

## Near-Infrared Reflectance Spectroscopy Prediction of Leaf and Mineral Concentrations in Alfalfa

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### ABSTRACT

Rapid and accurate prediction of leaf and mineral concentration of alfalfa (*Medicago sativa* L.) forage would be valuable for dried research and farm hay samples because current approaches are time-consuming and expensive. Our objective was to study the effectiveness of near-infrared reflectance spectroscopy (NIRS) in determining the leaf concentration in dried alfalfa forage and the concentration of total ash and minerals in leaves and stems. Leaf concentration in sun-cured hay samples from several years was accurately predicted using NIRS with standard errors of cross validation (SECVs) of 34 to 49 g kg<sup>-1</sup>, but equations had to be redeveloped with samples representing each year. Equations derived from dried research samples had SECVs of 37 to 39 g kg<sup>-1</sup> but still required equation redevelopment each year. Near-infrared reflectance spectroscopy accurately predicted total ash and Ca, K, and P of leaves and stems of alfalfa hay and research samples but was less consistent in prediction of Mg and S and micro-minerals Al, B, Fe, Mn, and Si in leaves and stems. We failed to develop any useful equations for Na. Leaves had higher concentrations of total ash and most minerals than stems, except for K, Cd, Cu, Ni, and Cr. Near-infrared reflectance spectroscopy is a rapid and accurate method for determining leaf, total ash, and Ca, K, and P concentration in sun-cured hay and oven-dried research samples. Near-infrared reflectance spectroscopy would be especially useful in an electrical generating facility using large quantities of alfalfa as a biofuel.

ALFALFA IS THE MOST important perennial forage legume grown in the Midwest. Alfalfa has potential to produce a high quality forage, to contribute N to subsequent crops in rotations, and to conserve soil. The Minnesota Valley Alfalfa Producers (MNVAP), a cooperative of hay producers in western Minnesota and South Dakota, studied use of alfalfa as a biomass fuel for generation of electricity (DeLong et al., 1995). In this process, leaves and stems of alfalfa hay were to be separated to produce a leaf pellet for high quality livestock feed and a stem pellet for gasification and production of electricity. The project to produce electricity from alfalfa was terminated under the economic and energy supply conditions of the late 1990s (Sheaffer et al., 2000), but the concept may again be viable in the 21st century as the USA explores alternative sources of energy and N fertilizer.

The proportion of leaves and stems in alfalfa hay can vary greatly, depending on maturity at harvest, handling, and rain damage (Sheaffer et al., 1988). For example, alfalfa at early bud may have 600 g kg<sup>-1</sup> leaves while

at early flower, the leaf concentration declines to 450 g kg<sup>-1</sup> (Sheaffer et al., 2000). To value alfalfa hay as a biofuel, or as a source of leaf protein, accurate prediction of the proportion of leaf and stem in the hay is necessary.

Hand separation of leaf and stem fractions of alfalfa may require 1 to 2 h per 250-g sample. Hill et al. (1988) used NIRS to rapidly and accurately ( $r^2 = 0.96$ ) analyze the leaf concentration in stratified alfalfa canopies and in esophageal extrusa of cattle grazing alfalfa pastures. Because accuracy of NIRS analysis was affected by cultivars, they recommended that NIRS prediction of leaf concentration be limited to predicting a closed population from which the calibration samples originated. This work demonstrated the potential of NIRS using freeze-dried forage from two alfalfa cultivars. To expand the utility of NIRS, there is a need to evaluate its use to predict leaf and stem fractions of open populations of sun-cured alfalfa hay produced on farms and of diverse populations of oven-dried alfalfa cultivars.

Gasification of stems for electricity generation results in by-product ash that can potentially serve as a fertilizer (Mozaffari et al., 2000). If ash contains significant amounts of undesirable minerals such as Cd and Na, it may represent a disposal challenge. In addition, because the minerals in biofuel do not incinerate when gasified, they will produce slag, which at high concentrations, can clog burners or decrease combustion efficiency. In addition to total ash content, the minerals most likely to increase slag are K, Na, and Ca (Mozaffari et al., 2000). Consequently, knowledge of mineral concentration in herbage is necessary to improve efficiency of the gasifier operation and reduce costs associated with excess slag production. Current techniques for determining mineral concentration in herbage are time-consuming and expensive (Munter, 1984). A fast and cost-effective method, such as NIRS, will be required for rapid, large-scale processing of stems. Near-infrared reflectance spectroscopy predicts forage components through the detection of rotational and vibrational energies associated with H bonding; consequently, NIRS cannot directly predict inorganic mineral concentrations (Clark et al., 1987). However, Shenk et al. (1981) reported that Ca in diverse hay samples could be predicted by NIRS with the same degree of accuracy as acid detergent fiber (ADF). Clark et al. (1987) also evaluated use of NIRS to determine mineral composition of diverse

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**Abbreviations:** CV, coefficient of variation; ICP, inductively coupled plasma; MNVAP, Minnesota Valley Alfalfa Producers; NIR, near infrared; NIRS, near-infrared reflectance spectroscopy; OM, organic matter; SEA, standard error of analysis; SECV, standard error of cross validation; SEL, standard error of laboratory analysis; SEP, standard error of prediction; SEP(C), standard error of prediction corrected for bias; 1 - VR, one minus the ratio of unexplained variance divided by variance.

