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Application of near to mid-infrared spectroscopy to estimation of grain nitrogen content in cowpea (*Vigna unguiculata*) grown under multiple environmental conditions

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Cowpea is an important protein primary source in West Africa. To improve both productivity and grain quality, a rapid, precise, robust, and cost-effective method of estimating the grain nitrogen content of samples grown under various conditions is needed for agronomic and breeding studies. Here, the researchers aimed to 1) develop robust calibration models to predict grain nitrogen content by near-infrared and mid-infrared spectroscopy; and 2) to evaluate the models' adaptability by testing variable samples of cowpea grown in three different agro-ecological zones of West Africa. A total of 251 germplasm accessions with wide variation in grain nitrogen content (2.97% to 5.04%) were used for calibration model development. The new models were validated by using 27 genotypes grown in three locations. The calibration model using both near-infrared and mid-infrared spectroscopy had reasonable accuracy (R^2 = 0.90, RMSECV = 0.07) in cross-validation, and it predicted the nitrogen content of the grain samples with acceptable accuracy for individual validation sets (R^2 = 0.90–0.92, RMSEP = 0.09–0.10, RPD = 3.13–3.46) and their combination (R^2 = 0.93, RMSEP = 0.10, RPD = 3.68). In contrast, the calibration model using near-infrared spectra alone had limited accuracy, especially for a sample grown in Ibadan, where there was a greater positive influence on grain nitrogen content than at the other two locations. NIR-MIR spectrometry is thus an adequate tool for agronomic study and breeding to predict grain nitrogen content in cowpea, and it has a reasonable balance between accuracy and applicability.

Key words: FT-IR, optical-phenotyping, crude protein, applicability, environmental effects.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a major staple grain legume widely cultivated in most tropical regions around the world, especially in wide range of agro-ecological zones in West Africa. The crop's importance is attributed to its tolerance to drought, nitrogen (N)-fixing ability, adaptability to different cropping systems, and nutritional and economic values, which help, in particular, small-scale farmers who have limited resources [1]. Also, the crop plays an important role as a primary source of protein, especially where consumption of animal proteins is precluded because of inaccessibility, poverty or dietary preferences. Cowpea shows wide genetic variation in nutritional quality, including crude protein content, which ranges from 15.3% to 28.3% (with N-to-protein conversion factor of 5.45 for cowpea) [2]. These high protein levels in the crop can contribute to overcome insufficient intake of protein and micronutrient (i.e. mineral

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and vitamin) for the malnourished populations in the West African region. The authors' recent research has shown that these high nutritional values can be coupled with the good agronomic characteristics such as high yield, tolerance to various stresses, and low concentrations of anti-nutritional factors owing to the crop's wide genetic variation in agronomic and nutritional traits and low genotypic correlation among these traits [2-4]. In contrast Moura *et al.* [5] have reported a significant negative correlation of grain yield with crude protein, Zn and Fe contents; they suggest that selection for higher yield may decrease these nutritional values in cowpea. In soybean, extensive yield-focused breeding efforts over the decades in USA led the significant decline of grain protein content in recently developed varieties; this may be the result of negative impact of yield increase on grain protein content [6,7]. Also, in cowpea, it has been reported that the environmental conditions greatly alter grain quality such as grain crude protein content and grain size [3,8,9]. These facts clearly indicate that, as part of the breeding process or of farm agronomic studies aimed at improving grain yield without losing other beneficial traits -especially nutritional quality- the researchers need to monitor the nutritional values of the product.

