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Near infrared reflectance spectroscopy estimation of ^{13}C discrimination in forages

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Abstract

Forage improvement programs often select for increased crude protein and dry matter digestibility. Additionally, breeding programs may be interested in selecting for enhanced transpiration efficiency or water use-efficiency. Forage crude protein and dry matter digestibility are commonly determined by near infrared reflectance spectroscopy (NIRS), whereas water use-efficiency is estimated from ^{13}C discrimination (Δ) values obtained from isotope-ratioing mass spectrometers. If NIRS could predict Δ, then W could be determined simultaneously with quality components at a much lower cost. To test this possibility, leaf samples of alfalfa (*Medicago sativa* L.) and several cool-season perennial grasses were analyzed with a dual-inlet, double collector gas isotope mass spectrometer, and values of Δ were calculated. Subsamples were scanned with monochromators that collected spectra from 400 to 2,500 nm or 1,100 to 2,500 nm, and absorption data were regressed with values of Δ. Standard errors of calibration for regressing Δ with NIRS absorption values were higher for grasses than for alfalfa. Coefficients of variation for all validation sample sets used for prediction of Δ by NIRS were less than 3%, and NIRS correctly identified 77 to 82% of the samples with the lowest Δ values as determined by mass spectrometer analysis. This level of predictability may be acceptable for identification of genotypes with high water use-efficiency during the early phases of forage improvement programs.

Key Words: spectral analysis, range grasses, crested wheatgrass, creeping foxtail, alfalfa, Russian wildrye, water-use efficiency

Farquhar et al. (1982) proposed that variation in ^{13}C discrimination (Δ) in C_3 plants depends on the ratio of leaf intercellular CO_2 concentration (C_i) to ambient CO_2 concentration (C_a), which is related to transpiration efficiency or water-use efficiency (amount dry matter produced per unit of water transpired). Johnson et al. (1993) and Johnson and Asay (1993) reviewed the use of Δ for determining water use-efficiency in cool-season forage grasses. Because a leaf incorporates carbon through time via photosynthesis, measurement of Δ integrates C_i/C_a which means Δ provides a potential means to select forage breeding populations with improved water use-efficiency (Johnson et al. 1990). Carbon isotope composition of plant tissues, from which Δ is determined, is typically analyzed with an isotope-ratioing mass spectrometer (Tieszen et al. 1983), which is expensive to purchase, operate, and maintain.

Near infrared reflectance spectroscopy (NIRS) is a rapid, precise, and nondestructive analysis method that measures moisture, oil, and protein concentration in grains (Hrushka and Norris, 1982). In addition, forage quality characteristics such as crude protein, acid and neutral detergent fiber, lignin, and in vitro dry matter disappearance have been successfully predicted by NIRS (Marten et al., 1983; Norris et al., 1976). This method has been certified by the Association of Official Analytical Chemists (AOAC) for the measurement of moisture, crude protein, and acid detergent fiber in forages (AOAC, 1990). As a result, NIRS is routinely used to evaluate forage quality characteristics in plant improvement programs.

When Okano et al. (1983) evaluated the potential of infrared absorption spectrometry for determining ^{13}C atom %, results were within 95 to 97% of the values obtained by mass spectrometry, and the relative standard deviation was less than 3%. They showed that infrared spectrometry could distinguish differences [about 14 per mil (‰)] in ^{13}C abundance between C_3 and C_4 plants. This suggests that spectrophotometric techniques might be useful in predicting Δ. This study was initiated to determine if NIRS can be reliably used to estimate Δ in a variety of forage species and genotypes.

Materials and Methods

Samples

The samples consisted of recently, fully expanded leaves of 30 alfalfa genotypes from 2 cultivars grown in Utah and 5 alfalfa cultivars grown in New Mexico, all under line-source sprinkler systems (Hanks et al. 1976). Alfalfa herbage was sampled from 4 cultivars grown under uniform rainfed conditions in South Dakota. In addition, flag leaves of 14 accessions from 9 Triticeae grass species were sampled from nurseries in Utah, Idaho, and Montana. We also harvested forage from 9 genotypes of crested wheatgrass [*Agropyron desertorum* (Fisch. ex Link) Schult.] grown in the greenhouse and 150 genotypes of creeping foxtail (*Alopecurus arundinaceus* Poiret) grown in South Dakota. Combinations of replicates, soil-water levels, and harvests pro-

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vided a total of 229 alfalfa and 358 grass samples. The Utah and New Mexico alfalfa and the greenhouse crested wheatgrass samples were dried at 70° C, whereas the South Dakota creeping foxtail and alfalfa and Triticeae grass samples were dried at 37° C. All samples were ground using a cyclone mill with a 1-mm screen.