forages but concluded that prediction accuracy was limited to Ca, P, K, and Mg. Additional minerals were evaluated for NIRS equation development using tall fescue (*Festuca arundinacea* Schreb.), crested wheatgrass [*Agropyron cristatum* (L.) Gaertn. and *A. desertorum* (Fisch. ex Link) Schult.], and alfalfa, with results differing among species (Clark et al., 1989). They suggested that NIRS indirectly measures the cations Ca, P, K, and Mg through their association with organic molecules. Although inorganic minerals theoretically do not absorb energy in the near-infrared (NIR) region, at least some forms of the inorganic mineral will be associated with organic compounds within the plant and will show a corresponding absorption within the NIR region as shown by Shenk et al. (1981) and Clark et al. (1987, 1989). Improved prediction of mineral concentration of alfalfa forage may now be possible because new NIRS instrumentation (e.g., Foss model 6500, Foss North America, Eden Prairie, MN)<sup>1</sup> has an increased spectral range of 400 to 2500 nm, significantly lower signal to noise ratio, and greater overall stability than previous instruments. The larger spectral range, particularly the 700- to 1100-nm segment, may contain useful information. In addition, new equation developmental software (Infrasoft International, Port Matilda, PA)<sup>1</sup> that uses modified partial least squares regression (Shenk and Westerhaus, 1991) provides lower standard errors and more stability than stepwise regression used in earlier reports. The new software also has standardization techniques that improve transferability of equations to other instruments. No other technology exists in the biofuel industry for real-time and rapid analysis of plant mineral concentration in large volumes of plant material.

The NIRS technique has been successfully used to predict forage chemical constituents such as crude protein and fiber (Sheaffer et al., 2000) and characteristics such as alfalfa fall dormancy (Kallenbach et al., 2001) and legume and grass composition of mixtures (Moore et al., 1990). However, the determination of leaf and stem proportion and mineral concentration of these fractions using NIRS has not been evaluated for a diverse array of dried alfalfa forage. Our objective was to study the effectiveness of NIRS to determine the leaf and stem concentration in dried alfalfa forage and the concentration of total ash and minerals in the leaf and stem fractions of alfalfa cultivars and hay from a large production region.

## MATERIALS AND METHODS

### Analysis of Leaf Concentration

#### Sun-Cured Hay

Samples were obtained from 103 alfalfa hay lots in the winter of 1993 from farms in west-central Minnesota. An additional 108 on-farm lots were sampled in 1997, and 66 lots were sampled in 1998 from the MNVAP alfalfa hay processing plant in Priam, MN. Hay lots represented a range in harvest maturity, degree and type of weathering, and storage. Bales were small (32 kg), midsize (455 kg), and large (910 kg) cuboid

bales as well as large cylinders (455 kg). Average size of the hay lots averaged 100, 50, and 25 bales, respectively. A hay probe (35 cm long with a 1.9-cm inside diameter; Forageurs, Lakeville, MN) mounted on an electric drill was used to collect samples from 25 to 30 bales per lot to a 35-cm depth. Only those bales within stacks that could be reached safely were cored. Combining 25 to 30 hay probe cores per hay lot created a composite sample of 250 g that represented a hay lot.

Whole hay core samples were dried in a forced-air oven at 65°C for 48 h and were separated into leaf and stem portions using U.S. standard brass test sieves (20.3-cm diam.). Sieves with the following mesh openings were stacked from top to bottom in the following order: no. 8 (2.36 mm), no. 10 (2.00 mm), no. 16 (1.85 mm), and no. 20 (0.85 mm); they were then fastened to a mechanical shaker. A bottom pan collected fractions passing through all sieves.

Samples were hand-massaged in plastic bags to break leaves into a fine powder, poured into the top sieve, and shaken at 300 rpm for 30 s. After shaking, the contents of sieves no. 8 and 10 were combined and designated pure stem. The contents of sieve no. 16 were massaged again and put on the shaker at 400 rpm for 20 s. The contents remaining on sieve no. 16 were combined with the contents of sieves no. 8 and 10, and the remaining material in sieve no. 20 and the bottom pan was designated pure leaf fraction. These portions were weighed to determine the leaf concentration in each hay sample. All fractions were ground through a cyclone mill with a 1-mm mesh screen. After grinding, portions of leaf and stem subsamples were recombined (dry matter basis) in ratios as occurred in the original hay sample and scanned for entry into the NIRS calibration set. Forty percent of the samples added into the leaf concentration calibration were pure stem or pure leaf to improve analysis of concentrated leaf and stem fractions. In the conversion of alfalfa to electricity, the biofuel will consist of concentrated stems, but this large-scale separation will allow the production of high-protein animal feed products (crude protein  $\approx 300 \text{ g kg}^{-1}$ ) using the concentrated leaf. We used leaf concentration values of  $1000 \text{ g kg}^{-1}$  for pure leaf fraction and  $0.1 \text{ g kg}^{-1}$  for pure stem fraction since the NIRS calibration program was set to ignore zero values. All samples were re-mixed using a sample tumbler to ensure homogeneity.