A combination of advantages such as speed, versatility, reliability, low cost and non-destructiveness has favored the use of near-infrared spectroscopy (NIRS) in various study areas [10]. For N content determination, use of NIRS is a cost and time effective option without using chemical regiments and gas, compared with standard methodologies, such as Kjeldahl and Dumas combustion methods. These advantages make this technique ideal for plant breeders and researchers as one of successful tools of optical-phenotyping, although caution is required because of the sensitivity of the NIRS calibrations to year, time of sowing, location and variety [11]. For wider application of the methodology to breeding and agronomic studies to aim at achieving a better combination and balance of good agronomic traits and higher grain quality in cowpea, the researchers need robust calibration models for evaluating grain nutritional components while minimizing the effects of environment factors. Broadening the variation in the calibration set is one way of widening the applicability of the calibration models to samples collected from different environments. Also, the use of mid-infrared spectroscopy (MIRS) for quantitative analysis may help to improve the accuracy and robustness of the models to predict protein content [12-14]. In this work, to develop a suitable method to predict crude protein content as a primary nutritional property in cowpea, the researchers attempted to develop calibration models using both near-infrared (NIR) and mid-infrared (MIR) spectra for the prediction of grain N content. The researchers then validated the applicability of the models by using samples with wide genetic variation and wide environmental effects on grain quality.

MATERIALS AND METHODS

Plant Materials and Sample Preparation

A total of 251 cowpea genotypes with wide genetic variation in physical, nutritional/anti-nutritional, and functional properties, including grain protein content, were collected from the IITA Genetic Resource Center and cowpea breeding program and used for the work (the work (for the researchers data on genetic diversity and traits, see [4]). For sample generation, 224 germplasm accessions were grown in Minjibir, Kano State, Nigeria in 2011 and 2012 with the application of inorganic fertilizer (N:P:K=15:15:15) at 100 kg/ha (Table 1). The researchers also selected an additional two locations, namely Ibadan, Oyo state, Nigeria and Toumnia, Zinder region, Niger Republic, to cover the agro-ecological zones where cowpea is extensively cultivated in West Africa, and 40 genotypes, comprising selected 13 germplasm, 23 breeding lines and 4 major local varieties were cultivated in the three locations during 2011 and 2012. To ensure variation in soil fertility, plots in each location were arranged in an alpha-lattice design with 2 fertilizer application rates and 2 replications. Pods were collected from each plot and threshed without contamination by soil after being air-dried. For the further analyses, grain samples were oven-dried at 40°C for 2 days and then ground with a mixer mill (MM200, Retsch, Germany) in a 25-mL Teflon grinding jar with a 15-mm-diameter zirconium oxide grinding ball.

Spectra Acquisition and Nitrogen Quantitative Analysis

The ground samples were scanned with a Fourier Transform Infrared Spectrometer (FT-IR 6100, JASCO, Japan) equipped with reflectance unit (DR PRO410-M), broadband KBr beam splitter (KBRBB-6000BS), and DLaTGS detector. NIR (4000 to 10000 cm^{-1}) and NIR-MIR (400 to 7000 cm^{-1}) spectra of the same samples were obtained by using a diffuse reflectance method at 4 cm^{-1} resolution (Figure 1). A halogen lamp was used as a light source for the NIR spectra, and a high-intensity ceramic light source was used for the NIR to MIR spectra. Every 7 samples, baseline data were obtained using an internal standard to prevent baseline shift.

Total grain N content was determined based on the Dumas combustion method [15]. Ground grain samples (approx. 40mg) were treated with automatic high sensitive NC analyzer (Sumigraph NC-22F, Sumika Chemical Analysis Service, Japan) to determine total N and carbon content. Acetanilide was used as standard to make calibration curve. The moisture content of each ground grain sample was measured after oven-drying at

Table 1: Characteristics of locations used for sample generation in 2011 and 2012.

Field	Country	AEZs*	Latitude	Longitude	Type of field	Fertilizer rate
Ibadan	Nigeria	Forest savanna transition	7°29'292"N	3°54'690"E	Experimental	Low: 0kg/ha High: 40kg/ha
Minjibir	Nigeria	Sudan savanna	12°08'448"N	8°40'069"E	Experimental	Low: 40kg/ha High: 100kg/ha
Toumnia	Niger	Sahel	13°58'747"N	9°01'698"E	Farmer	Low: 40kg/ha High: 100kg/ha

*AEZs: Agro-ecological zones.

