

Development of a Novel System to Estimate Protein Degradability in Legume and Grass Silages¹

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ABSTRACT

Five trials were conducted to develop a system to estimate rumen-undegradable protein (RUP) of legume and grass silages using near infrared reflectance spectroscopy. In situ procedures were the reference method used to determine silage RUP content. Trials 1, 2, and 3 were devoted to improving in situ procedures. In trial 1, alfalfa silage with and without heat treatment was incubated ruminally in 30 cows. The standard deviation of in situ RUP attributable to cow and diet was 0.82 and 3.80 g/10⁻¹ kg of crude protein (CP) for the unheated and heated alfalfa, respectively. Based on trial 1, it was determined that 8 cows would be required to establish RUP standards. In trial 2, low (13.3 g/10⁻¹ kg of CP) and high (44.5 g/10⁻¹ kg of CP) RUP standards were developed using eight ruminally cannulated cows.

In trial 3, 11 new RUP standards were developed by mixing trial 2 RUP standards together. The RUP standards were used to employ a calibration curve technique in ruminally cannulated cows. The technique was employed in four ruminally cannulated cows to estimate RUP contents of 121 silages, and RUP values were used for near-infrared reflectance spectroscopic analysis in trial 4. Trial 4 procedures yielded a calibration for RUP content of silages with an R² of 0.84 and a standard error of calibration of 1.55 g/10⁻¹ kg of CP. In trial 5, the equation was tested on 300 silage samples. The mean predicted RUP content was 21.8 g/10⁻¹ kg of CP. Data suggest near-infrared reflectance spectroscopy can predict RUP content of silages.

(**Key words:** silages, in situ, protein degradation, near-infrared)

Abbreviation key: CAS = common alfalfa silage, CCS = calibration curve standard, CPR = CP remaining, HT = heat treated, k_d = degradation rate, NIRS = near-infrared reflectance spectroscopy, TA = theoretical absolute.

INTRODUCTION

In some dairy regions of the United States, legume and grass silages supply the majority of CP, RDP, and RUP in dairy cow and heifer diets. Because no commercial test is available for RDP or RUP, our laboratory has investigated (4, 7, 8) the use of near-infrared reflectance spectroscopy (NIRS) as an unbiased predictor of the in situ-derived RUP content of legume and grass silages. Analytical challenges are numerous and require resolution before commercial application. To date, we have resolved the following analytical questions. First, legume and grass silages can be dried at 55°C without altering the RUP content (7). Resolution of this issue means that dried, ground legume and grass silage samples can be scanned via NIRS, which makes application universal across NIRS equipment. Second, we have developed techniques (4) to evaluate in situ sample residues efficaciously for microbial CP contamination, allowing for more accurate estimates of true CP degradation. Third, we have demonstrated that NIRS accurately predicts changes in RUP content of legume and grass silages created by proteolysis (7). Fourth, we have demonstrated that NIRS accurately predicts in situ CP fractions in legume and grass silages such as rapidly degraded protein (A), slowly degraded protein (B), undegradable protein (C), and in situ RUP content within a single experiment. Although encouraging, the results of our experiments (4, 7, 8) have not resulted in commercial application. Two major challenges remain. First, a streamlined in situ method must be developed, as opposed to a full kinetic approach (9). Development of an NIRS equation requires a minimum of 100 to 200 samples to represent a legume and grass silage population fairly.

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A full in situ kinetic evaluation (9) of 100 to 200 legume and grass silages would result in processing 6000 to 12,000 dacron bags if silages were evaluated in four ruminally cannulated cows. Maintenance of this effort is unrealistic. Second, between cow error associated with in situ methods (17) must be minimized. Resolution of this analytical issue would allow the NIRS equation developed from a single experiment to be upgraded, validated, and maintained because in situ analysis could be conducted in the future using different cows fed different diets and results would be similar to the original evaluation.

The purpose of the trials described in this paper was to attempt resolution of these remaining issues. A set of stepwise trials was implemented to resolve these issues to develop a commercially viable system to estimate RUP content in legume and grass silages.