Near-infrared reflectance spectra were collected on all samples using a scanning monochromator (Foss model 6500, Foss North America, Eden Prairie, MN) and chemometrics software (NIRS3 version 4.0, Infrasoft International, Port Matilda, PA). Reflectance ( $R$ ) data were recorded as  $\log 1/R$  at 2-nm intervals between 400- and 2500-nm wavelengths. The Infrasoft International program Calibrate with a math treatment of 1,4,4,1 (representing the derivative number, segment of data points to which the derivative was applied, and two smoothings of four data points and one data point, respectively) and the modified partial least squares option was used for all equation development (Shenk and Westerhaus, 1991). Each calibration was developed by running the Calibrate program twice to eliminate outliers with the criteria of minimizing the SECV and maximizing the portion of variance in lab values that is explained by NIRS-predicted values ( $1 - VR$ ). The Monitor program was used to compare conventional lab values to the spectral analysis using the NIRS equations on additional samples outside of the calibration.

Calibration equations were developed by regressing known leaf concentrations of the 103 samples collected in 1993 against NIRS spectra. Fifty-five samples from 1997 were used to monitor the equation and were then added into the 1993 calibration along with the remainder of the 1997 samples. In 1998, a new equation was developed by adding 66 samples to the 1993–1997 calibration set. Some samples were deleted from

<sup>1</sup> Mention of a proprietary product does not constitute a recommendation of the product by the University of Minnesota or the USDA-ARS.

each calibration during the equation development process due to elimination of both  $T$  and  $H$  statistic outliers. The critical  $T$  outlier value was set to the recommended default value of 2.5 and is calculated as 2.5 times the standard error of the equation. This was used to eliminate samples where there was a large difference between the lab and NIRS-predicted results. The critical  $H$  (Mahalanobis distance) outlier value was set to the recommended default value of 10. This criterion was used to eliminate samples whose spectra were more than 10 standard deviations from the mean of the samples in the file with a specific math treatment and scatter correction applied (Shenk and Westerhaus, 1991). Finally, two additional sets, independent from the calibration set, of 10 to 12 samples collected in June and July of 1998 were used to monitor the 1998 equation.

### Oven-Dried Herbage

Samples of first-flower alfalfa were obtained from field research trials in Minnesota and Wisconsin during 1991 to 1998. Samples were manually harvested to a 5-cm stubble height using hand shears from a 0.5-m<sup>2</sup> area. The samples (600-g wet weight) were dried in a forced-air oven at 65°C for 48 h in paper bags. After drying, the bags were hand-massaged, causing the leaves to break off. Leaves and stems were separated by hand, and each fraction was weighed. Leaves and stems were recombined to match actual field leaf percentage plus samples of pure leaf (1000 g kg<sup>-1</sup>) and pure stem (0.1 g kg<sup>-1</sup>) before grinding through a 1-mm screen using a cyclone mill and were remixed, and NIR spectra were collected as previously described.

Equations for leaf concentration were developed using spectra of 142 samples collected from 1991 to 1995. In 1996, 22 new samples were used to monitor the equation and were then added to the calibration set to create the 1996 equation. Twenty-two additional samples in 1997 were used to monitor the 1996 equation and then added to the calibration set to create the 1997 equation. Finally in 1998, 17 samples were used to monitor the previous year's equation and were added to the calibration set to create the 1998 equation. The population should be considered to be a closed population with the addition of each year's samples.

## Analysis of Mineral Constituents

### Sun-Cured Hay

Fifty-eight samples of sun-cured pure alfalfa hay were obtained in 1998 by sampling hay lots of 455- and 910-kg cuboid bales at the MNVAP processing plant in Priam, MN. Twenty-nine samples of overwintered 1997 hay were obtained in May, and 29 samples of hay made in the spring of 1998 were taken in July. At the MNVAP plant, each hay lot was mechanically chopped, dried, separated, and pelleted. Separation was accomplished by moving the dried chopped hay into a large perforated rotating drum, in which the leaves crumble and fall through the perforations. The separate stems were then expelled from the end of the drum by the force of rotation. The separated leaves and stems were pelleted, randomly subsampled ( $\approx 1000$  g), dried at 65°C for 48 h, and ground through a 1-mm screen using a cyclone mill.

### Oven-Dried Herbage

Research samples obtained from on-going alfalfa biomass research included harvests of alfalfa at bud, first flower, and pod stages at Rosemount, Morris, and Lamberton, MN (Sheaffer et al., 2000). The procedure for separation of leaves and stems was identical to that for oven-dried samples for leaf concentration described previously. Samples of leaves ( $n = 78$ ) and

stems ( $n = 72$ ) were ground through a 1-mm screen using a cyclone mill.

All oven-dried research and sun-cured hay samples were analyzed for total ash by combustion at 485°C for 12 h (Munter and Grande, 1981) at the University of Minnesota Soil Testing and Research Analytical Laboratory for P, K, Ca, Mg, Na, Al, Fe, Mn, Zn, Cu, B, Pb, Ni, Cr, and Cd. Single analyses were performed with a blank, a standard, and a duplicate analysis of the same subsample every 20 samples, to assure accuracy and repeatability of the analytical equipment on all sets, except sun-cured stems, which had duplicate analyses on all samples. Additionally, both oven-dried research and sun-cured hay stem samples were analyzed for Si, Ti, and Cl. Mineral analysis was accomplished using inductively coupled plasma (ICP) (Fisons, Valencia, CA) with pretreatment using the dry-ash method at 485°C (Munter and Grande, 1981) for the leaf samples and the ICP wet-ash method with microwave and nitric acid–hydrogen peroxide digestion (Gavlak et al., 1994) for the stem samples.

