is also presented in Table 2. The changes in CP contents of alfalfa and timothy at a maturity stage are consistent with other observations (8). Although not statistically evaluated, the CP content of silages made from an individual species and maturity across the six pre-ensiling wilting times were similar. This observation is consistent with other research (5), which suggests DM content at ensiling does not greatly alter CP content of the silage.

The effect of pre-ensiling wilting time on 24-h ruminal CP degradation for alfalfa and timothy silages is presented in Figures 3 and 4. The CP of NE alfalfa

and timothy remaining after 24 h of ruminal incubation for each maturity is also presented in Figures 3 and 4. Results of the statistical analysis revealed effects ( $P < 0.01$ ) of forage species, maturity, and wilting time on CP of the experimental silages remaining after 24 h. Because the intent of the experimental silage protocol was to develop a unique set of silage samples for NIRS evaluation, a detailed discussion of differences in CPR between the experimental silages will not be offered. A general discussion of silage and NE samples is as follows. Ruminal CP degradation of all silages was greater than any of their NE contemporaries, indicating that ensiling and proteolysis increased ruminal CP degrada-

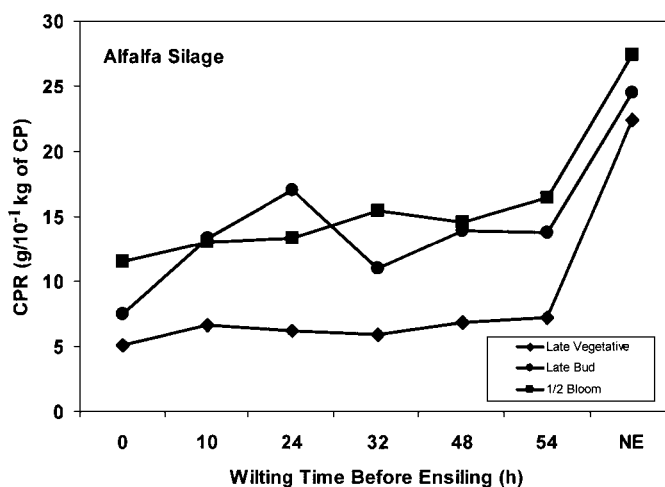


Figure 3. The effect of wilting time and forage maturity on the CP remaining (CPR) in trial 2 alfalfa silages after 24 h of ruminal incubation. NE = Not ensiled.

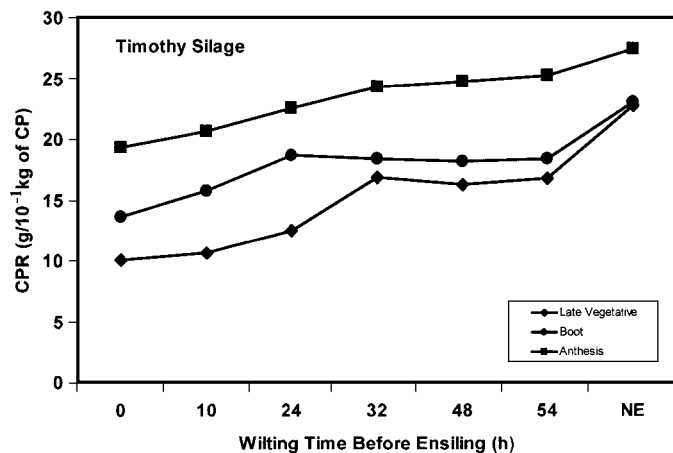


Figure 4. The effect of wilting time and forage maturity on the CP remaining (CPR) in trial 2 timothy silages after 24 h of ruminal incubation. NE = Not ensiled.

tion. Ruminal CP degradation of alfalfa silage harvested at the late vegetative stage was greater compared with silages harvested at late bud or mid-bloom. Similarly, ruminal CP degradation of alfalfa silage harvested at the late bud stage was greater than alfalfa silage harvested at the midbloom stage. The relationship between advancing forage maturity and decreased ruminal CP degradation observed in this experiment is consistent with previous reports (8). The effect of pre-ensiling wilting time and corresponding increased DM content of alfalfa silages

was negatively related to ruminal CP degradation. This relationship is also consistent with previous reports (5).

The effects of maturity and pre-ensiling wilting time on ruminal CP degradation of timothy silages are similar to the effects previously described for alfalfa silages. The only difference between timothy silages and alfalfa silages is CPR (grams per 10<sup>-1</sup> kg of CP) after 24 h of ruminal incubation was approximately five percentage units higher for timothy silage at comparative maturities and wilting times. The observation of less extensive ruminal CP degradation of timothy CP compared with alfalfa CP is also consistent with previous reports (8).

Results of NIRS determination of CP of experimental silages remaining after 24 h are presented in Table 3. The calibration, based on 41 centered and selected samples, had an R<sup>2</sup> of 0.91 and a standard error of calibration of 1.46 g/10<sup>-1</sup> kg of CP. Validation of the equation resulted in an r<sup>2</sup> of 0.94, a standard error of performance of 1.60 g/10<sup>-1</sup> kg of CP, and a low bias of 0.23 g/10<sup>-1</sup> kg of CP. The increase in the R<sup>2</sup> values between the calibration (R<sup>2</sup> = 0.91) and validation (r<sup>2</sup> = 0.94) is likely the simple result of more samples in the validation set.