Carbon Isotope Analysis

Ground samples (ca. 2 mg) were loaded into tin vessels and combusted in a C and N analyzer (Carlo Erba NA-1500; Fisons Instruments, Valencia, Calif.). The CO_2 and N_2 gases were separated at 50° C on a chromatographic column monitored by a thermal conductivity detector. The CO_2 gas from the C and N analyzer was trapped, cryogenically purified, and analyzed for $\sigma^{13}C$ (the ratio of $^{13}C/^{12}C$ relative to that of a Pee Dee Belemnite standard) using an isotopic ratioing mass spectrometer (SIRA 10; Fisons Instruments, Valencia, Calif.). Laboratory precision for σ13C exceeded 0.1 ‰. Standards 21 and 22 (NIST) were used routinely to verify accuracy of the working standards. The $\sigma^{13}C$ values were converted to Δ values as described by Farquhar et al. (1989), assuming a $\sigma^{13}C$ value for ambient air of -8.0 ‰ on the PDB scale (Mook et al. 1983). The Utah and New Mexico alfalfa and Triticeae grass samples were analyzed for Δ using similar procedures described above, except that the mass spectrometer was a Micromass 602E (VG Isotech, Middlewich, England) (Tieszen et al. 1983).

Near Infrared Determinations

Alfalfa and creeping foxtail samples from South Dakota were scanned with a scanning monochromator instrument (Model 5000, NIRSystems, Inc., Silver Spring, Md.) that collected spectra from 1,100 to 2,500 nm in 2 nm increments, whereas the remaining sample sets were scanned with a Model 6500 scanning monochromator that collected spectra from 400 to 2,500 nm in 2 nm increments. Both instruments used ISI software (Infrasoft International, Port Matilda, Penn.) to collect spectral data, develop calibration equations, and evaluate performance of calibration equations.

All 55 alfalfa and 137 creeping foxtail samples from South Dakota were identified for Δ analysis using the programs CENTER and SELECT (Shenk and Westerhaus, 1991), which are used to reduce the number of samples needed for calibration development. In theory, the CENTER program ranks samples in a file according to their Mahalanobis distance from the average spectrum of the file. This allows for the identification of samples that do not fit the population being examined. The SELECT program uses the "nearest neighbor" approach by examining the spectra of all samples and identifies groups or neighbors (similar to clustering). The program then selects a sample from each neighborhood for analysis by conventional laboratory procedures. This reduces the number of samples needed for chemical analyses by eliminating redundant samples.

The CENTER or SELECT programs were not used for the other sample sets because these samples all had been previously analyzed for Δ. The following sample sets were each split into 2 subgroups with the samples from 1 subgroup used for calibration and the other subgroup samples used for validation: New Mexico alfalfa samples; combined Utah and New Mexico alfalfa sample sets; greenhouse crested wheatgrass samples; combined Triticeae grass and greenhouse crested wheatgrass grass sample sets; and combined Utah and New Mexico alfalfa plus Triticeae grass and greenhouse crested wheatgrass grass sample sets. Spectral data from the foxtail and South Dakota alfalfa samples could not be combined with other studies because the spectra were obtained with different NIRS instruments.

