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Prediction of the chemical composition of *Cenchrus clandestinus* grass using Near Infrared Spectroscopy – NIRS

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ABSTRACT. Forage evaluation is important as milk production depends on the availability of highly nutritious forage. The aim of this study was to develop nutrient content prediction equations for kikuyu grass (*Cenchrus clandestinus*), a universally used forage in Colombian specialized dairy farms, with near-infrared spectroscopy (NIRS). Kikuyu samples obtained from two hundred dairy farms in the North of Antioquia were analyzed for DM, protein, NDF and ADF. Using three-step procedure (calibration, cross validation, and prediction), equations were developed in a NIRS equipment (NIRS DS 2500 monochromator, Foss-NIRsystem, Denmark). The absorbance values (logarithm (1/R), R = reflectance) were analyzed using the software WinISI version 4.8, performing mathematical treatments to generate several equations per chemical component analyzed. The R^2_v for protein content was 0.96 and SEP was 0.54 indicating an appropriate prediction equation. The R^2_v for NDF and ADF contents were 0.89 and 0.88 respectively, however the SEP value was lower for ADF (0.69) than NDF (1.88). Chemical composition for protein, NDF and ADF in kikuyu grass can reliably predicted using equations developed in NIRS. However, it was not possible to develop a prediction equation for kikuyu DM.

Keywords: chemometrics; forage quality; milk production.

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Introduction

In the tropics, beef and dairy cattle depends on forages as the main feeding source for bovines, and forage production changes in both quantity and quality in response to environmental factors, soil characteristics and management practices (Barahona & Sánchez, 2005). In dairy systems, the balance between dietary energy and crude protein (CP) determines milk production and composition, and when intake of energy and CP are low, diet digestibility diminishes, reducing animal productivity per unit area (Sandoval-Mejía et al., 2008).

One of the most important dairy regions in Colombia is the North of Antioquia, where farms are characterized for the rotational grazing of kikuyu grass (*Cenchrus clandestinus*) which presents great variations in both quantity and nutritional quality throughout the year. Regular monitoring of the nutrient content of this forage is important for the success of farmers. However, most farmers do not analyze forage samples due to the prohibitive cost and the amount of time it takes to receive the results.

Near-infrared spectroscopy (NIRS) has been used for the analysis of different products from the feed, food, chemical, biochemical, environmental, pharmaceutical and medical industries (Brogna et al., 2009) and has been reported to be a reliable technique to predict the content of protein, NDF and ADF of tropical grasses and legumes and other feeds, as well as for the analysis of the composition of feces (Landau et al., 2006). Moreover, it does not require the use of chemical reagents, therefore the cost of analysis decreases.

In the review carried out by Ortega Monsalve et al. (2024) on the use of images to evaluate the nutritional quality of pastures, they reported that NIR instruments are the most widely used in spectroscopy and many of the studies carried out in grasses focus on the use of this methodology, with good results. This leads to the idea that studies of grasses have evolved to include not only the visible part of the spectrum but also using the NIR region, which provides more information due to its greater number of spectral bands. Reports on the use of NIRS for the evaluation of tropical forage quality are restricted. It is then relevant to develop equations to predict the content of protein, NDF, ADF in grasses such as kikuyu, as well as to evaluate their accuracy and reliability, to provide

farmers with reliable nutritional information. A study was carried out to develop equations to predict the chemical composition of the kikuyu grass available in Colombian specialized dairy farms using NIRS.

Material and methods

Location

This study took place in the north of Antioquia municipalities of San Pedro de los Milagros, Belmira, Entrerríos, Donmatías, Bello, Santa Rosa de Osos, San José de la Montaña and Yarumal, in the Universidad Nacional de Colombia, Medellín campus facilities, and the Universidad de Antioquia, Robledo campus. In these municipalities, the research program "*Fortalecimiento de la cadena productiva lechera Del Distrito del Norte Antioqueño" [Strengthening the dairy production chain of the North of Antioquia District],* characterized 200 specialized dairy farms, through the application of surveys, milk monitoring and forage production programs.

