Use of near infrared spectroscopy for determining protein fractions in alfalfa

H. Hsu, A. Suleiman and G. Recinos-Diaz

Alberta Agriculture, Food and Rural Development, Edmonton, 6909–116 Street Alberta, Canada, T6H 4P2.

Introduction

Protein components in feed are important in ration balancing and development of feeding programs for ruminants, especially for meeting animal requirements for higher productivity, e.g. dairy cattle. Often, over half of the protein required is supplied by the forages. Nutritionists have known about the importance and use of protein fractions in forages for many years. However, they have not been able to make good use of this knowledge in formulating practical rations for on-farm use with any degree of certainty that they will work. Until recently, comprehensive, practical ration formation models had not been available. The Cornell Net Carbohydrate and Protein System (CNCPS) uses protein fractions in feedstuffs based on solubility in mineral solvents and detergent solutions. The greatest limitation to widespread practical application of what nutritionists know about protein nutrition, is poor information on the protein components of the feeds actually used on the farm, in particular, the forages. Although chemical *in vitro* or *in vivo* analysis for these important protein components is available commercially, the cost is prohibitive. Use of near infrared (NIR) spectroscopy would reduce the costs significantly.

NIR spectroscopy has been used to accurately predict crude protein (total N) content of feedstuffs as well as acid detergent and neutral detergent fibre portions (ADF and NDF) of forages.[1] Some protein fractions are associated with ADF and NDF. However, little information exists in the scientific literature on the use of NIR spectroscopy for prediction of the nutritionally important protein fractions of forages. The objectives of this study were to: (i) obtain values of various protein fractions in Alberta alfalfa hays and silages by wet chemistry and (ii) evaluate the use of NIR spectroscopy to predict protein fractions in alfalfa hay and silage.

Material and methods

Two different NIR spectroscopy calibration samples were developed, The first on alfalfa hay (AH) and the second on alfalfa silage (AS). The alfalfa hay calibration set was obtained from 1081 samples received by the Agricultural Soils and Animal Nutrition Laboratory (ASANL), in Edmonton, between April 1991 to December 1993. All AH and AS samples were dried at 60°C and ground through a Wiley mill to pass a 1 mm screen. Samples were scanned by the NIRSystems model 6500 instrument (NIRSystems Inc, Silver Spring, MD), and the spectra collected as log (1/*R*) from 400 nm to 2498 nm. The Center program was used to order all samples according to Mahalanobis distance *(H)* and discriminate outlier samples with a cut-off distance of global $H>3.0$. Three hundred and thirty five samples were chosen by Select program using a neighbourhood *H* cut-off of 0.6, thus eliminating spectrally similar samples. A subset of 25 samples from the selected 335 samples were chosen for validation. The alfalfa silage calibration set was represented by 95

samples chosen by SELECT program from 189 samples. A subset of 12 samples from the selected 95 samples was used for validation. Spectral distance calculations, selection of samples and calibration regressions were performed by the Center, Select and CAL programs developed by Infrasoft International NIRS 3 version 3.10 (NIRSystems Inc., Silver Spring, MD).

The selected samples were analysed by wet chemistry for crude protein (CP), acid-detergent fiber (ADF), neutral detergent fiber (NDF), acid detergent insoluble protein (ADIP), neutral detergent insoluble protein (NDIP), insoluble protein (IPROT) and soluble true protein (SPROT). Phosphate buffer soluble and insoluble protein were extracted using the method developed by Krishnamoorthy *et al.*[2] The Bradford Method[3] was used to determine soluble true protein (SPROT) from the phosphate buffer soluble protein extract. Wet chemistry results were used to develop NIR calibration equations for these components. The NIR calibrations were obtained using four cross-validation groups with modified partial least squares using every fifth wavelength between 400 and 2498 nm. The math treatment was 3,5,5,1 (third derivative) calculated with a difference of five data points followed by a 5-point smooth. Detrend was used to reduce the interference of light scatter and particle size of sample in the spectra. Downweight was used to remove samples with large *T* or *H* values.