Reflectance spectra for each wavelength were regressed with Δ values for the calibration samples from each sample set using a program that develops multiple regression equations utilizing methods similar to SAS® program PROC REG with the STEPWISE selection method. The program solves a regression equation in the form of:

$$Y = B_0 \pm B_1X_1 \pm B_2X_2 \pm B_3X_3 .... \quad (1)$$

where Y is Δ predicted by NIRS; X_1, X_2, and X_3 are absorption measurements or derivatives at wavelengths λ_1, λ_2, and λ_3, respectively; B_0 is the regression constant; and B_1, B_2, and B_3 are partial regression coefficients. Standard error of calibration (SEC) was calculated to assist in selection of the equation that best fit the Δ data:

$$SEC = [\Sigma(X_i-Y_j)^2/(N-p-1)]^{.5} \quad (2)$$

where X_i is the value determined by conventional analytical methods, Y_j is the predicted value from NIRS, N is the number of samples, and p is the number of dependent variables (wavelengths) in the calibration equation. The multiple coefficient of determination (R^2) and an F statistic (similar to the PROC REG SAS® procedure) also were used in selecting the best-fit equation. All F values for each independent variable in the equation had to be greater than 10 to be considered for the final equation.

Each of the calibration equations developed for each sample set were then tested (or validated) with a group of samples from the same sample set. None of the samples used in calibration development were used for validation. The standard error of prediction (SEP) was used to determine regression equation performance:

$$SEP = [\Sigma(X_i-Y_j)^2/(N-1)]^{.5} \quad (3)$$

where X_i, Y_j, and N are as previously defined (except that X_i and Y_j are from different populations). In addition, bias (mean reference analysis values minus mean NIRS-derived values), and a simple coefficient of determination (r^2) were used to evaluate regression equation performance. The SEP is also synonymous with √MSE, where MSE = mean square error. Coefficients of variation (CV) were also computed to compare results across the different sample sets:

$$CV = [\sqrt{MSE}/mean]*100) \quad (4)$$

where mean equals the NIRS predicted mean Δ for that particular sample set.

Results and Discussion

Table 1 shows the total number of samples in each sample set and the number of samples used for calibration and validation. Grass samples generally had higher mean Δ values and higher standard deviations than did alfalfa samples.

The standard errors of calibration (SEC), which includes errors associated with both chemical analyses and regression, were lower for alfalfa than grass samples (Table 2). The South Dakota sample set had the lowest SEC compared to the other alfalfa sets,

Table 1. Mean, range, and standard deviation (SD) of carbon isotope discrimination (Δ) for forage samples from various studies.

Sample Set	n[1]	Δ Mean	Δ Range	Δ SD
Alfalfa		‰	--‰--	
Utah (Ut.)	58 (58,0)	18.5	17.1-20.5	0.7
New Mexico (N.M.)	116 (50,66)	18.8	17.2-20.1	0.7
UT & NM combined	174 (60,114)	18.7	17.1-20.5	0.7
South Dakota	55 (55,0)	19.9	18.8-20.5	0.4
Grasses				
Triticeae grasses (TG)	78 (78,0)	20.2	17.3-21.8	0.9
Greenhouse crested wheatgrass (GCW)	106 (50,56)	23.1	19.9-24.8	1.2
TG & GCW combined	184 (52,132)	21.6	17.3-24.8	1.8
Creeping foxtail	137 (137,0)	19.4	17.3-21.2	0.7
Combined alfalfa and grass				
Ut. & N.M. alfalfa & TG & GCW grasses	358 (127,231)	20.3	17.1-24.8	2

[1] Number outside of parentheses is total number of samples in sample set; numbers in parentheses indicate the number of samples used for calibration and validation procedures, respectively, for a particular sample set.

which reflects the lower standard deviation obtained for the Δ values from the South Dakota set (Table 1). When the Utah and New Mexico alfalfa sample sets were combined into 1 group, the SEC increased and R^2 decreased compared to the individual sample sets (Table 2). Use of R^2 in NIRS regression development to determine accuracy can be misleading because this value can be affected by weak relationships between dependent and independent variables and/or minimal variation of the independent variables (Windham et al., 1989). Therefore, R^2 values must be interpreted with caution. The same logic applies during the validation process to the statistic, r^2.

Partial-least-squares (PLS) regression analysis also was performed with these sample sets and produced no differences in equation performance compared to STEPWISE procedures (data not shown). Wavelengths used for regression analysis are listed (Table 2) in decreasing order of F value. Wavelengths were not consistently selected among the sample sets.

