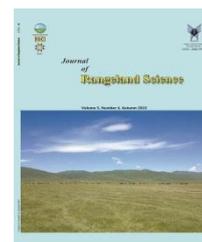




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**Research and Full Length Article:**

## **Estimating Nitrogen and Acid Detergent Fiber Contents of Grass Species using Near Infrared Reflectance Spectroscopy (NIRS)**

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**Abstract.** Chemical assessments of forage clearly determine the forage quality; however, traditional methods of analysis are somehow time consuming, costly, and technically demanding. Near Infrared Reflectance Spectroscopy (NIRS) has been reported as a method for evaluating chemical composition of agriculture products, food, and forage and has several advantages over chemical analyses such as conducting cost-effective and rapid analyses with non-destructive sampling and small number of samples. This study aims to estimate Nitrogen (N) and Acid Detergent Fiber (ADF) content of grass species using NIRS. A total of 171 samples of grasses (Poaceae) at vegetative, flowering, and seeding stages were collected from different regions in Iran. The samples were scanned in a NIRS DA 7200 (Perten instruments, Sweden) in a range of 950-1650 nm. The sample set consisted of 110 samples for calibration and 61 samples for validation was used to predict N and ADF. Samples were previously analyzed chemically for Nitrogen (N) and Acid Detergent Fiber (ADF) and then were scanned by NIRS. Calibration models between chemical data and NIRS were developed using partial least squares regression with the internal cross validation. The coefficients of determination ( $r^2$ ) of linear regression between chemical analyses and NIRS were 0.90 and 0.94 for N and ADF, respectively. The standard errors of prediction were 0.30% and 3.10% for N and ADF, respectively. The results achieved from this study indicated that NIRS has a potential to be used in the measurement of N and ADF contents regarding the forage samples.

**Key words:** Animal nutrition, Forage quality, Poaceae, NIRS

## Introduction

The successful adoption of forages in order to feed the animal husbandry requires knowledge of its nutritional quality for livestock as animal performance and health are highly dependent on nutritional composition of forages. This approach requires forage quality analysis while monitoring the proper feed and ration scheduling (Calderon *et al.*, 2009). Several parameters such as Crude Protein (CP) or total nitrogen (N), Acid Detergent Fiber (ADF), Neutral Detergent Fiber (NDF) and Metabolic Energy (ME) among the others are used to quantify forage quality analytically. Chemical assessments of forage clearly determine forage quality; however, traditional methods of analysis are time consuming, costly and technically demanding (Deaville and Flinn, 2000). Also, such parameters as N and ADF contents of plant species vary with respect to years and plant growth stages within a given growing season and require a constant evaluation (Arzani *et al.*, 2004; Ball *et al.*, 2001). As knowing the values of N, CP, ADF and NDF is essential for the controlled feeding of animals, several methods have been developed to estimate the digestible nutrient contents of forages. The principal methods are based on chemical composition (Andrieu *et al.*, 1981) that is an expensive and time-consuming process that requires large amounts of feeds. High sample replication and high costs associated with chemical analyses of tissue traits can limit the studies aimed at explaining how environmental conditions affect the plant traits or how variations in these traits affect the interactions among plants (Bain *et al.*, 2013). Near Infrared Reflectance Spectroscopy (NIRS) has been explored and reported as a method for evaluating chemical composition of agriculture products, foods, and forages by several authors (Aiken *et al.*, 2005; Moore *et al.*, 1990; Norris *et al.*, 1976; Reeves, 2012;

Ru and Glatz, 2000; Shenk and Westerhaus, 1993 and 1994; Gonzalez-Martin *et al.*, 2007). Unlike most conventional analytical methods, NIRS is rapid and nondestructive and it does not use chemicals or generate chemical wastes requiring disposal while it is multi-parametric; it means that several parameters can be determined simultaneously in the same measurement process (Eldin, 2011). The advantages of NIRS over conventional assessments include the accurate and cost-effective analysis, non-destructive sampling, minimal amount of samples required for testing and an increase in number of samples analyzed per unit of time (Givens and Deaville, 1999; Andrés *et al.*, 2005; Deaville and Flinn, 2000). Several authors have evaluated NIRS to determine forage nutrients content such as N, CP, and ADF (Givens and Deaville, 1999; Andrés *et al.*, 2005; Charehsaz *et al.*, 2010; Fassio *et al.*, 2009; Míka *et al.*, 2003; Scholtz *et al.*, 2009; Ward *et al.*, 2011; Arzani *et al.*, 2012).