MATERIALS AND METHODS

Pretrial Decisions

To streamline the NIRS reference method (in situ) for determination of RUP content of legume and grass silages, certain pretrial decisions were required. First, we abandoned measuring silages using a full kinetics approach (9) and opted to use a single (24 h) ruminal incubation time to serve as a predictor of legume and grass silage RUP content. This decision was made for two reasons. First and most importantly, a large database (more than 100) of legume and grass silage RUP contents could then be generated, which is required by NIRS to yield robust calibration equations. Second, the relationship between kinetically derived in situ RUP and CP remaining (**CPR**) after 24 h of ruminal incubation is high. The relationship between kinetically derived in situ RUP and CPR after 24 h of ruminal incubation has been observed to be $r^2 = 0.90$ and 0.93 in previous trials (8, 9). In both of those trials (8, 9), the relationship between CPR after 12 h of ruminal incubation and kinetically derived in situ RUP was better ($r^2 = 0.95$) than that after 24-h ruminal incubation and RUP, but the standard error of measurement for the 24-h ruminal incubation time was 3-fold less (P. C. Hoffman, S. J. Sievert, R. D. Shaver, D. A. Welch, and D. K. Combs, 1993, unpublished data) than the standard error of CPR after a 12-h ruminal incubation. Therefore, the 24-h ruminal incubation time was chosen to accommodate both accuracy and precision. The researchers understood the negative aspects of this decision, which were relinquishing the ability to predict the A, B, and C fractions and degradation rate (k_d) of CP in legume and grass silages using NIRS. Although these

protein fractions are an integral part of new ruminant nutrition models (15, 16), the industry has yet to produce a commercially viable system to estimate RUP content in legume and grass silages. The researchers, therefore, chose to work on an objective that was accomplishable. The second set of pretrial decisions pertained to issues associated with in situ measurement error.

Measuring CP degradation of legume and grass silages using in situ methods is plagued with potential error. This error would be present in any NIRS equation that uses in situ procedures for its reference method (20). Sources of in situ measurement error fall into two broad categories: laboratory technique and ruminal environment. Laboratory technique includes issues such as bag pore size, sample size to surface area ratio, grind size, rinsing technique, etc. In this and previous trials (4, 7, 8, 9), we followed guidelines for ruminal in situ digestion procedures as recommended by Nocek (17) to minimize laboratory technique error with the exception that bags were rinsed via a washing machine (5) upon removal from the cow and not rinsed by hand. For these trials, the more important source of in situ measurement error was ruminal environment, which is affected by cow and diet (17). This source of error is large and is especially problematic because results from one experiment cannot be completely compared with another. This problem meant that an NIRS equation for RUP content of legume and grass silages developed from our experiments could not be updated or maintained via future in situ analysis. We chose to employ a calibration curve technique (6) in ruminally cannulated cows to resolve this issue potentially. The following trials were implemented after these pretrial decisions were made.

Trial 1

To employ a calibration curve technique (6) in ruminally cannulated cows requires a set of standards with known or theoretical absolute (**TA**) RUP contents. Because none exist, we had to first develop these standards. Before developing these standards, we had to know how many ruminally cannulated cows fed different diets would theoretically represent the ruminal environments of all cows. To calculate the number of ruminally cannulated cows required to establish TA in situ RUP values for our proposed standards, we needed a general understanding of the variance attributable to cow and diet on results of in situ measurements. To assess these sources of variance, the following experimental protocol was implemented. A common alfalfa silage (**CAS**) (CP = 20.3%; NDF = 44.0%) sample (2 kg) was obtained

from an oxygen-limiting silo at the Marshfield Agricultural Research Station (WI). The silage was dried at 55°C for 48 h and ground through a Wiley mill (2-mm screen; Arthur H. Thomas, Philadelphia, PA). A second standard sample was created by heat treating dried, ground CAS for 90 m at 160°C. Dacron bags (20 × 10 cm; 52- μ m pore size) were filled with 5 g of CAS and heat-treated (HT) CAS. Duplicate sample bags were wetted with tepid water, placed in a mesh net, secured to the ruminal cannula via a nylon cord, and incubated in the ventral rumens of 30 ruminally cannulated cows for 24 h. The ruminally cannulated cows were at different stages of lactation, including 9 dry cows, and were consuming one of seven different diets. Bags were immediately immersed in ice water upon removal from the rumen to stop microbial activity. Bags were then washed according to the procedures of Cherney et al. (5). Bags were dried at 55°C for 48 h and weighed. The remaining DM was determined. Residue from the bags was removed, and the duplicates were composited for chemical analysis. Bag residue was analyzed for CP (2) and adjusted for microbial CP contamination using an NIRS calibration (4) that estimated ($R^2 = 0.81$) milligrams of RNA CP per gram of DM. Base chemistries for the NIRS calibration (4) were the procedures of Zinn and Owens (21) with modifications by Ahorani and Tagari (1).