Results and discussion

Statistical parameters of *SEC*, *SEP*, R^2 and the slope of the NIR regression equations are considered useful in the evaluation of accuracy of predicting the nutritionally important constituents of forages. Constituents studied were: crude protein (CP), soluble protein (SPROT), insoluble protein (IPROT), acid-detergent insoluble protein (ADIP) and neutral detergent insoluble protein (NDIP). Values of *SEC* and *SEP* for all these protein fractions obtained were low and R^2 values were high in both alfalfa hays and silages [Figure 1, (a)–(j)]. The slope for each fraction was close to 1. These statistics indicate acceptable calibration equations. The widest dispersion (over two standard deviations) from the calibration regression line was observed for soluble protein fractions in hays [SPROT, Figure 1(b)] and to a lesser extent in silages [see Figure 1(g)]. The reverse is true for NDIP and ADIP, the dispersion being narrower in hays than silages [Figure 1(d),(e),(i) and (j)]. This may be related to references in the literature that say, in alfalfa prior to harvest, 60–80% of total plant N is in the form of soluble protein,[4,5] and that after harvest, during wilting and ensiling, proteolysis by proteinase enzymes in plant cell hydrolyse a portion of the soluble protein (SP) to soluble non-protein N. Rapid proteolysis (ending within a few days) may contribute to changes in protein structure. In Alberta, hays are always wilted before being baled and most silages are wilted before ensiling. Thus, proteolysis will occur in both hays and silages during wilting and continue in silages until a significant drop in pH has occurred. The wider dispersion seen in silages for ADIP and NDIP may be associated with the variability that may exist in silages due to ensiling process resulting in conformational changes in proteins. These conformational changes may be quite variable . These calibrations should permit use of predicted values for the various protein fractions in alfalfa forage in ration balancing. This should enable nutritionists to accurately formulate rations to meet the needed amounts of different proteins fractions in growing and lactating cattle where protein supplementation is often required.

Important and relevant NIR regression statistics for hay calibration samples ($N = 290$) and validation samples ($N = 25$) and silage calibration samples ($N = 83$) and validation samples ($N = 12$) are given in Tables 1 and 2. The standard error of prediction in the calibration set (SEP_c) obtained for AH and AS respectively were low: 0.57 and 0.33 (CP); 0.16 and 0.05 (SPROT); 0.52 and 0.25 (IPROT); 0.94 and 2.94 (NDIP) and 0.46 and 0.77 (ADIP). The regression coefficients (R^2) obtained between actual and predicted results for hays and silages were high in the calibration set: 0.97 and 0.99 (CP); 0.86 and 0.94 (SPROT); 0.94 and 0.98 (IPROT); 0.96 and 0.91 (NDIP)