The objective of this study was to evaluate the use of NIRS as an alternative method to conduct the conventional chemical analyses in order to measure ADF and N contents in forage samples taken from Iranian rangelands.

## Materials and Methods

Samples were collected from 11 forage species of the Poaceae family (*Bromus tomentellus*, *Festuca ovina*, *Festuca rubra*, *Festuca sulcata*, *Poa trivialis*, *Alopecurus textilis*, *Stipa hohenackeriana*, *Aeluropus littoralis*, *Puccinellia distans*, *Koeleria cristata*, and *Agropyron trichophorum*). Forage samples were taken at three phenological stages (vegetative, flowering, and seeding) in three replications from different regions of Iranian rangelands, namely Ardabil, Isfahan, East Azarbaijan, West Azarbaijan and Zanjan provinces. One to four species were collected from each site in 2009, 2010, and 2011 to give

the total of 171 samples. For each sample, ten plants were randomly selected for collecting the samples concerning each species. Plants were cut at the height of 1 cm above ground.

Results from NIRS were compared with chemical data obtained by Arzani *et al.* (2011) who measured N content by Kjeldahl analysis following an AOAC procedure (Cunniff, 1995) and Acid Detergent Fiber (ADF) was measured by the procedure presented by Van Soest (1963) (Fibertec). Before conducting the forage analysis, samples were dried at 70°C in a forced-air oven for 24 hours and ground through a 2-mm mesh. About 10-15 g of each sample might be scanned by NIRS. Reflectance spectra were recorded in the scanning range of 950-1650 nm in a diode array instrument (DA 7200, Perten Instruments, Hägersten, Sweden), and the spectra were recorded as  $\log(1/R)$  at 2-nm intervals. Samples were scanned twice in the duplicate repacking.

Spectral data were exported into software for multivariate analysis (Unscrambler version 9.5 CAMO ASA, Oslo, Norway) (Cozzolino *et al.*, 2008). The samples were divided into two sets for each constituent, a larger set (calibration set) to develop the calibrations and a smaller set (validation set) to test the accuracy of calibrations. Calibrations (Cozzolino *et al.*, 2008; Alomar *et al.*, 2009; Batten, 1998; Calderon *et al.*, 2009) were developed by selecting 108 forage samples randomly and then validated (Stubbs *et al.*, 2010; Stuth *et al.*, 2003) with 63 samples.

Calibration equations were performed by the regression between spectral data and reference analysis data. The statistical procedures were developed using Partial Least Squares (PLS) regressions. The results from the calibration set were compared to the chemical values using the coefficient of determination in calibration ( $r^2_{cal}$ ) and

the Standard Error of Cross Validation (SECV). Residual Performance of Deviation (RPD) [defined as the ratio of Standard Deviation (SD) of the laboratory results to the Standard Error of Cross Validation (SECV)] was used to evaluate the calibration performance as suggested by Williams (2001) and Fearn (2002). If the RPD value is  $\geq 3$ , the NIRS calibrations can be considered adequate for the analytical purposes (Williams, 2001; Fearn, 2002). The validation sample set was used to test the calibration equation performance. Calibration performance was evaluated by examining the Standard Error of Prediction (SEP), bias and slope (Fearn, 2002). The ratio of Standard Deviation (SD) to the Standard Error of Prediction (SEP) was used as a criterion to evaluate the accuracy of desired equation and also as a basis for standardizing the SEP (Westerhaus *et al.*, 2004; Stubbs *et al.*, 2010). The coefficient of determination for cross validation (1-VR) was calculated. In this study, the Standard Normal Variate (SNV) was used for the pre-processing of methods or the normalization of data (Williams and Sobering, 1996; Brereton, 2003).