The CPR after 24 h of ruminal incubation was calculated for CAS and HT CAS for each cow. Summary statistics were performed on the data using SAS (18).

Trial 2

For implementation of a calibration curve technique, extremely low and extremely high in situ RUP standards are required (6); these were developed using the following procedures. A low in situ RUP standard was developed by harvesting 25 kg of stage 1 (10) early vegetative alfalfa, drying for 48 h at 55°C, and grinding through a Wiley mill (2-mm screen). The high RUP standard was developed by harvesting, drying, and grinding 25 kg of stage 8 (10) green seed pod alfalfa by the same procedures described for the low RUP standard. In addition, dried, ground, high RUP standard was placed 2.54 cm deep in the bottom of 20-cm × 20-cm aluminum pans and HT in an oven at 160°C for 90 m. All of the high RUP standard was HT in the manner. To establish TA RUP contents for the low and high RUP standards, dacron bags (20 × 10 cm; 52- μ m pore size) were filled with 5 g of either low or high RUP standard. Bags were handled and processed in a manner

identical to the procedures described in trial 1. Duplicate sample bags were incubated in the ventral rumens of eight ruminally cannulated cows for 0, 3, 6, 12, 24, 48, or 72 h and removed simultaneously. Zero-hour bags were soaked in tepid water for 0.5 h prior to removal of all bags for the period. Upon removal from the rumens, bags were processed and analyzed for CP and microbial CP in a manner identical to the procedures described in trial 1.

The CP degradation of the low and high RUP standards was analyzed using the NLIN procedure of SAS (18) and was fitted to the model of Mertens and Lofton (12):

$$FR = Pe^{-k(t-L)} + U \text{ when } t > L$$

and

$$FR = P + U \text{ when } 0 < t < L$$

where

$$\begin{aligned} FR &= \text{CPR at time } t, \\ P &= \text{potentially digested fraction (100 - U at a} \\ &\quad \text{fractional rate } k, k > 0), \\ U &= \text{fraction undigested at 72 h,} \\ L &= \text{discrete lag time, and} \\ t &= \text{incubation time (hours).} \end{aligned}$$

The RUP was estimated using the equation of NRC (14):

$$\text{RUP} = 100 - \{ \text{rapidly degraded protein A} \\ + B [k_d / (k_d + k_p)] \}$$

where k_p = ruminal passage rate (0.06/h).

Five of the ruminally cannulated cows were housed at the Marshfield Agricultural Research Station (Marshfield, WI), and three were housed at the Arlington Agricultural Research Station (Arlington, WI). Ruminally cannulated cows were lactating Holsteins housed in tie stalls and fed different diets to ensure that TA RUP values for the low and high RUP standards were established from a robust set of ruminal environments.

Trial 3

Upon determination of TA RUP content of the low and high RUP standards in trial 2, low and high RUP standards were physically mixed together to yield an expanded set of standards available to employ a calibration curve technique (6) in ruminally cannulated cows. Systematically mixing low and high RUP standards by increments of 10 percentage units (Table 1) yielded 11 calibration curve standards (CCS)

with an RUP ranging from 13.3 to 44.5 g/10⁻¹ kg of CP. Because CCS 1 and 11 were the end points of the calibration curve and because their TA RUP values were determined in trial 2, the RUP contents of CCS 2 to 10 were calculated, and determination was not required (6). Approximately 100 g of dried, ground (2 mm) material for each of the 11 CCS were prepared.

After CCS preparation, 121 legume and grass silage samples were collected from commercial forage testing laboratories. Approximately 100 g of each sample were dried at 55°C for 48 h and ground through a Wiley mill (2-mm screen). An in situ evaluation of each legume and grass silage was conducted over eight 1-d periods using the following procedures. Duplicate dacron bags (20 × 10 cm; 52- μ m pore size) filled with 5 g of silage were incubated for 24 h in the ventral rumens of four ruminally cannulated cows. Fifteen silages were evaluated during each period. Duplicate dacron bags containing 5 g of each CCS were also incubated in the ventral rumen of each ruminally cannulated cow for each period for 24 h. All pre and post incubation bag processing, drying, and rinsing procedures were identical to those described in trial 1. Determination of bag residues for CP and microbial CP contamination was also as per trial 1. The RUP content of legume and grass silages (n = 121) was determined using the following procedures. For each ruminally cannulated cow for each period, a first-order regression for the CCS was made where Y = TA RUP content of CCS, and x = CPR in CCS after 24 h of ruminal incubation.