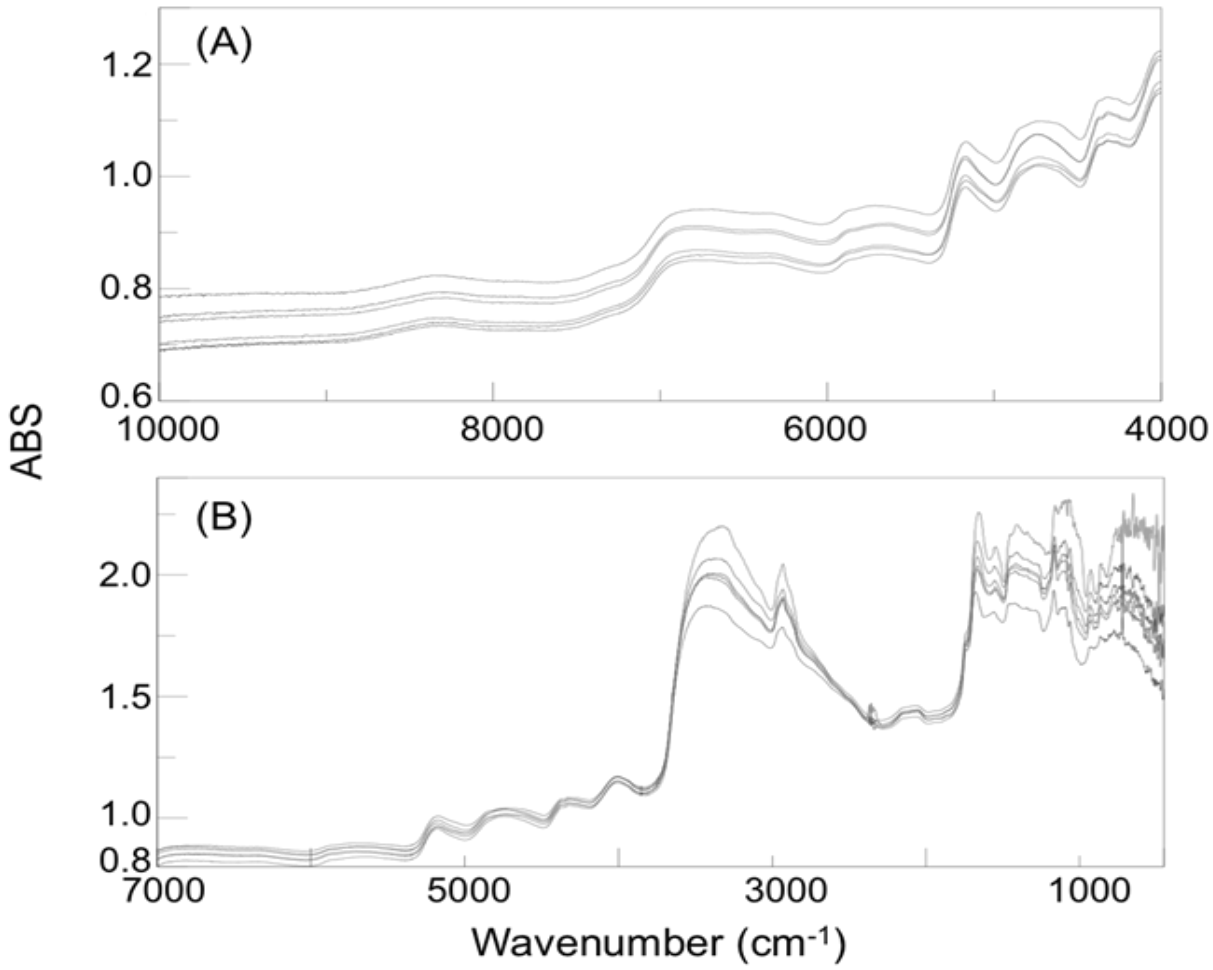

Figure 1. Near infrared (A) and near – mid infrared (B) spectra of samples collected from 6 germplasm accessions with wide variation in grain N content (3% to 4.9%).