Farm characterization

Farms had mostly wavy topography (61.4%), with only 17.4% of them possessing flat land and their average altitude was 2,525 m.a.s.l. Swards were mostly composed of kikuyu grass (*C. clandestinus*) managed under a rotational grazing system and chemical and/or organic fertilization, with re-growth periods oscillating between 35 to 70 d, depending on season.

Sample collection

Kikuyu samples were collected from all farms from March 2015 to September 2016, recording samples variability in terms of land type, fertility, plant age and sampling date. Samples were collected from pastures currently being grazed and those recently grazed, harvesting all fodder above grazing height (10-15 cm). Subsamples were obtained from representative points per pasture category, that later were homogenized and packed (*ca* 500 g) into bags previously identified and then sent to the Chemical and Bromatological Analysis Laboratory of the Universidad Nacional de Colombia, Medellín campus (certificate of accreditation ISOMEC 17025-2005). Once there, samples were dried in a forced-ventilation oven set at 60ºC for 48h and then ground to pass a 1 mm sieve in a Willey mill. Samples were analyzed for dry matter (DM) using the thermogravimetric method at 102 ± 2ºC; crude protein (CP) through the Kjeldahl method (N × 6.25); and neutral detergent (NDF) and acid detergent fiber (ADF) through the gravimetric method of Association of Official Analytical Chemists (AOAC, 2002).

Calibration, cross-validation, and prediction

Dry and ground kikuyu samples were kept in closed and adequately labeled jars, and then separated in three groups according to the equation development stage: calibration, cross-validation and prediction. For each nutrient, the average value, standard deviation, and minimum and maximum values are shown in Table 1. The frequency distribution of DM, protein, NDF y ADF contents was analyzed in each sample group and samples with low frequency were deleted, with the final calibration ranges being DM (13-23%), protein (15-29%), NDF (47-65%) and ADF (25-28%). For content lower than these, more samples must be collected to readjust the equations here generated.

Table 1. Sample number, mean, standard deviation and range by nutrient in calibration, cross-validation, and prediction groups.

	n	χ	σ	Range (%)
Calibration				
DM	85	17.10	3.13	10.5-26.8
Protein	153	22.55	3.89	14.5-29.6
NDF	441	56.62	5.21	43.9-73.3
ADF	316	23.76	2.51	17.3-31.2
Cross-validation				
DM	17	16.22	2.21	12.4-21.4
Protein	140	24.38	3.62	11.7-30.1
NDF	89	55.85	5.60	46.6-65.6
ADF	137	23.21	2.69	15.8-32.8
Prediction				
Protein	131	21.05	3.15	10.1-30.4
NDF	131	61.04	3.84	49.1-71.7
ADF	64	26.05	4.09	19.7-33.4

n: number, χ: mean, σ: standard deviation.

In the calibration step, nine g per sample was deposited in a circular quartz cuvette (70 mm diameter), pressure was applied to remove air, and the spectra were obtained by placing the cuvette in a NIRS DS 2500 monochromator equipment (Foss-NIRsystem, Dinamarca), set in reflectance measurement mode (R) and with silicon (400-1100 nm) and lead sulfide (1100 to 2500 nm) detectors. The absorbance values (logarithm (1/R), R = reflectance) obtained were analyzed with the software WinISI version 4.8 (Infrasoft International Software, Foss, Analytical A / S 69, Slangerupgade, Denmark). Using the SNV (Standard Normal Variable) and DETREND options, spectra were normalized and partially corrected for interferences caused by noise, humidity, or particle size. Then, a principal component analysis was conducted with the spectral information using the SCORE option, to select the calibration spectra based on an "H" value of 3, which represents a "*standard deviation*" for the average spectral data, using the Mahalanobis distance method. Subsequently, different mathematical treatments per nutrient (DM, protein, NDF, and ADF) were applied varying the range and interval of the wavelength as shown in Table 2, considering the first or second derivative and different gaps, first smooth and second smooth values, according to the procedure indicated by Kondal et al. (2024) and Ammeter et al. (2022). For the development of the equations, the Global Equations option was employed, which uses parameters with numerical values and data from 50 to 400 samples. Finally, a modified partial least square (MPLS) regression method was applied.