Calibration and validation statistics indicate NIRS predicted CPR in silages after 24 h of ruminal incubation very accurately in this study. This concept is presented in Figure 5. Tremblay et al. (21) predicted RUP in roasted soybeans using NIRS, but the performance of the equation (r<sup>2</sup> = 0.70) was lower than that in this trial. The study of Tremblay et al. (21) used the inhibitor in vitro system of Broderick (4) to

TABLE 3. Results of near infrared reflectance spectroscopic determination of CP remaining (CPR) after 24 h of ruminal incubation of the experimental silages.

Item <sup>1</sup>	
Calibration	
n	41
Minimum	4.9
Maximum	24.9
Mean	13.6
R <sup>2</sup>	0.91
SEC <sup>2</sup>	1.46
Validation	
n	66
Minimum	5.0
Maximum	26.6
Mean	14.9
r <sup>2</sup>	0.94
SEP <sup>3</sup>	1.60
Bias	0.23

<sup>1</sup>Except for n and the R<sup>2</sup> value, CPR units are grams per 10<sup>-1</sup> kg of CP.

<sup>2</sup>Standard error of calibration.

<sup>3</sup>Standard error of performance.

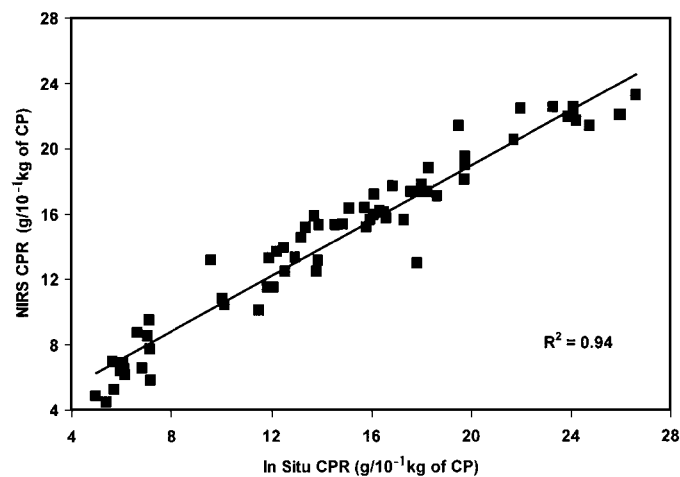


Figure 5. Relationship between the CP remaining (CPR) after 24 h of ruminal incubation of trial 2 silages as predicted by in situ or near-infrared spectroscopy (NIRS) methods.

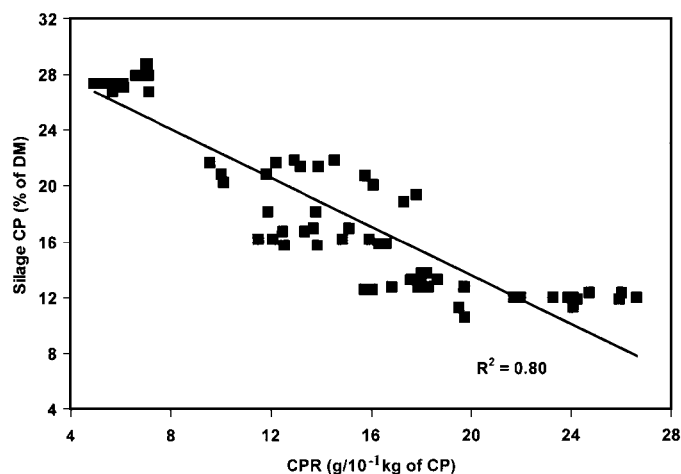


Figure 6. Relationship between CP and the CP remaining (CPR) after 24 h of ruminal incubation of trial 2 silages.

estimate RUP content of roasted soybeans, and the laboratory error associated with this procedure could be responsible for the lower  $r^2$  of the NIRS soybean RUP validation. In the study of Tremblay et al. (21), NIRS was used to predict RUP differences in soybeans that theoretically varied only in the level of heat treatment and, correspondingly, RUP. Because soybeans are relatively consistent in CP content and other chemical constituents, NIRS could only detect the absorption bands of molecular bonds associated with maillard products and could not use differences in other protein molecular structures, possibly related to RUP, to strengthen the calibration. Even though we tried to minimize the influence of forage species and maturity on the CPR in silage in this study, a large portion of the variance in CPR in our study still could be due to forage species and maturity. This concept is visually presented in Figure 6. The simple relationship between silage CP content and CPR accounts for 80% of CPR variance in our experiment. The NIRS determination in our study theoretically found 14 additional units of CPR variance, which is 70% of the remaining 20 units. Although this rationale is crude, we estimate that NIRS found approximately 70% of the variance in CPR created by silage proteolysis in this experiment. This value compares closely with the ability of NIRS to predict the variance of RUP in soybeans ( $r^2 = 0.70$ ) as observed by Tremblay et al. (21).

### CONCLUSIONS

The viability of NIRS to predict ruminal CP degradation of silages depends on many factors. These

experiments explored two critical factors. First, simple preparatory issues, such as drying and grinding of silage, should not alter CP degradation of silages. Our data suggest the common practice of oven-drying for 48 h at 55°C does not greatly affect ruminal CP degradation of silages. Secondly, NIRS should have the ability to predict changes in silage CP degradation created by proteolysis. In this study, CP degradation of alfalfa and timothy silages were altered by ensiling at different moisture contents (wilting). Near infrared reflectance spectroscopy accounted for the majority ( $R^2 = 0.91$ ) of CPR variance in the experimental silages after 24 h of ruminal incubation. The NIRS calibration developed from this experiment is not robust enough for commercial application, but results suggest routine NIRS procedures have the potential to predict accurately the ruminal CP degradation in silages.

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