Figures 1a and 1b show the validation statistics and prediction equation with resulting regression lines for the individual New Mexico and the combined Utah and New Mexico alfalfa sample sets. The standard error of prediction (√MSE) estimates how well the calibration equation will perform on similar samples (error of

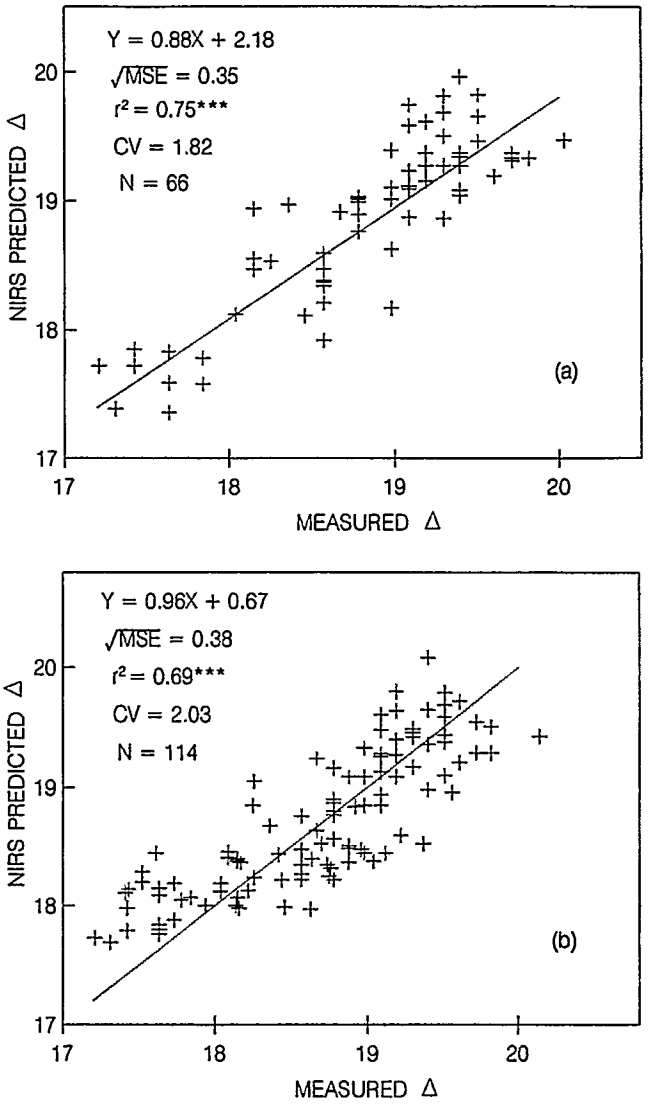
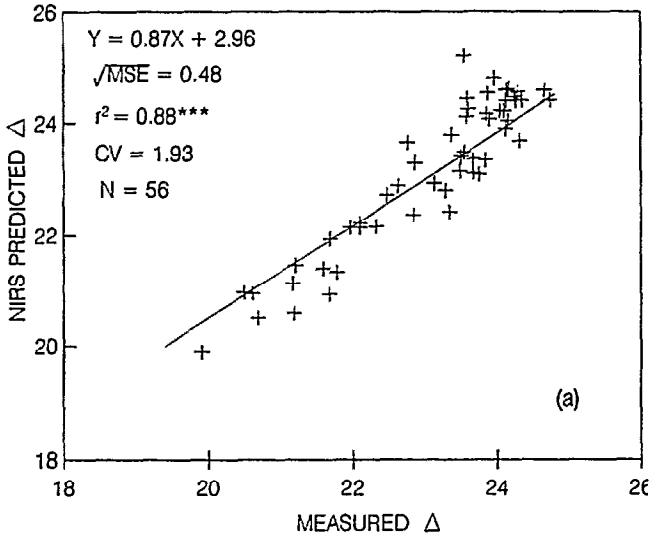


Fig. 1. Relationship between D predicted by NIRS and Δ measured by mass spectrometry (√MSE=standard error of prediction, r^2=simple coefficient of determination, CV=coefficient of variation, and N=number of samples). (a) New Mexico alfalfa (N.M). samples used for validation. (b) Combined Utah and New Mexico alfalfa samples (Ut. & N.M.) used for validation.