Table 2. Mathematical treatments, wavelength, interval and number of components used per nutrient.

Constituent	Mathematical treatment				Wavelength (nm)	Int.	Comp. No.
	Derivate	Gap	Smooth	Smooth2			
DM1	1	4	4	1	858-2492	2	15
DM2	1	4	3	1	858-2492	2	11
DM3	1	4	2	1	858-2492	2	11
DM4	1	4	4	1	858-2492	4	11
DM5	1	4	4	1	858-2492	8	11
DM6	1	4	4	1	858-2492	6	11
DM7	2	4	4	1	858-2492	6	29
Protein1	1	4	4	1	1108-2492	8	18
Protein2	1	4	3	1	1108-2492	6	18
Protein3	1	4	3	1	1108-2492	8	18
Protein4	1	4	4	1	1108-2492	4	18
Protein5	1	4	3	1	1108-2492	6	27
NDF1	1	4	3	1	858-2492	8	24
NDF2	1	4	3	1	858-2492	8	27
NDF3	1	4	3	1	858-2492	4	28
NDF4	1	4	4	1	858-2492	8	24
NDF5	1	4	3	1	858-2492	6	26
NDF6	1	4	3	1	858-2492	8	31
ADF1	1	4	4	1	858-2492	8	28
ADF2	1	4	4	1	858-2492	8	28
ADF3	1	4	4	1	858-2492	8	28
ADF4	1	4	3	1	858-2492	6	30
ADF5	2	10	10	1	858-2492	8	32
ADF6	2	10	10	1	858-2492	6	32

Int: interval, Comp. No.: component number.

Three parameters were used to eliminate samples at the development stage of the equations: (a) "T" critical of 2.5, used to eliminate samples when there is a large difference between the laboratory value and the NIRS prediction; (b) Critical "GH" of 3.0, used to eliminate samples that do not fit the population and (c) critical "X" of 10.0, used to eliminate samples considered rare but that have not been identified within the critical groups "T" or " GH " (Burns et al., 1994).

For each equation the following statistical parameters were recorded: (a) standard error of calibration (SEC), which corresponds to the differences between the value of the conventional chemistry in the laboratory and the value predicted by NIRS; (b) coefficient of determination (R^2_c), which describes the linear relationship between the reference data of the calibration samples and their predicted values; and (c) standard cross-validation error (SECV), which measures the predictive capacity of the equation and the 1-VR corresponding to the coefficient of determination of the cross-validation and explains how much of the variation of the reference data is explained by the equation generated. These parameters were used to select equations according to the lowest value of SEC and SECV and the values closest to 1 for R^2_c and 1-VR and with those equations selected, proceed to perform cross-validation (Burns et al., 1994).

In the cross-validation step, randomly selected samples were used by scanning and obtaining their spectra as in the calibration step. The spectra obtained with their respective reference data was validated in WinISI version 4.8 software using the "Monitor" option, which compares the values predicted with the selected equation with their laboratory values. At this stage, the following statistical parameters were recorded: (i) standard error of prediction (SEP), which corresponds to the error of the differences between the results predicted by NIRS and the reference results of the group of cross validation samples and it is accepted up to 1.3 times the SEC of the selected equation; (b) the final validation determination coefficient (R^2_v), and (c) the bias, which corresponds to the difference between the reference values and the values calculated by the equation generated, for which acceptable values are up to 0.6 times the SEC of the selected equation. With these parameters, the best equation per component was selected based on the lowest SEP value and R^2_v was close to 1 (Burns et al., 1994). Once equations were selected, each equation was adjusted to obtain the slope, intercept, SEP, R^2_v and bias values.