Near-infrared reflectance spectroscopy calibration equations were developed by regressing total ash and specific mineral concentration values against the spectra. It has been our experience that combining data sets for NIR equation development will make a more robust equation, but it is usually accompanied by higher standard errors. In the case of mineral analysis, we wanted the lowest possible errors for each specific data set. Procedures for NIRS equation development were similar to those described previously for determination of leaf and stem concentration of hay. In the case of total ash, we most likely were calibrating for organic matter (OM) rather than inorganic mineral concentration, which does not have a direct relationship to the spectra. Total ash is equal to 100 minus OM, and therefore either total ash or OM could be reported, but in plant analysis, total ash is preferred. Coefficient of variation (CV) was calculated by using either SECV or standard error of laboratory (SEL) for each mineral where  $CV = [(SECV \text{ or } SEL)/\text{mean}] \times 100$  and  $SEL = \{[\sum_i \sum_j (X_{ij} - \bar{X}_i)^2 / (R - 1)] / N\}^{0.5}$ , where  $X_{ij}$  is the  $j$ th replicate of the  $i$ th sample,  $\bar{X}_i$  is the reference method mean value of all the replicates of the  $i$ th sample,  $R$  is the number of replicates, and  $N$  is the number of samples (Marten et al., 1989). The standard error (SECV) and the measure of explained variance ( $1 - VR$ ) as reported in Infrasoft International software are routinely used to determine the usefulness of NIRS equations. Although  $1 - VR$  for the mineral equations may not reach the commonly accepted limits as seen for protein and fiber (Marten et al., 1989), the equations developed will be of value in the biofuel industry. Clark et al. (1989) proposed using CVs, where in this case  $CV = [\text{standard error of prediction (SEP)/mean}] \times 100$ , as a tool in evaluating NIRS equation performance across elemental parameters. They also suggested using consistency of CVs across populations as an indication of successful NIRS analysis. Clark et al. (1987, 1989) reported useful NIRS mineral equations for Ca, P, K, Mg, Al, and S, with CVs  $\{[\text{standard error of analysis (SEA) or SEP}/\text{mean}] \times 100\}$  ranging from 11 to 28 across three forage data sets. The simple correlation squared ( $r^2$ ) of Clark et al.'s validation sets of stated useful mineral equations ranged from 0.56 to 0.88. The SECV reported here is a measurement of validation error and is conceptually similar to Clark et al.'s SEA or SEP; the  $1 - VR$  reported here is a measurement of explained variance and is again conceptually similar to Clark's validation  $r^2$ . We, therefore, used similar criteria as Clark et al. (1987, 1989) for determination of useful mineral equations: CVs of approximately 20 or less, a general consistency of CVs for the four equations (sun-cured leaf and stem; oven-dried leaf and stem) for each

mineral, and a  $1 - VR$  value  $\geq 0.6$ , where 60% or more of variation in lab values is explained by NIRS-predicted values.

## RESULTS AND DISCUSSION

All data sets were kept separate and not pooled for NIR equation development for several reasons: (i) Pooling of data increases robustness of the equation but usually results in higher standard errors. In this research, robustness was less important than achieving the highest possible accuracy in the analysis; (ii) the alfalfa pelleting plant needed sun-cured equations for the two separate products: leaf meal for animal feed and stem cubes for gasification; and (iii) plant breeders needed NIRS equations for research samples that were only oven-dried and wanted to specifically look at either leaves or stems for analysis.

### Analysis of Leaf Concentration

#### Sun-Cured Hay

Hay leaf concentration for all sun-cured samples ranged from 112 to 778 g kg<sup>-1</sup>, with a mean of 476 g kg<sup>-1</sup>, and was accurately predicted using NIRS when equations were recalibrated using each year's samples. The SECV for leaf concentration in sun-cured alfalfa ranged from 34 to 49 g kg<sup>-1</sup> and increased each time a new year's sample set was added to the calibration (Table 1). The coefficient of determination ( $R^2$ ) was high (0.99) for all equations due to the large range of leaf concentrations in samples that included pure leaves (1000 g kg<sup>-1</sup>) and stems (0.01 g kg<sup>-1</sup>). However, the accuracy of prediction was poor when equations developed from previous years' samples were used to predict the leaf concentration of an open population of samples from a new year (Table 2). The standard errors of prediction for the monitoring sets, corrected for bias [SEP(C)], were 94 and 172 g kg<sup>-1</sup> in 1997 and 1998, respectively, which were two to four times higher than the SECVs for equation development. For the 1997 monitoring, the average global  $H$  was 3.3, which is above the recommended limit of 3.0 (Shenk and Westerhaus, 1991), and indicated the equation needed updating. A global  $H$  is the standardized  $H$  distance from the mean and is used as a boundary for spectral outliers, whereas neighborhood  $H$  is the distance between a sample and

the closest sample in the calibration. The 1998 monitoring set had a reasonable global  $H$  of 1.9 but a high neighborhood  $H$  of 1.3, which is two times higher than the recommended level of 0.6 (Shenk and Westerhaus, 1991). This again indicated the need for updating the equation. Monitoring Set 2 also had a bias or difference between means from conventional and NIRS analysis of 60 g kg<sup>-1</sup>. Additional monitoring sets for 1998 samples using a redeveloped equation containing samples from the same year (1998) showed improved SEP(C) and lower bias.