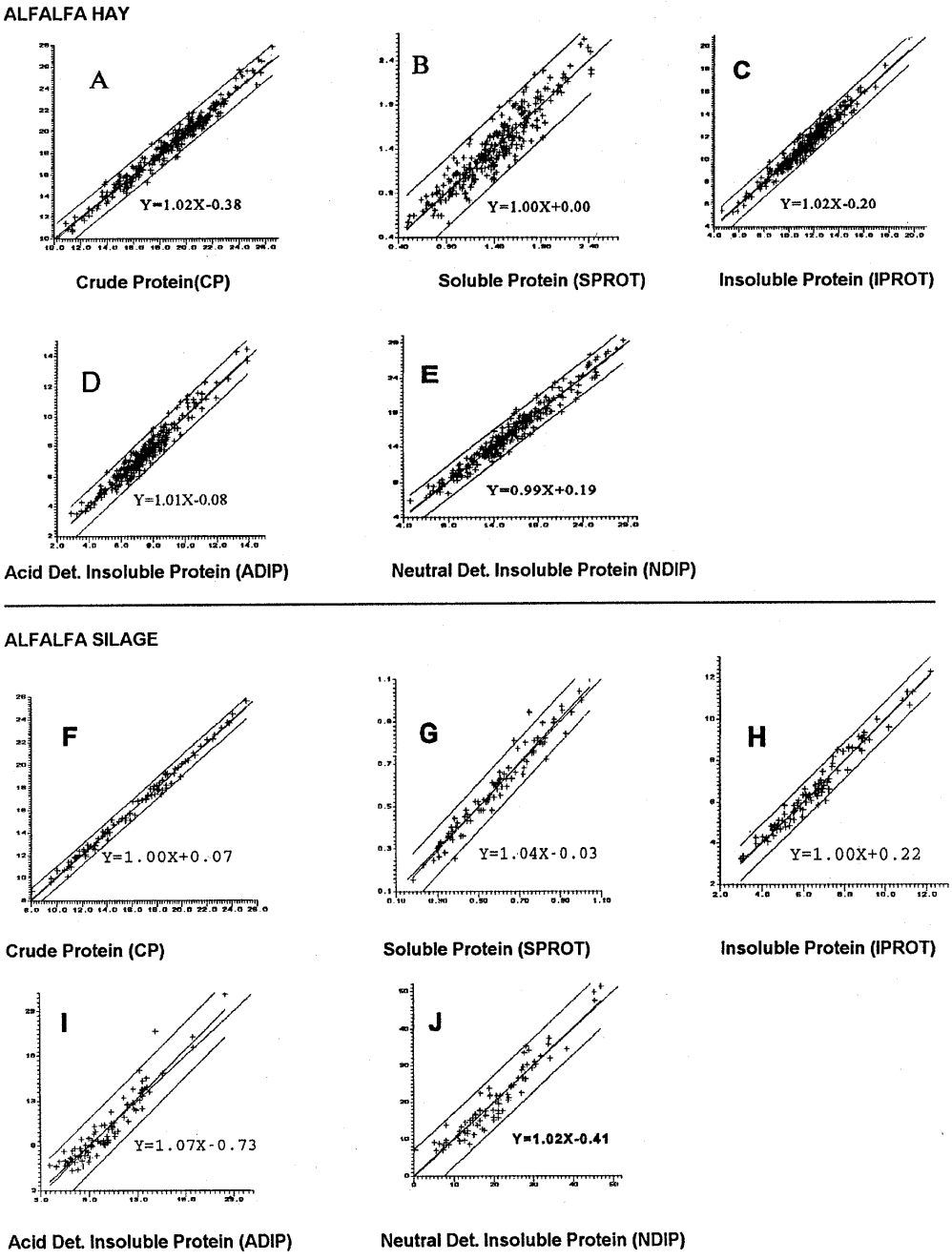

Figure 1. NIR regression equations of protein fractions for Alfalfa hay and Alfalfa silage. Lab versus NIR results.

Table 1. NIR regression statistics for alfalfa hay.

	N_c	R^2_c	SEP_c	Range$_c$	N_v	SEP_v	R^2_v	RPD[b]
CP	280	0.97	0.57	10.0–27.8	25	0.84	0.98	5.61
SPROT	283	0.86	0.16	0.5–2.7	25	0.21	0.92	2.63
IPROT	282	0.94	0.52	5.2–20.8	25	0.67	0.97	4.27
NDIP[a]	275	0.95	0.94	6.3–29.2	25	1.56	0.96	4.74
ADIP[a]	278	0.93	0.46	3.4–14.4	25	0.57	0.88	3.96

[a]Percent of CP bound with fibers.
[b]*RPD* is the ratio of *SD* lab data to *SEP* from the calibration set (over 2.5 is acceptable).
SEP_c = standard error of prediction calibration set.

Table 2. NIR regression statistics for alfalfa silage.

	N_c	R^2_c	SEP_c	Range$_c$	N_v	SEP_v	R^2_v	RPD_c[b]
CP	81	0.99	0.33	8.7–27.8	12	0.64	0.95	11.39
SPROT	78	0.93	0.05	0.5–1.1	12	0.09	0.83	4.40
IPROT	80	0.98	0.25	3.2–12.2	12	0.44	0.91	8.04
NDIP[a]	80	0.91	2.94	6.7–51.2	12	1.66	0.92	3.41
ADIP[a]	79	0.96	0.77	5.1–24.8	12	1.29	0.91	5.11

[a]Percent of CP bound with fibers.
[b]*RPD* is the ratio of *SD* lab data to *SEP* from the calibration set (over 2.5 is acceptable).
SEP_c = standard error of prediction calibration set.

Table 3. Comparison of lab and NIR predicted values for alfalfa hays in the validation set.