80°C for 48h, and used to calibrate the grain N content to a dry matter basis.

Calibration Model Development

Spectral analysis and development of calibration models were conducted by using the partial least squares (PLS) method with Imaging-Model Analysis Program (JASCO, Japan). A total of 925 spectral data obtained from the 224 germplasm accessions grown in Minjibir and the 13 germplasm accessions selected from the three locations with different soil fertilities were subjected to the

development of calibration models and cross-validation. The NIRS and MIR calibration model was developed with a total of 919 of spectrum, after removal of 6 samples as outliers using NIR spectra (4000 to 10000 cm^{-1}) and NIR-MIR (400 to 7000 cm^{-1}) spectra obtained. Also, the two more calibration models were developed by using information from only the NIR region (MIRS-N) or the MIR region (MIRS-M) of the NIR-MIR spectra. All of the developed models were cross-validated with the separate data set (n=129) to prevent over fitting of the model.

The calibration models were assessed by the coefficient of determination in calibration (R^2) and the root mean square error of cross-validation (RMSECV) with the optimum calibration model being chosen on the basis of the minimum RMSECV and maximum R^2. RMSECV and the root mean square error of prediction (RMSEP) were calculated according to Eq. (1) [16].

$$RMSECV/P = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_{pi}-y_i)^2}{n}} \quad (1)$$

Where y_{Pi} = NIRS/MIRS predicted values, y_i = true values, and n = the number of samples.

Model validation

Developed calibration models for MIR and NIR spectra were evaluated against validation sets consisted by the grain samples of 27 genotypes (24 breeding lines and 3 local varieties) collected from Minjibir (n=212), Ibadan (n=212), and Toumnia (n=212), and their combination (n=636). Each validation process was evaluated by using R^2, RMSEP, and the ratio of performance deviation (RPD). RPD was calculated according to Eq. (2), (3) and (4) [10,17].

$$bias = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_{pi}-y_i)}{n}} \quad (2)$$

$$SEP_{biascor} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_{pi}-y_i-bias)^2}{(n-1)}} \quad (3)$$

$$RPD = \frac{S_{ref}}{SEP_{biascor}} \quad (4)$$

Where $SEP_{biascor}$ = the standard error of prediction after bias correction and S_{ref} = the standard deviation of the reference values.

RESULTS

The N contents of all samples used to develop the calibration model ranged from 2.97% to 5.04% with mean value of 4.04% (Table 2). The range of grain N contents observed in the calibration set covered the variation of those observed in the validation sets consisting of the 27 genotypes grown in different agro-ecological zones over 2 years (3.04% to 5.01%).The average N content of the Ibadan validation set was markedly higher than that at other locations, as were the minimum and maximum N contents. Although the researchers observed effects of fertilizer application rate and growing year on grain N content at some locations, the effect was smaller than the effects of location (data not shown). NIRS and MIRS calibration models were developed with cross-validation and evaluated by using three independent validation sets. The best NIRS calibration models were achieved in the cross-validation process using multiplicative scattering correction (MSC) pretreated NIR spectra ranging from 4000 to 9000 cm^{-1} with the mathematical treatment of Savitzky-Golay smoothing (convolution width 11) and mean centering of the spectra and concentration. For MIRS calibration model, the minimum RMSECV and maximum R^2 in the cross-validation process were obtained using both NIR (4500 to 4985 cm^{-1}) and MIR (1400 to 2246 cm^{-1}) of original NIR-MIR spectra with the mathematical treatment of Savitzky-Golay smoothing (convolution width 5) and mean centering of the concentration. The number of factors was 12 for both NIRS and MIRS calibration models.