The CPR after 24 h of ruminal incubation (x) for each legume and grass silage (n = 121) was used in the appropriate cow, period first-order equation in which the silage was evaluated, and its corresponding RUP (Y) was determined. The final RUP content of each legume grass silage was the mean of Y for all four ruminally cannulated cows.

In addition to RUP evaluation of the 121 legume and grass silage samples, an evaluation of the calibration curve technique was made in a concurrent phase of trial 3. The CCS 3, 6, and 9 (Table 1) were treated as unknowns, and RUP content was reestimated using the calibration curve technique to ascertain whether TA RUP values generated via trial 2 could be repeated when evaluated (in situ) in different ruminally cannulated cows fed different diets (trial 3). Duplicate dacron bags containing 5 g of CCS 3, 6, and 9 (Table 1) were incubated in the ventral rumens in each of the four ruminally cannulated cows in periods 1 to 4 in trial 3. In situ procedures and analyses of in situ residues were as previ-

TABLE 1. The methodology used to develop calibration curve standards.¹

Calibration curve standard	Low RUP	High RUP	TA RUP
	— (g per standard) —		
1	100	0	13.3
2	90	10	16.4
3	80	20	19.5
4	70	30	22.7
5	60	40	25.8
6	50	50	28.9
7	40	60	32.0
8	30	70	35.1
9	20	80	38.2
10	10	90	41.3
11	0	100	44.5

¹TA = Theoretical absolute (grams per 10⁻¹ kg of CP); TA RUP values for calibration curve standards 1 and 11 were determined in trial 2. The TA RUP values for calibration curve standards 2 through 10 were calculated.

ously described. The RUP content of CCS 3, 6, and 9 were derived for each cow for each period (n = 16) using the same calibration curve technique used on the 121 legume and grass silage samples. The mean and standard deviation of CCS 3, 6, and 9 were determined and compared with the TA RUP values for CCS 3, 6, and 9 generated in trial 2.

Trial 4

Dried, ground legume and grass silage samples (n = 121) evaluated in trial 3 were reground through a UDY mill (1-mm screen; UDY Corp., Boulder, CO). Samples were packed into a cylindrical sample holder equipped with a quartz window and scanned according to the procedures of Marten et al. (11) on a near infrared reflectance spectrophotometer (model 6500; NIR Systems, Perstop Analytical, Silver Spring, MD) with a spinning cup holder. Spectra were saved with center and select procedures implemented using Infracsoft International[®] software [version 2.0 (19)]. Spectra from 60 samples (calibration set) were used to develop a calibration equation for RUP content of legume and grass silages. Calibration was made using a modified least squares regression method, and the number of terms in the equation was varied until no significant or relevant improvement in the coefficient of determination or standard error of calibration could be determined. Different math transformations (19) were explored, and the 1, 4, 4, 1 math transformation with six terms in the equation offered the best prediction of RUP content of the silages. The remain-

ing samples ($n = 61$) were used as a validation set to evaluate the standard error of performance of the equation.

Trial 5

The NIRS RUP equation for legume and grass silages developed in trial 4 was field tested over a 3-mo period in spring 1998 at the University of Wisconsin Soil and Forage Analysis Laboratory (Marshfield). Legume and grass silage samples ($n = 300$) were procured from dairy farms via nutrition consultants, veterinarians, educators, and dairy producers and evaluated for RUP using the NIRS equation developed in trial 4. The silages were also evaluated for DM, CP, ADF, and NDF using NIRS equations from NIR Systems. Summary statistics of the predicted RUP values for the field study legume and grass silages were generated using SAS (18). Correlations between RUP and DM, CP, ADF, and NDF were also made (18).