Table 2. Near infrared reflectance calibration statistics for determining carbon isotope discrimination (Δ).

Sample Set	n[1]	SEC	R^2	Math[2]	Wavelengths[3]
Alfalfa					----------nm----------
Utah (Ut.)	58	0.30	0.80	1, 5, 5,1	2384,480,2264,1652,2032,2336
New Mexico (N.M.)	50	0.29	0.83	1, 5, 5,1	1724,1436
Ut & N.M. combined	60	0.40	0.66	2,10,10,1	2192,2392,2312
South Dakota	55	0.19	0.76	2,10,10,1	2236,2068,1228,2364,1708
Grasses					
Triticeae grasses (TG)	78	0.52	0.60	2,10,10,1	1372,1276,2144
Greenhouse crested wheatgrass (GCW)	50	0.56	0.80	1,10,10,1	2208,2448,2264,1476,1252
TG & GCW combined	52	0.61	0.89	1, 5, 5,1	464,1164,1180,1716
Creeping foxtail	137	0.44	0.61	2,10,10,1	1508,1652,1604,1476,1868,1700
Combined alfalfa and grass					
Ut. & N.M. alfalfa & TG & GCW grasses	127	0.55	0.93	1, 5, 5,1	2352,1460,2360,1772,2264,2336

[1] n = number of samples; SEC = standard error of calibration for Δ (‰); and R^2 = Multiple coefficient of determination.



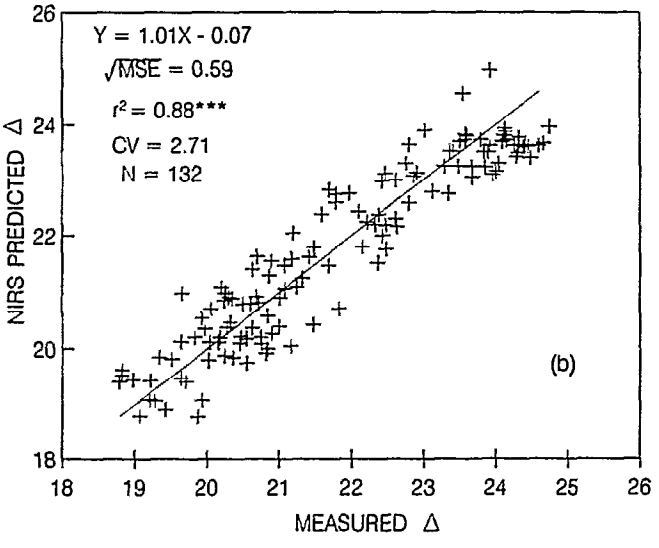


Fig. 2. Relationship between Δ predicted by NIRS and Δ measured by mass spectrometry (√MSE=standard error of prediction, r²=simple coefficient of determination, CV=coefficient of variation, and N=number of samples). (a) Greenhouse crested wheatgrass (GCW) samples used for validation. (b) Combined Triticeae (TG) and greenhouse crested wheatgrass grass samples (TG & GCW) used for validation.

prediction). Coefficients of variation (CV) were computed to compare validation errors across sample sets because magnitudes of Δ varied among sample sets. Values of Δ predicted by NIRS agreed quite well with actual Δ values for the New Mexico alfalfa samples (r^2=0.75***, CV=1.82, √MSE=0.35) (Fig. 1a). When the New Mexico and Utah sample sets were combined, NIRS did not predict Δ as well (r^2=0.69***, CV=2.03) even though the standard errors of prediction were similar.