Hill et al. (1988) reported success in NIRS calibration for leaf concentration of alfalfa but used two hand-harvested, freeze-dried cultivars and made NIRS equations specific for each cultivar. We concluded that NIRS can accurately predict leaf concentration in an open population of sun-cured hay samples with a yearly recalibration of the equation using a small number of the new year's samples. No attempt was made to represent all of the yearly variation of the hay production region, and therefore the population was still considered to be an open population. Representation of additional variation of years, seasonal cuttings, and weathering may improve the validity of NIRS analysis for leaf concentration and lessen the need for yearly recalibration but may also increase the standard error of the equation.

#### Oven-Dried Herbage

Leaf concentration in oven-dried research samples ranged from 302 to 750 g kg<sup>-1</sup> with a mean of 496 g kg<sup>-1</sup>. The oven-dried equations successfully predicted alfalfa leaf concentration of herbage with low standard errors (SECV) and a stable standard error when samples from the current year were included in the equation development (Table 1). Our experience has shown that NIRS equations for oven-dried leaf concentration with an SECV < 50 g kg<sup>-1</sup> provided a satisfactory level of prediction. However, as with sun-cured samples, the leaf concentration equations made from oven-dried herbage were not effective on open populations where the previous year's equation was used to predict the current year's samples. Three monitoring sets from 1996 to 1998 with 12 to 22 samples resulted in standard errors [SEP(C)] from an acceptable 29 g kg<sup>-1</sup> to an unacceptable high of 101 g kg<sup>-1</sup>. In addition, there was a high bias (-84

**Table 1. Equation development statistics for near-infrared reflectance spectroscopy (NIRS) prediction of leaf concentration in sun-cured alfalfa hay and oven-dried alfalfa herbage.**

Forage population	Variable	N	Range	Mean	Calibration		Cross-validation	
					SEC†	R <sup>2</sup> ‡	SECV§	1 - VR¶
				g kg <sup>-1</sup>			g kg <sup>-1</sup>	
Sun-cured	1993 leaf	100	77-1000#	534	28	0.99	34	0.99
	1993-1997 leaf	210	0.1††-1000	515	35	0.99	41	0.99
	1993-1998 leaf	244	0.1-1000	534	44	0.99	49	0.99
Oven-dried	1991-1995 leaf	138	0.1-1000	500	29	0.99	37	0.99
	1991-1996 leaf	150	0.1-1000	508	31	0.99	37	0.99
	1991-1997 leaf	167	0.1-1000	501	31	0.99	37	0.99
	1991-1998 leaf	190	0.1-1000	493	33	0.99	39	0.99

† Standard error of calibration.

‡ Coefficient of determination.

§ Standard error of cross-validation.

¶ One minus the ratio of unexplained variance divided by variance.

# Value of 1000 is equivalent to pure leaf.

†† Value of 0.1 is equivalent to pure stem. The NIRS equation development software was set to a default whereby a zero value represented missing data.

**Table 2. Monitoring statistics for leaf concentration in sun-cured alfalfa hay.**

Equation year	Monitoring year	N	Range	Mean	Bias†	SEP(C)‡	Slope§	R²
g kg⁻¹								
1993	1997	55	0.1  –1000#	509	–12	94	1.15	0.98
1993–1997	1998	66	0.1–1000	575	60	172	0.99	0.82
1993–1998	1998	12	200–800	504	–29	38	1.01	0.97
1993–1998	1998	10	360–480	428	40	25	0.80	0.78

† Bias = average difference between laboratory and near-infrared-predicted values.

‡ Standard error of prediction corrected for bias.

§ Slope (*b*) is the steepness of a straight line curve expressed as  $Y = a + bX$  and is measured by the increase in *Y* for each unit of *X*, where *Y* represents conventional analysis and *X* represents near-infrared predictions.

|| Value of 0.1 is equivalent to pure stem. The NIRS equation development software was set to a default whereby a zero value represented missing data.

# Value of 1000 is equivalent to pure leaf.

and  $-89 \text{ g kg}^{-1}$ ) in two of three sets. Hill et al. (1988) also reported a high standard error of  $108 \text{ g kg}^{-1}$  for determining leaf concentration of samples not represented in NIRS calibration. Equations developed from oven-dried samples performed better with lower standard errors than those developed from sun-cured hay samples but still required equation redevelopment by adding samples from the current year. Sample variation was reduced in the hand-harvested, oven-dried plant material by eliminating unknown variation due to weathering and weed contamination effects that influence sun-cured alfalfa hay. However, for accurate prediction of leaf concentration, equations must be annually updated with spectra from current samples because of environmental effects on plant growth.