In the prediction stage, the equation selected with the intercept and slope for each component (CP, NDF and ADF) was imported into the software operator of the NIRS equipment (MOSAIC and ISIscan) and the spectra of the sample group were randomly selected for this stage. With the results generated in the NIRS and each component was correlated with the results of the reference method. For this procedure, the PROC CORR option of the statistical software SAS version 9.4 was used. In the final phase of the study, the equations selected after calibration, cross-validation and prediction were used in the NIRS DS 2500 equipment to determine the protein, NDF and ADF values of 396 kikuyo samples collected between July and October 2015 on dairy farms from Antioquia.

Results and discussion

In this study it was possible to develop prediction equations for protein, NDF and ADF of kikuyu grass. Table 3 shows the statistical parameters of the equations developed for DM, protein, NDF and ADF, applying different mathematical treatments. The highest coefficients of determination of cross-validation (1-VR) were obtained for protein, NDF and ADF, whereas for DM this value was low. There is less variation in the residual error (SEC) for the protein and ADF components.

Table 3. Accuracy of NIR spectroscopy calibrations for equations selected by nutrient.

Constituent	*SEC*	R^2_c	*SECV*	*1-VR*
DM1	1.407	0.799	1.834	0.654
DM2	1.481	0.775	1.861	0.642
DM3	1.536	0.761	1.897	0.631
DM4	1.406	0.799	1.759	0.682
DM5	1.430	0.793	1.843	0.652
DM6	1.432	0.798	1.724	0.704
DM7	1.251	0.840	1.817	0.658
Protein1	0.331	0.991	0.426	0.986
Protein2	0.315	0.992	0.417	0.987
Protein3	0.324	0.992	0.438	0.985
Protein4	0.304	0.993	0.439	0.986
Protein5	0.340	0.991	0.424	0.987
NDF1	1.485	0.889	1.70	0.855
NDF2	1.437	0.894	1.68	0.856
NDF3	1.544	0.880	1.70	0.854
NDF4	1.541	0.880	1.71	0.852
NDF5	1.532	0.879	1.74	0.844
NDF6	1.444	0.893	1.77	0.838
ADF1	0.781	0.879	0.884	0.844
ADF2	0.830	0.852	0.885	0.831
ADF3	0.844	0.855	0.904	0.833
ADF4	0.798	0.866	0.865	0.842
ADF5	0.768	0.872	0.834	0.848
ADF6	0.778	0.867	0.834	0.846

SEC: standard error of calibration; R^2_c : coefficient of determination of calibration; SECV: standard error of cross validation ; 1-VR: coefficient of determination of cross validation.

The statistical parameters of cross-validation for each of the equations generated (DM, protein, NDF and ADF) are presented in Table 4. A low coefficient of determination of cross-validation (R^2_v) was observed in all equations generated for DM, so it was not possible to select an equation to predict this component. The statistical parameters of cross validation SEP and R^2_v for protein, NDF and ADF indicate acceptable values for the prediction equations. Equations selected by their lower values of SEP and R^2_v values close to 1, were the equation protein4 for the crude protein, NDF6 for NDF and ADF6 for ADF.

Table 4. Accuracy of NIR spectroscopy prediction equations by nutrient.

Constituent	*SEP*	R^2_v	*Bias*
DM1	2.11	0.354	0.317
DM2	1.73	0.450	0.435
DM3	1.64	0.529	0.834
DM4	2.11	0.356	0.386
DM5	1.94	0.391	0.495
DM6	1.82	0.436	1.101
DM7	2.28	0.118	2.214
Protein1	0.93	0.91	-0.462
Protein2	1.14	0.89	-0.143
Protein3	0.94	0.91	-0.528
Protein4	0.89	0.91	-0.504
Protein5	0.95	0.91	-0.354
NDF1	2.95	0.79	-0.035
NDF2	2.48	0.78	0.087
NDF3	2.54	0.77	-0.200
NDF4	2.52	0.77	-0.005
NDF5	2.46	0.78	-0.021
NDF6	2.42	0.79	0.418
ADF1	0.771	0.862	0.085
ADF2	0.812	0.849	0.084
ADF3	0.889	0.819	-0.003
ADF4	0.793	0.857	0.083
ADF5	0.760	0.864	0.279
ADF6	0.758	0.865	0.311

SEP: standard error of prediction; R^2_v: coefficient of determination of validation.