## Results

The mean and range for N and ADF contents were measured in 171 forage samples by NIRS and chemical determinations are reported (Table 1). The average N compositions of all samples were given as 1.54 and 1.50% by NIRS determination and chemical determination, respectively. The average ADF compositions of all samples were 42.14 and 44.72% by the means of NIRS determination and chemical determination, respectively.

**Table 1.** Overall mean and range of nitrogen and acid digestible fiber concentrations in all 171 samples analyzed by NIRS or conventional chemical laboratory procedures for forage grasses

Variable	Mean		Range	
	NIRS	Chemical	NIRS	Chemical
concentration as % dry weight				
N	1.54	1.50	0.31 - 4.19	0.33 - 4.17
ADF	42.14	44.72	26.25 - 50.9	24.77 - 61.84

N, nitrogen; ADF, acid detergent fiber; Range, low to high values in the determinations of N or ADF

The descriptive statistics (mean, range, and standard deviation) for N and ADF were measured in the forage samples and the analyses conducted for the calibration and validation of NIRS analyses are presented in Table 2. High variation (SD) in chemical composition was observed due to the different growth stages of

forage samples (vegetative, flowering, and seeding) as well as the differences in environmental conditions in different conditions of sampling sites (soil, temperature, and topography). Therefore, a wide range in chemical composition was obtained to develop NIR calibrations (Dardenne *et al.*, 2000).

**Table 2.** Descriptive statistics for nitrogen and acid detergent fiber measured in forage samples by NIRS and conventional chemical analyses of 108 samples for calibration and 63 samples for validation of NIRS procedure

Variable	n	Mean	SD	Range
concentration as % dry matter				
NIRS analyses				
Calibration				
N	108	1.53	0.82	3.88
ADF	108	42.82	5.55	26.13
Validation				
N	63	1.56	0.58	2.44
ADF	63	40.99	6.18	24.95
Chemical analyses				
Calibration				
N	108	1.49	0.87	3.84
ADF	108	44.84	7.57	31.44
Validation				
N	63	1.53	0.62	2.74
ADF	63	44.50	8.98	35.23

N: Nitrogen; ADF: Acid Detergent Fiber; n: number of samples; SD: Standard Deviation: Range, low to high values of N or ADF

The calibration statistics including the Standard Error of Calibration (SEC), Standard Error of Cross Validation (SECV), coefficient of determination ( $r^2$ ), coefficient of determination in cross validation (1-VR) and the RPD values for each analyzed parameter are shown in Table 3. The  $r^2$  and SECV values for N

were 0.90 (SECV 0.30 %) and for ADF, it was given as 0.95 (SECV 1.85 %). The RPD values (residual performance of deviation) obtained in the calibration set for the chemical analyzed parameters were 3.02 and 4.00 for N and ADF, respectively.

**Table 3.** Near infrared reflectance calibration statistics for nitrogen and acid detergent fiber measured in the forage samples from Iran

Variable	n	Mean % DM	SEC	SECV	$r^2$	1-VR	RPD
N	108	1.49	0.29	0.30	0.90	0.89	3.02
ADF	108	45.27	1.79	1.85	0.94	0.93	4.00

N: nitrogen; ADF: acid detergent fiber; n: number of samples in calibration; SEC: standard error of calibration; SECV: standard error of cross validation;  $r^2$ : coefficient of determination for calibration; 1-VR: coefficient of determination for cross validation; RPD (residual performance of deviation); SD/SECV

Table 4 shows the validation statistics for the NIRS calibration models developed regarding NIRS data and chemical analyses. The  $r^2$  and SEP for N were 0.89 (SEP: 0.30%) and for AD, it was estimated as 0.95 (SEP: 3.105%). The

predictive accuracy for the NIR models was considered intermediate as judged by the RPD values. The RPD values in the validation were 2.03 and 2.66 for N and ADF, respectively.