MIRS-N calibration model showed best result in cross-validation process using the spectra information ranging from 4000 to 7000 cm^{-1} of original NIR-MIR spectra with the mathematical treatment of Savitzky-Golay smoothing (convolution width 11) and centering of the spectra and concentration. For MIRS-M calibration model, the minimum RMSECV and maximum R^2 in cross-validation process were obtained using MIR (1150 to 3660 cm^{-1}) of original NIR-MIR spectra with the mathematical treatment of Savitzky-Golay smoothing (convolution width 15) and centering of the spectra and concentration. The factor numbers used were 9 and 13 for MIRS-N and MIRS-M respectively. The statistics for the cross-validations of the calibration models developed for the estimating of grain N content are shown in Table 3. In the NIRS calibration process, the R^2 was 0.90 and the root mean square error of calibration (RMSEC) was 0.34, whereas R^2 was 0.87 and RMSECV was 0.08 in the cross-validation process. For the MIRS calibration model, R^2 was 0.91 and 0.90 for the calibration and cross-validation processes respectively, whereas RMSEC was 0.34 and RMSECV was 0.07. The R^2 values were 0.89 for MIRS-N and 0.87 for MIRS-M calibration models in calibration process and 0.88 for MIRS-N and 0.84 for MIRS-M in the cross-validation processes.

The N content predicted by using the NIRS calibration model had R^2 value of 0.88, 0.90, and 0.91 in comparison with the actual N values in the case of the validation sets from Ibadan, Minjibir, and Toumnia respectively (Table 4). The R^2 for the combined set was 0.92 (Figure 2). RPD

Table 2: Distribution of N content (%) in cowpea grain from populations used for the calibration and validation processes.

		N content (%)			
Data set	**n**	**Mean**	**SD**	**Min**	**Max**
Calibration	925	4.04	0.34	2.97	5.04
Ibadan	212	4.17	0.28	3.58	5.01
Validation Minjibir	212	3.77	0.31	3.12	4.52
Tounmia	212	3.91	0.36	3.04	4.82

Table 3: Calibration and cross-validation statistics for the models developed on NIR and MIR spectra to determine grain N content (%).

				Calibration			Cross-validation	
	Wavenumber range (cm^{-1})		**n**	**factor**	**R^2**	**RMSEC**	**R^2**	**RMSECV**
NIRS		4000 – 9000	919	12	0.90	0.34	0.87	0.08
MIRS	1400 – 2290	4000 – 4985	919	12	0.91	0.34	0.90	0.07
MIRS-N		4000 – 5230	919	10	0.89	0.34	0.88	0.08
MIRS-M	1150 – 3660		919	13	0.87	0.33	0.84	0.09

Table 4: Validation statistics of the NIRS and MIRS calibration models for estimating N content (%) in ground grain samples from individual locations and for all three locations combined.

Validation	**NIRS**			**MIRS**			**MIRS-N**			**MIRS-M**		
set	**R^2**	**RMSEP**	**RPD**	**R^2**	**RMSEP**	**RPD**	**R^2**	**RMSEP**	**RPD**	**R^2**	**RMSEP**	**RPD**
Ibadan	0.88	0.10	2.90	0.90	0.09	3.13	0.80	0.13	2.25	0.85	0.11	2.52
Minjibir	0.90	0.10	3.20	0.92	0.09	3.40	0.86	0.12	2.63	0.85	0.12	2.55
Toumnia	0.91	0.11	3.38	0.92	0.10	3.46	0.89	0.12	2.97	0.86	0.14	2.62
Combined	0.92	0.10	3.47	0.93	0.10	3.68	0.88	0.12	2.93	0.89	0.12	3.01

ranged from 2.90 to 3.47 for the three validation sets and the combined set. The MIRS calibration model predicted grain N content with R^2 values of 0.90, 0.92, and 0.92 for the validation sets from Ibadan, Minjibir, and Toumnia respectively, whereas the RMSEP ranged from 0.09 to 0.10 (Table 4 and Figure 3). Slightly lower RPD was obtained for the Ibadan validation set than for the other two sets, whereas the highest RPD of 3.68 was obtained for the combined set containing all of the samples collected across all agro-ecological zones. R^2 values of MIRS-N and MIR-M calibration models were ranged from 0.80 to 0.89 and from 0.85 to 0.89 for all validation sets, respectively.