RESULTS AND DISCUSSION

Trial 1

The distributions of CPR in CAS and HT CAS after a 24-h ruminal incubation in 30 ruminally cannulated cows were reasonably normal (data not presented); therefore, the standard deviation and mean of the subpopulation ($n = 30$) should have fairly represented σ and μ , respectively. The mean CPR after 24 h of ruminal incubation for CAS and HT CAS were 8.74 and 46.29 g/10⁻¹ kg of CP, respectively. The standard deviation of CPR after 24 h of ruminal incubation for CAS and HT CAS were 0.82 and 3.80 g/10⁻¹ kg of CP. The minimum number of ruminally cannulated cows required to estimate RUP μ using in situ procedures for any given legume and grass silage was estimated by the following equation (3):

$$n = \left[\frac{Z_{\alpha/2}(SD)}{\bar{X}(ME)} \right]^2$$

where ME = tolerable measurement error. We assumed $\alpha = 0.10$ ($Z_{0.10/2} = 1.64$) (3) and tolerable measurement error = 5.0%. Therefore, the minimum number of ruminally cannulated cows to estimate RUP μ for CAS and HT CAS would have been 9.5 and 7.3, respectively.

Based on these observations, we chose to use eight ruminally cannulated cows in trial 2 to establish TA RUP values for our low and high RUP standards, which were required to implement our proposed calibration curve technique.

Trial 2

Background information on the eight ruminally cannulated cows used to establish the TA RUP content of the low and high RUP standards is presented in Table 2. The ruminally cannulated cows represented a range of lactations (1 to 5) and DIM (30 to 276), providing a robust set of ruminal environments. Diets fed to the eight ruminally cannulated cows were not comprehensively evaluated but contained different forages, grains, and forage to grain ratios (Table 2). Diets contained feed ingredients commonly fed in the upper Midwest region of the United States. Given the impracticality of acquiring and feeding diets from multiple geographic locations, we fed diets containing readily accessible feed ingredients. Therefore, our attempt to establish TA RUP values for the low and high RUP standards probably is biased to ruminal environments of lactating cows found in the upper Midwest region of the United States or other areas in which similar feedstuffs are fed.

The A, B, C, k_d , and RUP contents of the low and high RUP standards for each of the ruminally cannulated cows are also presented in Table 2. There was modest variation among cannulated cows in the amount of A, B, and C. The variation was more pronounced for the high RUP standard. There was a large variation in k_d among cannulated cows. Some variation in k_d was expected, but the extremely wide range in k_d was not and may in part be due to inherent error of the measurement (17). The mean RUP content of the low and high RUP standard for the eight ruminally cannulated cows was designated as TA RUP and was 13.3 and 44.5 g/10⁻¹ kg of CP, respectively.

Trial 3

Upon determination of TA RUP content of the low and high RUP standards (trial 2), the low and high RUP standards were physically mixed together by decreasing and increasing 10 percentage units per sample of low and high RUP standard, respectively, to yield the CCS (Table 1). The methodology resulted in 11 CCS with TA RUP values starting at 13.3 g/10⁻¹ kg of CP, increasing 3.1 g/10⁻¹ kg of CP per CCS to a high CCS containing 44.5 g/10⁻¹ kg of CP (Table 1). As previously described, the CCS were used in trial 3 ruminally cannulated cows to derive RUP values for 121 legume and grass silage samples. Because the calibration curve technique was a method used in these studies and not the focus of the studies, a detailed evaluation was not conducted. As a result,

TABLE 2. Background information on ruminally cannulated cows, their diets, and the effect on CP degradability of low and high RUP standards (trial 2).¹

Item	Cow no.								\bar{X}	SD
	1	2	3	4	5	6	7	8		
General										
Lactation number	2	2	4	1	3	2	5	4		
DIM	30	118	116	277	244	276	184	167		
Diet ²										
Alfalfa silage	29.5	30.7	32.6	73.0	52.0	52.0	18.9	18.9		
Corn silage	15.9	13.2	13.0	27.2	27.2		
Small grain silage	9.6		
Shelled corn	32.8	39.2	43.6	33.3	33.3		
Grain mix	27.0	48.0	48.0		
Soybean meal	9.2	13.4	7.9	8.9	8.9		
Distillers grains		
Cottonseed	8.5	8.5		
Blood meal	...	1.0	0.8	0.9	0.9		
Vitamins and minerals	2.9	2.5	2.0	1.2	1.2		
CP	17.0	18.1	17.5	17.0	16.5	16.5	18.1	18.1		
NDF	33.0	29.0	28.0	34.0	29.0	29.0	29.5	29.5		
Low RUP standard										
A, % of CP	59.9	60.0	59.4	61.3	60.1	60.3	59.2	58.6	59.9	0.76
B, % of CP	36.0	35.8	36.8	34.0	34.7	35.3	35.5	36.6	35.6	0.87
C, % of CP	4.1	4.2	3.8	4.7	5.2	4.4	5.5	4.4	4.5	0.53
k_d , 1/h	0.300	0.247	0.164	0.155	0.119	0.119	0.314	0.199	0.202	0.0724
RUP, ³ % of CP	10.1	11.2	13.7	14.2	16.8	16.2	11.0	13.3	13.3	2.3
High RUP standard										
A, % of CP	32.2	36.0	30.9	33.5	31.7	32.5	33.8	33.3	33.0	1.46
B, % of CP	53.5	46.8	55.4	54.1	53.8	48.7	44.5	45.1	50.2	4.16
C, % of CP	14.3	17.2	13.7	12.4	14.5	18.8	21.7	21.6	16.8	3.39
k_d , 1/h	0.084	0.091	0.044	0.025	0.029	0.041	0.061	0.61	0.055	0.0234
RUP, % of CP	36.6	35.8	45.7	50.6	50.8	48.3	43.8	44.0	44.5	5.4