	CP%		SPROT%		IPROT%		NDIP%		ADIP%	
Sample	Lab	NIR	Lab	NIR	Lab	NIR	Lab	NIR	Lab	NIR
1	24.77	24.68	1.96	2.26	14.57	14.68	10.00	9.57	4.56	4.44
2	20.81	21.52	1.47	1.99	13.96	14.49	15.47	14.41	7.29	6.52
3	18.75	19.52	1.60	1.61	12.09	11.72	13.56	12.84	6.75	6.89
4	14.10	13.77	1.19	1.16	7.59	8.92	16.38	16.31	7.66	7.89
5	20.24	20.02	1.53	1.63	12.04	12.64	13.50	14.33	6.64	6.65

Table 3. Comparison of lab and NIR predicted values for alfalfa hays in the validation set (continued).

	CP%		SPROT%		IPROT%		NDIP%		ADIP%	
Sample	Lab	NIR	Lab	NIR	Lab	NIR	Lab	NIR	Lab	NIR
6	19.58	18.85	1.56	1.36	12.12	11.72	13.07	14.05	6.49	6.89
7	20.56	20.04	1.25	1.31	13.52	13.12	15.52	15.41	6.40	6.82
8	17.72	18.61	0.99	1.04	13.09	13.35	21.70	21.26	8.92	8.90
9	21.43	20.74	1.30	1.53	12.36	12.72	13.84	13.48	6.75	5.93
10	18.28	18.36	1.33	1.26	11.56	11.07	19.20	19.34	9.54	9.46
11	19.78	20.93	1.47	1.61	9.51	9.81	15.64	16.36	6.62	7.33
12	15.56	16.75	0.57	0.73	9.76	11.50	21.48	19.44	9.36	9.01
13	19.61	19.15	1.88	1.53	11.73	11.30	15.29	15.36	6.55	7.23
14	23.00	24.36	2.02	2.20	14.26	13.98	11.12	7.32	5.50	4.08
15	15.43	16.16	1.96	1.67	9.39	9.71	14.31	13.04	7.21	6.85
16	24.10	23.89	1.75	1.79	12.16	11.46	7.40	6.37	5.25	4.62
17	24.64	24.42	2.69	2.33	12.28	12.15	8.90	8.99	4.55	4.61
18	18.38	18.71	1.61	1.72	10.69	10.19	9.61	9.56	6.96	6.31
19	20.53	20.64	1.81	1.76	11.05	11.22	14.82	14.00	7.94	7.43
20	13.91	13.85	1.06	1.05	9.54	9.45	26.14	23.61	11.15	10.36
21	18.32	18.23	1.57	1.52	11.15	10.58	11.57	12.08	5.12	5.59
22	11.13	12.19	0.65	0.72	6.53	8.20	22.98	17.72	8.34	7.29
23	26.45	24.32	2.57	2.17	15.25	14.69	6.65	6.96	2.97	2.87
24	21.88	20.38	2.37	2.20	12.77	12.49	7.57	7.41	3.88	4.10
25	20.68	19.69	1.72	1.62	8.77	9.26	7.56	8.21	5.65	5.81
Mean	19.59	19.59	1.60	1.59	11.51	11.62	14.13	13.50	6.72	6.55
SD	3.66	3.35	0.52	0.45	2.16	1.84	5.17	4.68	1.87	1.81
Slope	1.06		1.05		1.12		1.06		0.99	

and 0.94 and 0.97 (ADIP) respectively. The regression coefficients (R^2) obtained between actual and predicted results for silages and hays were high in the validation set: 0.95 and 0.98 (CP); 0.83 and 0.92 (SPROT); 0.91 and 0.97 (IPROT); 0.92 and 0.96 (NDIP) and 0.91 and 0.88 (ADIP) respectively. Wet chemistry values compared to NIR predicted values were very similar in the validation set for both alfalfa hays and silages (See Tables 3 and 4). The average results in the validation set for NDIP (21.96%) and ADIP (9.93%) were higher in silages compared to hays (NDIP, 13.5% and ADIP, 6.55%). The reverse was true for soluble and insoluble proteins: 1.59% and 11.62% for hays and 0.57% and 6.69% for silages, respectively. These validation sets for hay and silage were representative of the population. The average CP values for hay (1081 samples) and silage (189 samples) were, respectively, 19.05% and 15.85% versus 19.59% and 15.84% for the validation sets. These values compare reasonably with the ten year average[6] composition of alfalfa hay (CP 18.1%) and silage (CP 16.7%), indicating that the samples used in this study were similar to what is normally available in Alberta. The *RPD* statistic, which is the ratio of the standard error of prediction (*SEP*) to the standard deviation (*SD*) in the original calibration data, should be as high as possible in order to decide if a calibration equation is acceptable for predicting a