**Table 4.** Near infrared reflectance validation statistics for nitrogen and acid detergent fiber measured in the forage samples from Iran

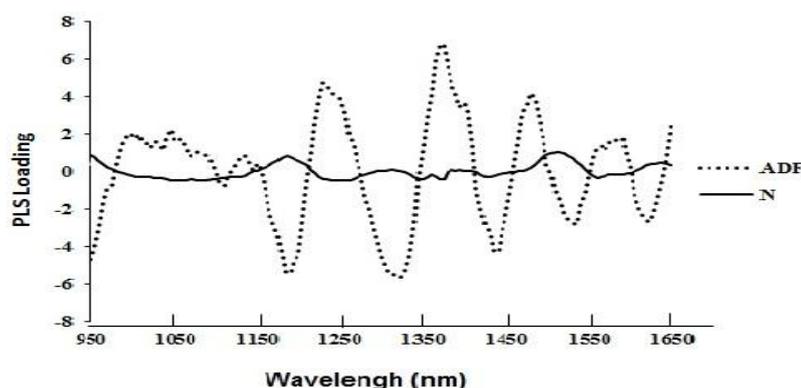
Variable	n	SEP	Bias	$r^2$	Slope	Offset	RPD
N	63	0.30	-0.001	0.89	0.79	0.23	2.03
ADF	63	3.105	0.02	0.824	0.89	4.70	2.66

N: nitrogen; ADF: acid detergent fiber; n: number of samples in validation; SEP: standard error of prediction; Bias: average between reference and NIRS values; Slope: slope of reference vs. NIRS; Offset: the point where a regression line crosses the ordinate (y-axis); RPD (residual performance of deviation): SD/SEP

Examination of the regression coefficients (or loadings) is very important as they indicate specific wavelengths or regions in the NIR spectra related to the measured parameters. The regression coefficients for the partial least squares models developed for ADF and N using NIR spectroscopy are shown in Fig 1. Some similarities were observed in the NIR region at wavelengths between 1350 and 1450 nm associated with O-H overtones

(water). The main differences were observed in the NIR regions between 1400 and 1550 nm related to N-H aromatic amine with O-H and C-H aromatic groups associated with nitrogen, cellulose and water.

Differences between 1500 and 1650 nm are related to C-H, O-H stretching, C-C stretching bonds, and CONH<sub>2</sub> associated with nitrogen, cellulose and other chemical compounds present in the matrix and associated to specific varieties.



**Fig. 1.** Coefficients of regression for the partial least squares models developed for ADF (-----) and N (—) by NIR spectroscopy

The RPD values obtained for the validation were less than those obtained for the calibration. The prediction accuracy (as indicated by the RPD values in the validation) is less than the desirable value for the analytical

purposes; however, the NIRS calibrations allow the screening of screen samples with respect to the quality in term of low, medium, and high for N content.

## Discussion

The results of coefficients of determination ( $r^2$ ) for ADF was high ( $R^2=0.94$ ), but for the validation set, it was relatively low ( $R^2=0.82$ ). Estimation of ( $r^2$ ) for N by the means of calibrations was also high ( $R^2=0.90$ ), but the estimation for the validation set was relatively low ( $R^2=0.89$ ). Similar results were obtained by Aiken *et al.* (2005), Stubbs *et al.* (2010) and Arzani *et al.* (2012). The SEP value of ADF in current study was relatively high (SEP=3.10). However, this result is comparable with SEP values of ADF (1.91; 2.03 and 2.45 respectively) pointed out by the other researchers (Garcia Ciudad *et al.*, 2004; Richardson and Reeves, 2005; Arzani *et al.*, 2012).

It has been suggested that RPD values lower than 2 indicate unsuitable calibration whereas the values greater than 10 are excellent for a routine analysis (Williams, 2001; Fearn, 2002). Stubbs *et al.* (2010) suggested that SD/SECV ratios higher than 3.0 are acceptable for the quantitative prediction with ratios between 2.5 and 3.0 indicating the equations that might be useful for screening, and ratios lower than 2.5 indicating the threshold where an equation is not useful. According to the RPD values obtained in this study, Partial Least Squares (PLS) calibrations can be used for the quantitative prediction of N and ADF contents in a routine analysis (Williams, 2001; Fearn, 2002). Similar results were reported by the other authors when forage and rangeland samples were analyzed using NIR spectroscopy (Alomar *et al.*, 1999; Andrés *et al.*, 2005; Calderon *et al.*, 2009; Cozzolino *et al.*, 2006; Roberts *et al.*, 2004; Stubbs *et al.*, 2010; Woolnough and Foley, 2002).