¹A = Rapidly degraded protein, B = slowly degraded protein, C = undegradable protein, and k_d = fractional degradation rate of B.

²All values are expressed as percentages of DM; CP and NDF values are approximate and were obtained from ration formulation documents.

³Calculated as $100 - \{A + B[k_d/(k_d + k_p)]\}$, where A, B, and k_d are as defined previously and k_p = ruminal passage rate of 0.06/h.

we are offering a condensed demonstration of the calibration curve technique in Table 3. The individual samples representing the minimum, maximum, and median between cow error in estimating RUP content of the 121 legume and grass silages are presented in Table 3. In our demonstration (Table 3), between cow error is simply defined as the range in RUP values among the four ruminally cannulated cows for a given legume and grass silage sample. Using the calibration curve technique on silage sample 13 resulted in the smallest range of RUP values among cows (0.2 g/10⁻¹ kg of CP). For sample 13, the range in CPR after 24 h of ruminal incubation among ruminally cannulated cows was 2.7 g/10⁻¹ kg of CP, and the calibration curve technique reduced between cow error 13-fold. In contrast, the calibration curve technique increased the range in RUP values (6.2 g/10⁻¹ kg of CP) among cows for sample 114 as compared with the CPR after 24 h of ruminal incubation, the

range for which was 2.3 g/10⁻¹ kg of CP. For sample 114, the calibration curve technique increased cow-to-cow error when evaluating RUP using in situ techniques. The median between cow error was represented by sample 42 with a range among cows of 1.9 g/10⁻¹ kg of CP. For sample 42, the between cow error of CPR after 24 h of ruminal incubation was 4.4 g/10⁻¹ kg of CP, indicating that the calibration curve technique reduced the between cow in situ measurement error approximately 2-fold.

When comparing the between cow error (range) in CPR after 24 h of ruminal incubation and calibration curve-generated RUP (data not presented), between cow error was reduced for 82 of the legume and grass silages and increased for 39 legume and grass silages. On average, employing a calibration curve in ruminally cannulated cows reduced between cow error 2-fold but did not guarantee in situ measurement error reduction on every legume and grass silage

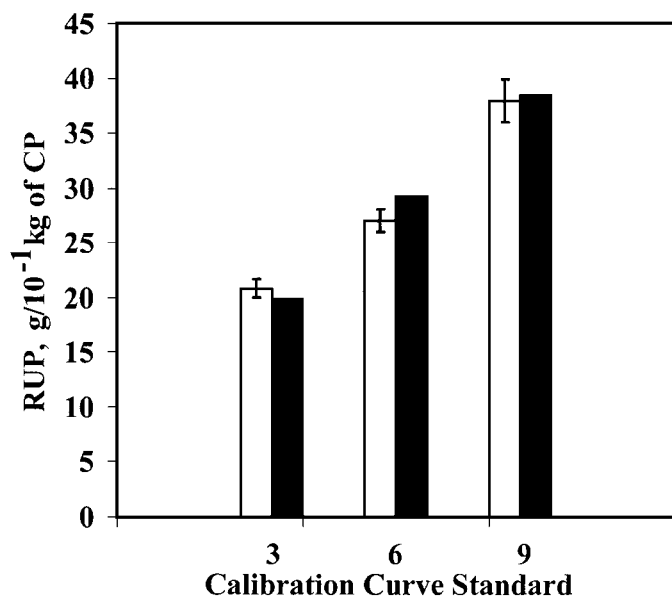


Figure 1. A comparison of RUP content of calibration curve standards 3, 6, and 9 determined in trial 3 to their original theoretical absolute (TA) RUP values determined in trial 2. Trial 3 RUP (grams per 10⁻¹ kg of CP) (□); TA RUP (grams per 10⁻¹ kg of CP) (■). Error bars denote standard deviations.

sample. These data suggest that the calibration curve technique aids in reducing in situ measurement error, but it is still prudent to use multiple ruminally cannulated cows to establish mean in situ RUP values.