DISCUSSIONS AND CONCLUSIONS

The applicability and accuracy of calibration depend on the population used in calibration model development. Genetic variation in the chemical composition of samples greatly influences the accuracy of the models developed, depending on the type of sample, and target traits. Also, environmental factors such as growing location, weather conditions, and soil fertility may affect the chemical composition of samples and decrease the accuracy of the calibrations. NIRS calibrations are sensitive to year, time of sowing, location, and various environmental factors [11,18]. In the case of cowpea leaf using NIRS (1100–2498 nm), Towett *et al.* [19] reported that their model for protein content showed reduced prediction ability in the case of some samples from different growing environments. In this work, the researchers aimed to develop the robust calibration model using NIR and MIR spectra to estimate N content of the grain samples generated in diverse environments for easier application to breeding and agronomic studies. To this purpose, for calibration model development, the researchers used 224 germplasm accessions representing the genetic diversity of the grain characteristics of cowpea, as well as 13 selected germplasm accessions grown in multiple

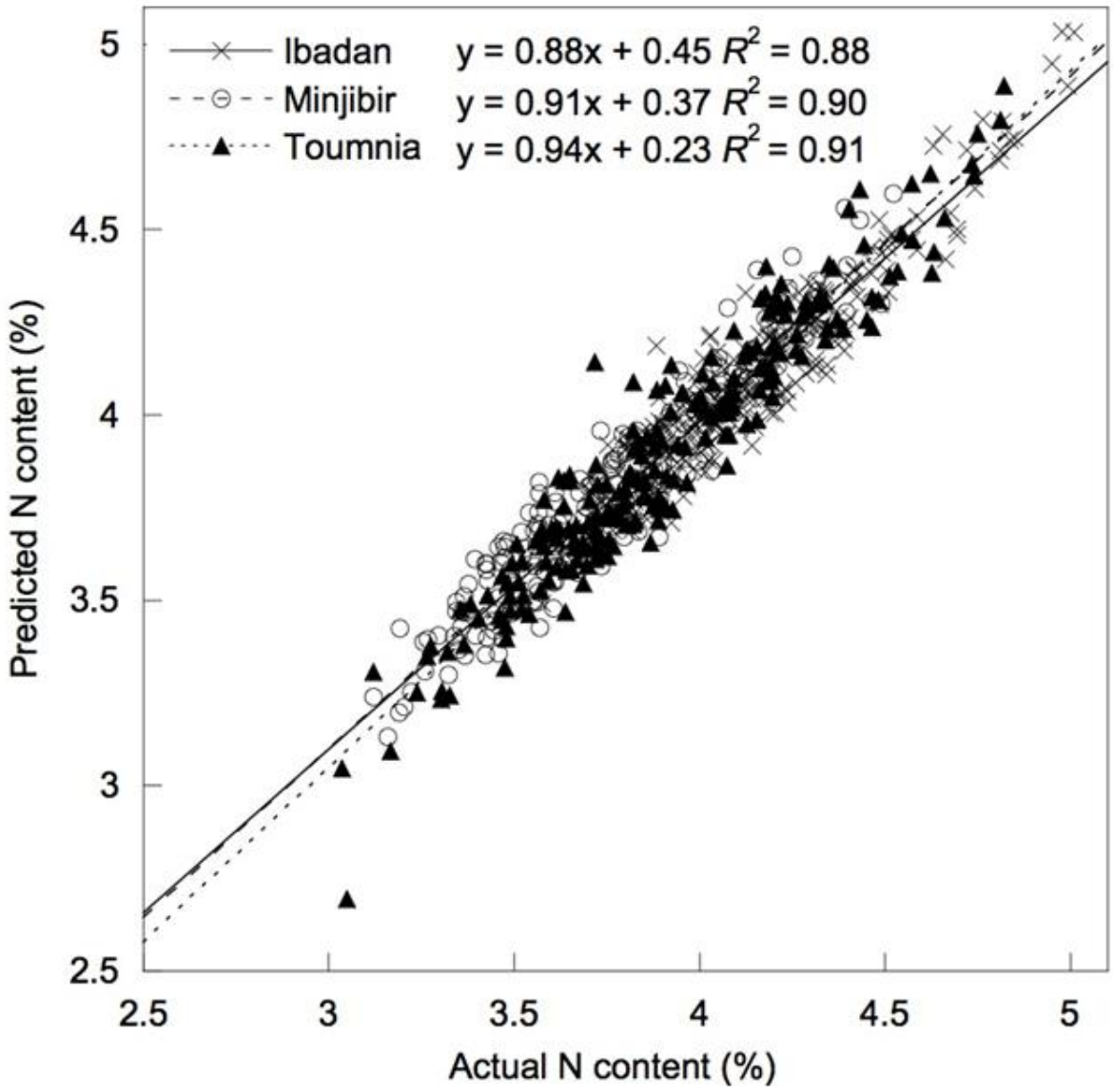

Figure 2. Validation of the NIRS calibration model for the N content of grain samples collected from Ibadan, Minjibir, and Toumnia, in different agro-ecological zones (total n = 636).