It was not possible to obtain reliable equations for the prediction of *C. clandestinus* DM content, probably because the number of samples used for this component in this study was low. In addition, there are several factors associated to the process of sample collection that generate errors and add to the DM prediction uncertainty. One of these factors is the differential time for different samples to reach the laboratory, as an important portion of farms were located at a great distance from the laboratory. Samples collected in these farms could suffer alteration in moisture content, more so considering that an adequate sample cooling system was not available. Another factor that may have affected is the particle size of the sample after grinding and the type and time of storage of the ground sample, since the larger particles absorb more infrared radiation. Also, when the sample storage period is prolonged, samples can gain moisture.

It must also be pointed out that the hydrogen and oxygen bonds are those that more strongly absorb infrared radiation (Cozzolino, 2002). Windham (1987) suggested that, if the grinding process is adequate and allows a homogeneous particle size, NIRS detects variation in moisture content at the wavelength 1450 to 1930 nm of the spectrum. Cozzolino (2002) suggested that the most suitable reference method for determining moisture in the samples used to generate DM equations in forages is the titration method (Karl Fisher) or lyophilization method. In contrast, there are reports that indicate that the oven drying method for forage samples is the most suitable for calibration in NIRS and that errors in calibration would be associated with the concentration of volatile compounds, as is the case of preserved forages such as silage (Corson et al., 1999). In this study, the method used for drying and grinding was thermogravimetric and has been used in other studies for the development of prediction equations for hay and concentrate, with calibration coefficient (R^2_c) of 0.94- 0.99 and standard cross-validation error (SECV) of 4.5-7.1, indicating that it is possible to develop prediction equations of this component with reliable results (Landau et al., 2006).

In the present study, R^2_v of the equation generated for protein of kikuyu grass was 0.96 with a SEP value of 0.54, and a bias smaller than SEP, indicating that it is a reliable equation to estimate this component. The R^2_v

for NDF and ADF were 0.89, 0.88 respectively, however in fiber the lowest SEP was 0.69 for ADF and 1.88 for NDF. Additionally, the NDF SEP value was higher compared to protein and ADF. However, the bias values for both components were lower than those for protein and lower than their respective SEP values. Accepted bias values are 0.6 times the SEC of the selected equation, and this is the statistical centrality criterion that shows the difference between the mean of the reference method and the mean estimated by the prediction equation, having an acceptable value for all the components in this study.

The calibration results for the protein, NDF and ADF components of this study are consistent with the calibration results for forages reported by Corson et al. (1999), which were for R^2_c of 0.99, 0.95 for protein and NDF respectively and SECV of 0.95, 2.79 for protein and NDF. In the same way Marten et al. (1984) in legumes like alfalfa report R^2_c of 0.93, 0.96, 0.97 for protein, NDF and ADF respectively. In kikuyu grass, the values of R^2_c reported by Herrero et al. (1996) were 0.94 and 0.87 for protein and NDF, values like those found in this study. However, our study had lower SEC values for protein and higher for NDF, a possible consequence of the difference in the number of samples and the range used for the calibration of each component. Previous studies have indicated that the magnitude of the range of tenor values for a given nutrient, influences the calibration coefficient and the standard validation error (Brown et al., 1987). In the same way, Williams (1975) for more than four decades observed that by extending the range used in the calibration of protein in cereals, the standard calibration error was reduced.

The results of the correlation of each of the selected equations are shown in Table 5. A high correlation (0.98) was observed between the protein results calculated with the equation and the laboratory results.

Table 5. Correlation of NIR spectroscopy equation and conventional methodology.

Constituent	*n*	Conventional Methodology		NIRS		*CORR.*	*P*
		χ	σ	χ	σ		
PB	131	21.05	3.15	21.10	3.08	0.98	< 0.0001
NDF	131	61.04	3.84	59.63	3.81	0.88	< 0.0001
ADF	64	26.07	2.54	25.3	1.82	0.78	< 0.0001

n = number; χ = mean; σ = standard deviation; *CORR* = correlation.