The second and concurrent phase of trial 3 was to determine whether the TA RUP values developed for the CCS in trial 2 could be repeated in trial 3, in which different ruminally cannulated cows were used. A comparison of the RUP values of CCS 3, 6, and 9 as determined in trial 3 with their TA RUP values is presented in Figure 1. In general, the RUP values for CCS 3, 6, and 9 determined in trial 3 using the calibration curve technique compared favorably with the trial 2 TA RUP values. The largest difference between RUP (trial 3) and TA RUP (trial 2) was for CCS 6 at 2.2 g/10⁻¹ kg of CP. The trial 3 RUP and TA RUP values for CCS 3 and 9 were, however, nearly identical. Although not conclusive, these data suggest that generating similar in situ RUP values between experiments is feasible.

Trial 4

Calibration and validation statistics of the NIRS analysis of the 121 legume and grass silages are presented in Table 4. The minimum and maximum RUP content of the legume and grass silage data set was 16.1 and 49.6 g/10⁻¹ kg of CP, respectively, with a mean RUP content of 22.2 g/10⁻¹ kg of CP. The best NIRS RUP calibration resulted from 1, 4, 4, 1 math transformation using six terms in the model. The R² of 0.84 and the standard error of calibration of 1.55 g/10⁻¹ kg of CP indicated reasonable accuracy in predicting legume and grass silage RUP content us-

TABLE 3. Minimum, maximum, and median error¹ associated with the calibration curve technique employed on 121 legume and grass silage samples in trial 3.²

Error	Sample	Cow no.	CPR After 24 h	CCS		RUP
				Intercept	Slope	
				(g/10 ⁻¹ kg of CP)		
Minimum	13	1	17.1	10.8	0.89	26.1
		2	17.0	11.0	0.89	26.1
		3	14.4	10.4	1.08	25.9
		4	15.2	11.2	0.98	25.9
		X	15.9	10.7	0.96	25.9
		Range	2.7	0.8	0.19	0.2
Maximum	114	1	27.3	10.9	0.90	35.5
		2	25.0	10.5	1.11	38.4
		3	25.8	10.7	0.83	32.2
		4	26.4	10.9	0.98	36.6
		X	26.1	10.7	0.95	35.7
		Range	2.3	0.4	0.28	6.2
Median	42	1	8.3	11.1	1.02	19.5
		2	9.8	10.8	0.91	19.7
		3	12.7	10.9	0.82	21.4
		4	11.3	10.9	0.92	21.2
		X	10.4	10.9	0.92	20.5
		Range	4.4	0.3	0.20	1.9

¹Range in estimated legume and grass silage RUP contents among cows.

²CPR = CP remaining; CCS = calibration curve standards.

TABLE 4. Calibration and validation statistics for near infrared spectroscopy analysis of legume and grass silages (trial 4).¹

Item	RUP (g/10 ⁻¹ kg of CP)	CP — (% of DM) —	NDF
Data set (n = 121)			
Minimum	16.1	9.4	33.2
Maximum	49.6	24.3	73.2
X	22.2	17.7	49.8
SD	4.41	7.15	3.28
Calibration (n = 60)			
Transformation ²	1, 4, 4, 1
PLS, terms	6
R ²	0.84
SEC, g/10 ⁻¹ kg of CP	1.55
Validation (n = 61)			
r ²	0.85
SEP, g/10 ⁻¹ kg of CP	1.51
Bias, g/10 ⁻¹ kg of CP	0.21

¹PLS = Partial least squares, SEC = standard error of calibration, and SEP = standard error of performance.