### Analysis of Mineral Constituents

#### Total Ash

Equations for prediction of total ash concentration were successfully developed for leaves and stems of both

**Table 4. Equation development statistics for near-infrared reflectance spectroscopy (NIRS) prediction of mineral concentration (dry weight basis) in sun-cured alfalfa leaves.**

Variable	N	SD†	Mean	Calibration		Cross-validation		CV††
				SEC‡	R²§	SECV¶	1 – VR#	
mg kg⁻¹								
<b>Macrominerals</b>								
Ca	49	3487	27 067	1229	0.88	1623	0.79	6
K	52	4156	24 561	1547	0.86	2481	0.66	10
Mg	49	642	4 729	374	0.66	542	0.33	11
Na	55	178	335	163	0.16	323	0.01	96
P	46	506	2 979	150	0.91	175	0.88	6
S	55	823	4 574	518	0.60	603	0.48	13
<b>Microminerals</b>								
Al	51	162	272	74	0.79	93	0.67	34
B	56	12	68	6.4	0.70	7.4	0.63	11
Cd	56	0.05	0.2	0.04	0.33	0.05	0.18	25
Cr	49	0.3	1.1	0.2	0.76	0.2	0.59	18
Cu	52	11	25	7	0.57	8	0.38	32
Fe	56	178	316	71	0.84	92	0.73	29
Mn	51	16	72	7	0.76	10	0.60	14
Ni	52	0.9	2.9	0.8	0.14	0.9	0.03	31
Pb	55	0.7	2.8	0.4	0.63	0.7	0.19	25
Zn	51	6	32	3	0.77	4	0.59	13

† Standard deviation of the mean.

‡ Standard error of calibration.

§ Coefficient of determination.

¶ Standard error of cross-validation.

# One minus the ratio of unexplained variance divided by variance.

†† Coefficient of variation.

**Table 3. Equation development statistics for near-infrared reflectance spectroscopy (NIRS) prediction of ash concentration in leaves and stems of sun-cured alfalfa hay and oven-dried alfalfa herbage.**

Total ash	N	Min.	Max.	Mean	Calibration		Cross-validation	
					SEC†	R²‡	SECV§	1 – VR¶
g kg⁻¹								
<b>Sun-cured</b>								
Leaves	51	120	190	140	4	0.89	7	0.75
Stems	56	50	120	90	5	0.84	7	0.70
<b>Oven-dried</b>								
Leaves	71	90	140	110	4	0.89	5	0.81
Stems	68	40	80	60	2	0.94	5	0.91

† Standard error of calibration.

‡ Coefficient of determination of calibration equation.

§ Standard error of cross-validation.

¶ One minus the ratio of unexplained variance divided by variance.

sun-cured hay and oven-dried herbage. Our standard errors of 4 to 7  $\text{g kg}^{-1}$  (Table 3) were lower than values reported by Windham et al. (1991) of  $9.8 \text{ g kg}^{-1}$  but similar to those reported by Vazquez de Aldana et al. (1996) for grassland communities in Spain. Although Windham et al. (1991) preferred  $\log(1/R)$  as the best math treatment for an NIRS ash equation for alfalfa and tall fescue pasture samples, we used a first derivative with good results, which also avoided a baseline shift in the NIR spectra. Equations for total ash content of oven-dried leaf and stem had lower SECV and higher explained variance ( $1 - \text{VR}$ ) than those for sun-cured hay although all equations were acceptable for future NIRS analysis.

Sun-cured hay had higher total ash values for both leaf and stem than oven-dried samples. This may be due to the mechanical handling of the sun-cured hay that exposed the herbage to greater direct contact with the soil and to dust generated by mechanical harvesting. Hand harvesting and oven drying should reduce soil contamination although some contamination of alfalfa herbage can result from splashing during heavy rainfall events.

**Table 5. Equation development statistics for near-infrared reflectance spectroscopy (NIRS) prediction of mineral concentration (dry weight basis) in sun-cured alfalfa stems.**

Variable	N	SD†	Mean	Calibration		Cross-validation		CV‡†
				SEC‡	R²§	SECV¶	1 - VR#	
			mg kg⁻¹			mg kg⁻¹		%
<b>Macrominerals</b>								
Ca	50	2514	11 131	1028	0.83	1396	0.69	13
Cl	48	1556	5 072	1131	0.47	1198	0.42	24
K	55	6360	28 402	2308	0.87	3433	0.72	12
Mg	45	517	3 194	291	0.68	344	0.58	11
Na	55	301	388	289	0.08	452	0.03	22
P	49	531	2 392	278	0.73	333	0.61	14
S	52	326	1 638	179	0.70	198	0.65	12
<b>Microminerals</b>								
Al	53	42	82	26	0.60	32	0.46	39
B	52	5	28	1.5	0.91	2.3	0.80	8
Cd	56	0.03	0.3	0.03	0.05	0.03	-0.04	10
Cr	44	0.04	0.6	0.03	0.47	0.03	0.33	5
Cu	48	3	11	2.5	0.29	2.8	0.19	25
Fe	49	32	97	14	0.81	19	0.68	20
Mn	51	7	24	3.5	0.72	4.5	0.54	19
Ni	45	0.6	2	0.5	0.46	0.5	0.34	25
Pb	54	0.04	3.6	0.02	0.65	0.03	0.45	1
Si	41	105	196	37	0.87	53	0.75	27
Ti	44	0.45	1.2	0.14	0.91	0.2	0.77	19
Zn	50	4	22	2.3	0.61	2.7	0.46	12

† Standard deviation of the mean.

‡ Standard error of calibration.

§ Coefficient of determination.

¶ Standard error of cross-validation.

# One minus the ratio of unexplained variance divided by variance.

‡† Coefficient of variation.