Table 4. Comparison of lab and NIR predicted values for alfalfa silages in the validation set.

	CP%		SPROT%		IPROT%		NDIP%		ADIP%	
Sample	Lab	NIR	Lab	NIR	Lab	NIR	Lab	NIR	Lab	NIR
1	13.94	14.11	0.45	0.48	7.48	7.78	27.80	29.42	12.77	11.84
2	10.08	11.88	0.34	0.43	5.47	5.49	31.86	32.27	13.93	13.59
3	18.57	18.23	0.55	0.58	5.80	5.63	10.52	10.18	7.31	6.54
4	18.88	20.04	0.65	0.75	6.92	6.91	15.29	14.61	8.51	7.70
5	20.03	19.71	0.63	0.65	6.24	5.90	10.19	9.09	7.60	5.95
6	20.23	20.31	0.66	0.71	7.65	7.14	17.79	14.31	8.76	6.99
7	12.22	12.13	0.36	0.50	8.53	8.26	31.56	30.56	11.98	13.47
8	18.36	18.40	0.53	0.64	10.88	10.25	25.50	26.11	9.76	10.81
9	22.26	22.17	0.76	0.78	9.13	8.23	11.37	12.44	6.55	5.50
10	11.98	12.05	0.36	0.49	5.10	5.52	26.43	27.65	13.79	12.11
11	9.92	9.70	0.29	0.43	4.81	4.60	32.01	30.32	14.01	12.74
12	11.33	11.31	0.35	0.43	4.03	4.58	23.36	26.45	13.50	11.85
Mean	15.65	15.84	0.49	0.57	6.84	6.69	21.97	21.96	10.71	9.93
SD	4.48	4.37	0.16	0.13	2.00	1.71	8.54	8.97	2.91	3.12
Slope	1.02		1.15		1.15		0.94		0.88	

constituent accurately.[7] Based on the coefficient of determination (R^2), the standard error of prediction (*SEP*) and the *RPD* values, we conclude that the equations developed in this study are acceptable and capable of predicting those parameters accurately.

Implications

This study has shown that NIR can be used to rapidly and accurately determine protein fractions in alfalfa hays and silages grown in Alberta. Availability of such predicted values will enable nutritionists to accurately formulate rations to supply the needed amounts of different protein fractions in growing and lactating cattle. This will be one more tool that nutritionists can use in practical feeding programs for ruminants.

References

1. J.L. Shenk and M.O. Westerhaus, "The Application of Near Infrared Reflectance Spectroscopy (NIRS) to Forage Analysis", in *Forage Quality, Evaluation, and Utilization*, Ed by G.C. Fahey Jr. American Society of Agronomy, Inc., Crop Science Society of America Inc. and Soil Science Society of America, Inc., Madison, WI, USA, p. 406–449 (1994).
2. U. Krishnamoorthy, T.V. Muscato, C.J. Sniffen and P.J. Van Soest, *J. Dairy Sci.* **65,** 217 (1982).
3. G.L. Peterson, *Methods of Enzymology* **91,** 95 (1983).
4. J.L. Hansen *et al., Crop Science* **32,** 879 (1992).
5. N.F. Makoni *et al., J. Dairy Sci.* **77,** 1598 (1994).
6. A. Suleiman, *Ten Year Average Analyses of Alberta Feeds 1984–1994.* Research Support Group, Animal Industry, Alberta Agriculture Food and Rural Development (1995).
7. P.C. Williams and D.C. Sobering, *J. Near Infrared Spectrosc.***1,** 25 (1993).