²Order of derivative function, segment length (nanometers), segment length (nanometers) of first smoothing, and segment length (nanometers) of second smoothing.

ing NIRS. Validation statistics indicated good performance of the equation with an r^2 of 0.85 and a standard error of prediction of 1.51 g/10⁻¹ kg of CP. These data support our previous observations (7, 8) that NIRS can predict in situ RUP content of legume and grass silages. In previous studies (7, 8), NIRS RUP calibrations were developed on preplanned (7) or small (8) (n = 32) legume and grass silage data sets. In the present study, the NIRS calibration was developed on a much larger data set of legume and grass silages (n = 121), which may have the potential for commercial application (19).

Trial 5

The NIRS RUP calibration equation developed in trial 4 was field tested on 300 legume and grass silage samples obtained from commercial dairy farms in the upper Midwest region of the United States. The RUP, DM, CP, ADF, and NDF contents of the field test silages were determined, and data are presented in Table 5. The mean DM, CP, ADF, and NDF contents of the field test silages were 40.3, 20.1, 35.2, and 46.5%, respectively. The mean RUP content of the field test silages was predicted to be 21.8 g/10⁻¹ kg of CP with a minimum and maximum predicted RUP of 14.2 and 36.6 g/10⁻¹ kg of CP, respectively. The RUP contents predicted by the NIRS equation compare closely with tabular RUP values (15, 16) for legume and grass silages of similar quality. Correlations between RUP and DM, CP, ADF, and NDF are also presented in Table 5. The RUP values predicted by the NIRS equation were negatively correlated ($P < 0.05$) with CP and positively correlated ($P < 0.05$) with ADF and NDF. These relationships are similar to those defined in previous research (9, 16). There was a positive correlation ($P > 0.05$) between the NIRS predicted RUP and DM content of the field test silages. Because silage DM content effects proteolysis and ultimately silage RUP content (7), the relationship between RUP and DM content of silages was expected to be stronger. Because the RUP and DM relationship might have been colinear with CP, ADF, or NDF RUP relationships, we developed a multiple regression (18) of CP and NDF on RUP ($R^2 = 0.76$) for the field test silages and plotted the residuals against the DM content of silages (data not presented). This procedure resulted in a somewhat stronger

TABLE 5. Summary statistics and correlations of nutrients in legume and grass silages (n = 300) evaluated by near infrared reflectance spectroscopy in trial 5.

Item	DM (%)	CP ————— (% of DM) —————	ADF ————— (% of DM) —————	NDF ————— (% of DM) —————	RUP (g/10 ⁻¹ kg of CP)
Data set (n = 300)					
Minimum	15.1	12.5	26.7	34.6	14.2
Maximum	83.8	26.4	47.9	64.3	36.6
X	40.3	20.1	35.2	46.5	21.8
SD	10.31	2.60	3.41	5.54	3.66
					r =
Correlation					
RUP vs. DM	0.14
RUP vs. CP	-0.66*
RUP vs. ADF	0.68*
RUP vs. NDF	0.74*

* $P < 0.05$.

r^2 of 0.17 and a second-order relationship ($P < 0.03$) between silage DM and NIRS predicted RUP. Data suggest that silage DM has little effect on RUP content when legume and grass silages are ensiled at less than 50% DM, but, when legume and grass silages contain greater than 50% DM, the RUP content increases. This relationship is logical because ensiling at high DM contents may result in increased heat of fermentation, which may result in increased silage RUP content as compared with ensiling at a lower DM content (13). Based on the results of trial 5, the NIRS equation developed to predict RUP content of legume and grass silages appears to do so within industry norms (15, 16) and according to preestablished relationships (9, 13, 16) between other legume and grass silage nutrients and RUP.

CONCLUSIONS

The objective of this research was to explore the development of a commercially viable system to estimate RUP content of legume and grass silages using NIRS technology. We attempted to accomplish this objective but understand that our methods did not result in a perfect system. We were able to reduce error in our chosen NIRS RUP reference method (in situ) significantly by employing a calibration curve technique in ruminally cannulated cows. The calibration curve technique does not, however, completely eliminate between cow error when evaluating RUP content of legume and grass silages. Despite these and all of the shortcomings of the in situ technique, we were able to develop a reasonably good NIRS calibration equation to predict in situ RUP content of legume and grass silages. This equation appeared to perform acceptably under field conditions.

The major problem in developing this system was that we had to decide on acceptable levels of measurement error. Because no guidelines exist, we used our collective experiences and judgment. We make no attempt to defend our decisions and judgments and rather offer this novel approach in hope that future investigations may improve upon our efforts.

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