**Mineral Constituents**

The range in mineral concentration as indicated by standard deviation was greater in sun-cured leaves and stems than in oven-dried research samples, except for Mg, Na, and B in leaves and Cl, Mg, Cu, Ni, and Pb in stems (Tables 4–7). The sun-cured means were also higher, except for P in leaves and Cd in stems. Means for Na and Cd in leaves and Cd, Cr, and Pb for stems were the same or similar between curing methods. Leaves had higher concentrations of minerals than

stems, except for K, Na, Cd, and Pb. This agrees with Rominger et al. (1975), who reported that concentrations of most minerals were greater in leaves than in stems but who also found K concentration to be greater in stems than leaves. They reported Al and Na concentrations of stems and leaves to be similar, whereas we found Al to be two to three times higher in leaves and Na to be fractionally higher in stems. Our range of values for oven-dried samples usually encompassed those of Rominger et al. (1975). Our sun-cured hay

**Table 6. Equation development statistics for near-infrared reflectance spectroscopy (NIRS) prediction of mineral concentration (dry weight basis) in oven-dried alfalfa leaves.**

Variable	N	SD†	Mean	Calibration		Cross-validation		CV‡†
				SEC‡	R²§	SECV¶	1 - VR#	
			mg kg⁻¹			mg kg⁻¹		%
<b>Macrominerals</b>								
Ca	71	3342	24 962	1245	0.86	1601	0.77	6
K	66	3799	19 596	902	0.94	1204	0.90	6
Mg	71	819	4 584	278	0.89	357	0.81	8
Na	64	205	338	125	0.63	147	0.48	43
P	70	387	3 098	143	0.86	202	0.74	7
S	68	464	3 920	421	0.18	433	0.12	11
<b>Microminerals</b>								
Al	60	20	52	10	0.76	11	0.67	21
B	73	22	62	9.9	0.80	13	0	21
Cd	71	0.03	0.2	0.03	0.19	0.03	0.07	15
Cr	70	0.08	0.7	0.05	0.56	0.06	0.43	9
Cu	68	3	11	2	0.39	2.3	0.29	21
Fe	62	14	107	9.8	0.52	11	0.38	10
Mn	70	11	50	9	0.37	10	0.27	20
Ni	67	0.7	2.1	0.5	0.53	0.6	0.37	29
Pb	66	0.05	1.8	0.03	0.54	0.04	0.32	2
Zn	70	3	25	2	0.32	3	0.21	12

† Standard deviation of the mean.

‡ Standard error of calibration.

§ Coefficient of determination.

¶ Standard error of cross-validation.

# One minus the ratio of unexplained variance divided by variance.

‡† Coefficient of variation.

**Table 7. Equation development statistics for near-infrared reflectance spectroscopy (NIRS) prediction of mineral concentrations (dry weight basis) in oven-dried alfalfa stems.**

Variable	N	SD†	Mean	Calibration		Cross-validation		CV‡†
				SEC‡	R²§	SECV¶	1 - VR#	
			mg kg⁻¹			mg kg⁻¹		%
<b>Macrominerals</b>								
Ca	67	1121	7 958	439	0.85	676	0.63	8
Cl	58	1706	3 846	406	0.94	690	0.84	18
K	68	5034	19 742	1090	0.95	1384	0.93	7
Mg	67	726	2 252	197	0.93	289	0.84	13
Na	61	293	550	143	0.76	192	0.57	35
P	59	368	2 009	172	0.78	224	0.63	11
S	69	186	994	80	0.82	95	0.74	10
<b>Microminerals</b>								
Al	63	14	25	5	0.85	6.6	0.77	26
B	70	3	20	1	0.83	1.7	0.65	9
Cd	63	0.004	0.3	0.004	0.27	0.004	0.22	1
Cr	66	0.01	0.6	0.01	0.05	0.01	-0.09	2
Cu	58	3	8	1.7	0.75	2	0.64	25
Fe	61	14	50	7	0.77	8.8	0.63	18
Mn	62	3	13	1.4	0.81	1.5	0.77	12
Ni	62	0.8	1.5	0.3	0.87	0.4	0.73	27
Pb	69	0.08	4	0.07	0.27	0.07	0.21	2
Si	56	21	33	5.9	0.92	8.7	0.83	26
Ti	57	0.02	0.7	0.01	0.74	0.01	0.50	1
Zn	59	4	14	1.7	0.82	2.1	0.74	15

† Standard deviation of the mean.

‡ Standard error of calibration.

§ Coefficient of determination.

¶ Standard error of cross-validation.

# One minus the ratio of unexplained variance divided by variance.

‡† Coefficient of variation.

samples represented diverse alfalfa cultivars grown in a large production region, whereas Rominger et al. (1975) used only oven-dried 'Vernal' alfalfa, fertilized and unfertilized, harvested at bud stage, and separated into 10-cm lengths above stubble height.