The results of the equations obtained in the validation of prediction equation stage in this study coincide with several studies that mention the reliability in the prediction of protein and fiber in forages applying the prediction equations developed in the NIRS (Jung et al., 1998; Brogna et al., 2009). Herrero et al. (1996) reported in Kikuyo values of R^2_v of 0.98, 0.94, 0.94 for protein, NDF and ADF respectively and SEP of 0.42, 1.46, 1.30, as well as a high correlation of the NIRS results and the results by the conventional analysis method (0.99, 0.98, 0.99 for protein, NDF and ADF). In this study R^2_v for protein was similar to previous reports, however for NDF and ADF the values are below 0.90, which indicates that there were some factors that influenced the results of the calibration step. Studies report less accuracy in the prediction of cell wall components such as cellulose and lignin or other components, because these components have greater variation with the physiological age of the plant compared to the protein components that have been shown to be more stable (Burns et al., 1994). In addition, the results of the prediction equations for NDF and ADF can be altered by the same factors mentioned for the DM component, with grinding being a cause of variation in the analytical results, because depending on the mill used in the preparation of the sample, the particle size may vary, affecting the absorbance of the spectrum (Jiménez Torres, 2007).

The kikuyu grass of the dairy farms of several municipalities of the Antioquia state was evaluated with the equations validated in the NIRS for protein, NDF and ADF content, showing values similar to previous reports for forage quality evaluations performed in the same pasture and in the same region (Gómez-Urrego et al., 2014; Sossa-Sánchez et al., 2015; Arias-Ortiz et al., 2023). Studies published by Correa et al. (2012), reported average protein contents of 20.5% and a range of 15.4 to 27.1% and average NDF contents of 58.1% with a range of 51.7 to 66.9%; while average ADF was 30.3% with a range of 28.3 to 32.8%. Likewise, Castro et al. (2015) in kikuyu grown at the Savannah of Bogotá reported average values for protein of 18.4%; NDF of 57.5% and ADF of 25.7% and in the department of Nariño at different altitude ranges (2800 - 3200 m) found protein values obetween 18.47-21.77%; NDF between 60.98-63.35% and ADF between 35.04-30.14% (Apráez Guerrero et al., 2012). The greater variation reported for the fibrous component of kikuyu, may be associated with factors such as soil type and grazing management, as well as fertilization practices and protection from winds (Barahona & Sanchez, 2005).

For NDF and ADF, with a prediction correlation inferior to 0.90, it is necessary to continue the collection of samples considering all factors responsible for nutrient content variation (age, time of year, fertilization, among others), to increase the reliability of the equations. The results of chemical composition prediction using NIRS on 396 kikuyo samples from dairy farms in the Antioquia state are shown in Table 6. A wide range of nutrient content is reported in Table 6, with ranges very similar than those reported in the laboratory analysis, yet generally wider.

Table 6. NIR spectroscopic prediction of the chemical composition of kikuyo in Dairy Farm of Antioquia - Colombia.

	n	χ	σ	*Max.*	*Min.*
Protein	396	22.58	3.15	29.17	9.73
NDF	396	55.47	5.09	74.30	40.76
ADF	396	23.37	2.17	30.58	17.46

n: number; χ: mean; σ: standard deviation; Max.: maximum; Min.: minimum.

It is necessary to continue increasing the reliability of these equations, increasing the number of samples of the region, at different times of the year, mainly focused on the fibrous components. In addition, the sample collection process, sample preparation in terms of the type of mill used and the type and time of storage of the sample should be standardized in order to minimize errors during the calibration and validation steps.

Conclusion

The chemical composition of protein, NDF and ADF of Kikuyo grass (*C. clandestinus*) can be determined by the equations generated thru the NIRS methodology. It is necessary to continue collecting samples to increase the reliability of the equations developed for NDF and ADF in kikuyo. Kikuyo DM prediction was not possible using the NIRS methodology.

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