The results indicated that the NIR calibrations can be useful as a screening tool for ADF in the set of analyzed samples while for N, the calibrations will be marginal. High correlations between NIRS and reference data for N and ADF

were mentioned in this study. However, only the NIR models in the set of samples can be considered useful to measure ADF in a routine analysis. Overall, the results from the present study are in agreement with those reported by the other authors using similar species or rangeland conditions (Garcia Ciudad *et al.*, 1999 and 2004; Richardson and Reeves, 2005; Scholtz *et al.*, 2009; Ruiz-Barrera *et al.*, 2005; Valdés *et al.*, 2006; Pilon *et al.*, 2010; Arzani *et al.*, 2012).

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## برآورد میزان نیتروژن (N) و الیاف نامحلول در شوینده اسیدی (ADF) در گرامینه‌های مرتعی با استفاده از تکنولوژی طیف‌سنجی مادون قرمز نزدیک (NIRS)

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**چکیده.** روش‌های تجزیه شیمیایی به وضوح کیفیت علوفه را تعیین می‌کنند، با این حال، روش‌های سنتی تجزیه شیمیایی کیفیت علوفه زمانبر، پرهزینه و از لحاظ فنی طاقت فرسا هستند. روش طیف‌سنجی مادون قرمز نزدیک انعکاسی (NIRS) به عنوان روشی برای ارزیابی ترکیبات شیمیایی محصولات کشاورزی، غذایی و علوفه‌ای گزارش شده است و دارای مزایای متعددی نسبت به روش تجزیه شیمیایی از قبیل هزینه کم، آنالیز سریع و غیر مخرب نمونه‌ها و مقدار کم نمونه‌های مورد نیاز برای آنالیز می‌باشد. هدف از این مطالعه برآورد میزان نیتروژن (N) و الیاف نامحلول در شوینده اسیدی (ADF) در گرامینه‌های مرتعی با استفاده از تکنولوژی طیف‌سنجی مادون قرمز نزدیک بود. در این مطالعه ۱۷۱ نمونه گیاهی از خانواده گندمیان در سه مرحله رویشی، گلدهی و بذردهی از مناطق مختلف ایران انتخاب شد. نمونه‌ها با استفاده از دستگاه NIRS DA 7200 در محدوده طول موج ۹۵۰-۱۶۵۰ نانومتر اسکن شدند. از مجموع ۱۷۱ نمونه گیاهی، ۱۱۰ نمونه برای ایجاد کالیبراسیون و ۶۱ نمونه برای ارزیابی صحت بکار گرفته شدند. در ابتدا مقادیر نیتروژن (N) و الیاف نامحلول در شوینده اسیدی (ADF) نمونه‌های گیاهی با استفاده از روش تجزیه شیمیایی اندازه‌گیری شدند سپس این نمونه‌ها بوسیله دستگاه NIRS اسکن شدند. مدل کالیبراسیون بین داده‌های شیمیایی و NIRS با استفاده از مدل رگرسیون حداقل مربعات جزئی صورت گرفت. ضریب تعیین ( $R^2$ ) رگرسیون خطی بین تجزیه شیمیایی و روش NIRS، ۰/۹۰ برای N و ۰/۹۴ برای ADF بود. مقدار خطای استاندارد پیش بینی برای N، ۰/۳۰٪ و برای ADF ۳/۱٪ بود. نتایج این مطالعه نشان داد که روش NIRS توانایی لازم برای ارزیابی مقادیر N و ADF نمونه‌های گیاهی را دارد.

**کلمات کلیدی:** تغذیه دام، کیفیت علوفه، گندمیان، NIRS