Separate NIRS equations were developed for both sun-cured alfalfa hay leaves and stems and oven-dried leaves and stems (Tables 4–7). We chose not to pool the sun-cured and oven-dry data sets to develop equa-

**Table 8. Standard errors of laboratory (SEL) from duplicate inductively coupled plasma (ICP) mineral analysis of sun-cured alfalfa stems, and coefficient of variation (CV) based on SEL.**

Variable	SEL†	Mean	CV‡
	mg kg⁻¹		%
<b>Macrominerals§</b>			
Ca	626	9 972	6
Cl	25	3 905	1
K	368	25 975	1
Mg	90	2 873	3
Na	24	385	6
P	66	2 240	3
S	8	1 193	1
<b>Microminerals§</b>			
Al	15	83	18
B	1.4	25	6
Cd	0.1	0.2	30
Cr	0.2	0.6	25
Cu	5	12	42
Fe	20	97	20
Mn	1.5	23	7
Ni	2	2	84
Pb	2	4	47
Zn	10	22	44

† The SEL is defined as  $\{\sum_i[\sum_j(X_{ij} - \bar{X}_j)^2/(R - 1)]/N\}^{0.5}$ , where  $X_{ij}$  is the  $j$ th replicate of the  $i$ th sample,  $\bar{X}_j$  is the reference method mean value of all the replicates of the  $i$ th sample,  $R$  is the number of replicates, and  $N$  is the number of samples.‡ Coefficient of variation where  $CV = (SEL/mean) \times 100$ .§ All minerals had 51 samples run in duplicate except S ( $N = 14$ ) and Cl ( $N = 8$ ).

tions with the lowest possible prediction errors. We were challenged in choosing criteria to determine usefulness of the NIRS mineral equations. A commonly accepted criterion for useful NIRS equations is that the SEP (or with new equation development methods, SECV) should be less than two times the SEL (Marten et al., 1989). Sun-cured stems had duplicate analyses, and therefore SEL could be calculated (Table 8). Of the macrominerals, only Ca met this criterion, but all of the microminerals except Mn qualified. However, many of these equations had very low  $1 - VR$  values and still met the criterion of SECV being less than two times SEL; therefore, this criterion was not very useful.

In general, mineral equations for oven-dried forage were superior to those for sun-cured forage with lower SECVs, higher  $1 - VR$ s, and lower CVs (based on SECV). Equations were developed for macrominerals Ca, K, Mg, P, and S and microminerals B, Cr, Fe, Mn, and Zn in each plant fraction and curing type that had low CVs  $\approx 20$  and that were consistent across the data sets (sun cured, oven cured, leaf, and stem). Equations that met the criterion of  $1 - VR \geq 0.6$  over all data sets were macrominerals Ca, K, and P; and for three of four data sets, microminerals B and Fe. Aluminum had three of four equations with satisfactory  $1 - VR$  but had high CVs in both sun-cured leaves and stems. Clark et al. (1989) also reported a high CV for Al in alfalfa. Magnesium, Mn, and S had two equations with  $1 - VR$ s  $\geq 0.6$  and one additional equation with  $1 - VR \approx 0.5$ . Chlorine, Si, and Ti were subject to ICP analysis only for stems. Both Si stem equations had somewhat higher CVs but had higher  $1 - VR$ s of  $\approx 0.8$ . Chlorine and Ti had only oven-dried stem equations with acceptable criteria. Copper and Ni equations had high CVs with

only one equation having an acceptable  $1 - VR$ . Cadmium, Cr, and Pb had acceptable CVs, but all equations had low  $1 - VR$  values. We failed to develop any useful equations for Na, which is important in creation of slag formed during biofuel incineration.

In general, our validation errors (SECV) were lower than or similar to those of Clark et al. (1987, 1989), except for our Na equations, which had two to three times higher validation errors. In sun-cured leaves, Al and S equations had higher validation errors than Clark et al.'s (1987, 1989). We concur with Clark et al. (1987, 1989) that useful NIRS equations can be developed for Ca, P, K, Mg, S, and Al. We also found additional NIRS equations for Fe, Mn, and Si to be potentially useful. Minerals not previously reported were B, Cd, Cl, Cr, and Ti. Of these, only B had a potentially useful equation.

In an attempt to find factors that influence the success of NIRS equation development, we compared means and CVs, where  $CV = (SEL/mean) \times 100$ , with the appropriate SEL of duplicate ICP mineral analysis of sun-cured stems (Table 8). Coefficients of variation (based on SEL) were between 1 and 6 for the macrominerals, but of the microminerals, only B and Mn had CVs (SEL/mean) similar to the macrominerals. These same minerals were all previously considered to have made useful NIRS equations. Aluminum and Fe, for which we also had developed useful NIRS equations, had higher CVs (based on SEL) of 18 and 20, respectively. All of the other microminerals that had duplicate analysis (Cd, Cr, Cu, Ni, Pb, and Zn) had higher CVs (based on SEL), ranging from 25 to 84, which may help to explain the higher failure rate of NIRS equation development for prediction of microminerals. Other obvious problems can be traced back to the small range and magnitude of some of the microminerals and, in general, to the insensitivity of NIRS spectra to the minerals.

In conclusion, NIRS accurately predicted leaf and stem concentration, total ash, and macrominerals Ca, K, and P. Less success was found in prediction of macrominerals Mg and S and microminerals Al, B, Fe, Mn, and Si concentrations in leaves and stems so as to be useful in evaluating alfalfa as biofuel for electrical power production. Additional samples will need to be taken for future verification of the equations and for addition to the calibration to make the equations more robust. The speed of NIRS allows analysis of the massive amounts of biomass required daily in a power plant for adjustment of burner efficiency in real time. A higher level of accuracy for minerals is available in ICP laboratory methods for research or farm samples, but NIRS analysis can be useful when speed of analysis and per-sample costs are a consideration.

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