environments, as the calibration set (Table 1). The variation in these samples was expected to reflect wide genetic variation and the wide range of the effects of growing environments and to thus increase the applicability of the calibration models. Also, to evaluate the applicability of the researchers developed calibration models, on the basis of the results of their earlier reports [3,4], they developed validation sets by using 27 genotypes showing substantial variation in physical and nutritional properties, grown at three locations in different agro-ecological zones.

As reported by Boukar *et al*. [3]; Bliss [8] and Oluwatosin [9], there was a significant influence of location ($P < 0.01$) on the N content of the grain samples collected from the three locations (Table 2). The growing conditions at Ibadan seemed to have a positive effect on grain N content. The researchers also observed considerable effects on grain colour and size within the same genotype across the environments (data not shown). The difference in average N content between Ibadan and Minjibir was 0.41%; this is considered substantial compared to the genetic variation (1.43 to 1.78%) observed at the three locations among the 27 genotypes. On the other hand, the range of N content in the calibration set 2.97% to 5.04% (equivalent to 16.2% to 27.5% protein with a conversion factor of 5.45 [4]) was larger than that of the validation sets (Table 2). The statistical results of the calibration and cross-validation process showed that the MIRS calibration model had better applicability to the estimation of N contents than did the NIRS calibration model developed on the same calibration set (Table 3). Williams *et al*. [20] reported the