The SEC values were similar among individual grass sample sets and the combined Triticeae grass and greenhouse crested wheatgrass sample set (Table 2). The R^2 values for the Triticeae grass and creeping foxtail sample sets were somewhat lower than the other sample sets. Values of Δ were predicted very well by NIRS for the greenhouse crested wheatgrass sample set (Fig. 2a). Combining the Triticeae grass sample set with the greenhouse

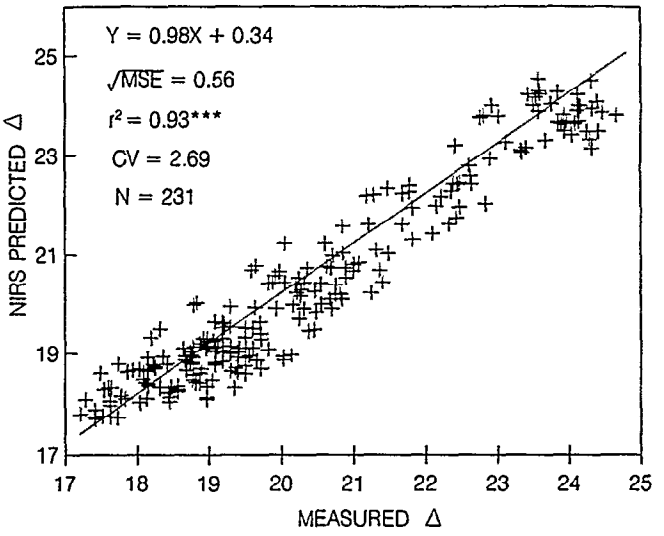


Fig. 3. Relationship between Δ predicted by NIRS and Δ measured by mass spectrometry (√MSE=standard error of prediction, r²=simple coefficient of determination, CV=coefficient of variation, and N=number of samples). Data points represent a combination of Utah and New Mexico alfalfa samples and Triticeae and greenhouse crested wheatgrass samples (Ut. & N.M. alfalfa & TG & GCW grass) used for validation.

crested wheatgrass sample set increased the standard error and coefficient of variation compared to the greenhouse crested wheatgrass sample set (Fig. 2b), whereas the r^2 remained the same.

The R^2 for the combined sample set (Utah and New Mexico alfalfa, plus Triticeae grass and greenhouse crested wheatgrass grass) was 0.93 with an SEC of 0.55 (Table 2). The increased R^2 in this combined sample set was probably caused by the greater diversity in spectra and Δ values than for the individual sample sets. The standard error of prediction and coefficient of variation for this combined sample set (Fig. 3) were similar to the combined Triticeae grass and greenhouse crested wheatgrass sample set (Fig. 2b).

The standard errors of prediction in this study were similar to those reported by Okano et al (1983). The coefficient of variations for our studies were less than 3%, which is comparable to results reported by Mayland et al. (1993). They found a significant relationship between ash concentration and Δ in genotypes of crested wheatgrass (r = 0.69). Windham et al. (1991) reported that NIRS could be used to measure ash in forage, esophageal, and fecal samples. They also noted that ash concentration in their samples was related to spectral peaks of silicon dioxide. Clark et al. (1989) reported, however, that elemental silica estimations with NIRS were variable (coefficient of variations ranged from 11 to 33%) in alfalfa, crested wheatgrass, and tall fescue (*Festuca arundinacea* Schreb.).

In breeding programs for forage grasses, NIRS sometimes is used to identify breeding lines with high forage quality (Starr et al. 1981). Because the entire spectral scan is stored in the computer, breeding lines could be identified simultaneously for both forage quality and high water-use efficiency (low Δ) using proper calibration equations. For the 5 sample sets used for validation (Figs. 1-3), we identified 20% of the samples in each set that NIRS predicted would have low Δ values, and compared these

samples with actual Δ values determined by mass spectrometer analysis. We found that NIRS analysis agreed with between 77 and 82% of the reference values for these samples. In the early stages of a breeding program where the number of samples and costs prohibit analyses of Δ by mass spectrometry, this level of predictability may be acceptable. At more advanced phases of a breeding program, however, where there may be few samples and accurate Δ analysis is required, this level of predictability may not be acceptable.

In summary, NIRS may be useful in predicting Δ in both grass and alfalfa samples. This ability may be related to ash concentration (Mayland et al. 1993, Windham et al. 1991). Results from our study indicate that coefficient of variations for NIRS estimation of Δ generally are quite low and are only slightly higher than coefficient of variations obtained with an isotope ratioing mass spectrometer. Also, the NIRS method requires less time for analysis, costs less to purchase or maintain, and does not require technicians with considerable training and expertise in chemistry. As a result, for some applications, NIRS may be a suitable alternative for determining Δ.

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