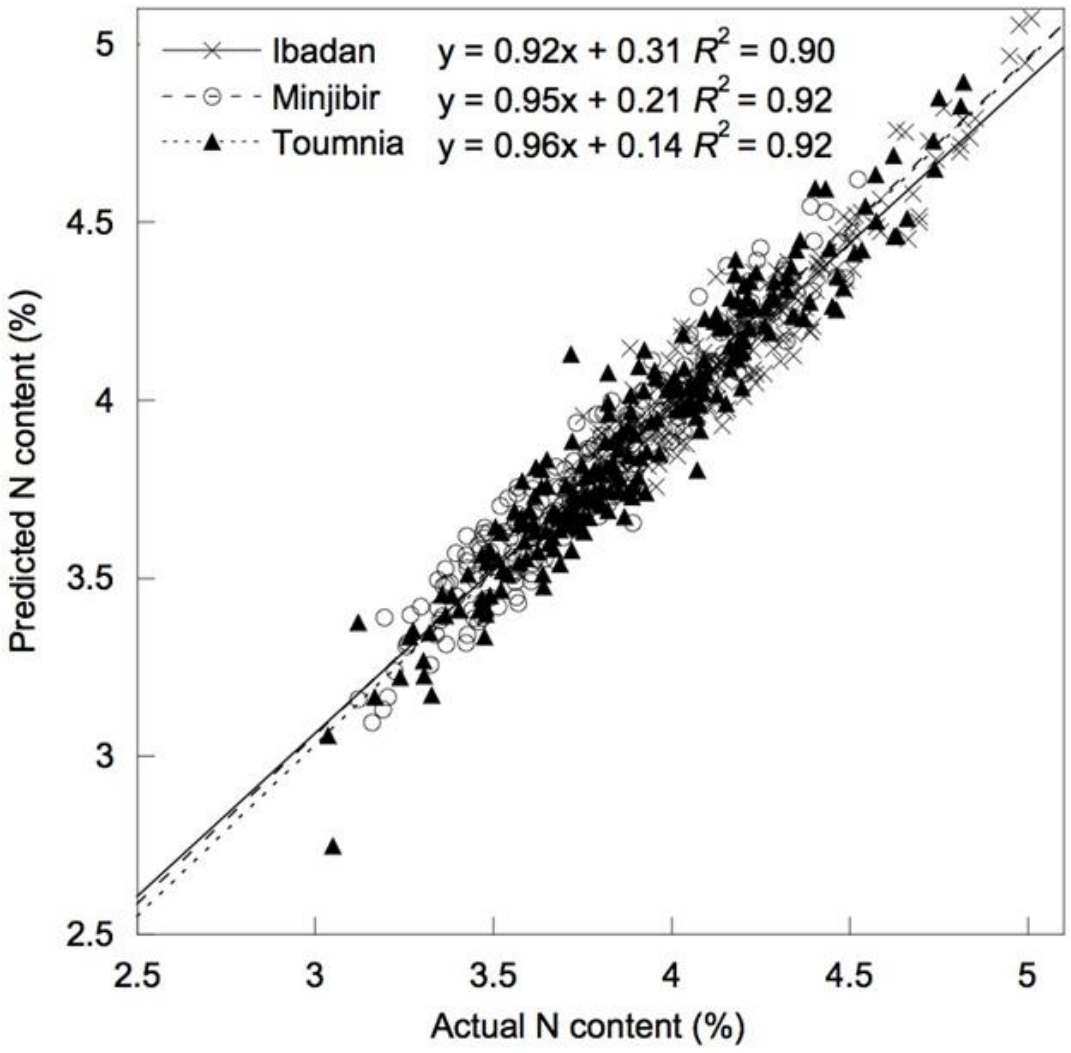

Figure 3. Validation of the MIRS calibration model for the N content of grain samples collected from Ibadan, Minjibir, and Toumnia, in different agro-ecological zones (total n = 636).

NIRS calibration models for crude protein contents in various pulses (e.g. broad bean, faba bean, chickpea, pigeon pea, lentil, vetch and dry pea) with R^2 ranging from 0.89 to 0.96. The R^2 value (0.93) of the researchers MIRS calibration model was reasonably high. The estimated values for the MIRS calibration model for all validation sets from different environments also had good R^2 values (0.90 to 0.92) and RMSEP ranges (0.09 to 0.10) in comparison with the actual reference values for grain N content, and similar values (R^2 = 0.93 and RMSEP = 0.10) were obtained across all environments combined. Although RPD interpretations depend on the number and distributions of the reference values, generally RPD>3 is used as an indicator of acceptable accuracy [10,17]. The RPD ranged from 3.13 to 3.68 was obtained for the samples from the three locations, and combined samples across agro-ecological zones, while slightly lower R^2 and RPD values were observed for the Ibadan validation set. Even though the validation sets that we used for the experiment cannot represent all possible variations, the results showed that the MIRS calibration model was reasonably applicable to estimations of the N content of grain samples from different agro-ecological zones in West Africa (Figure 3). Contrastingly, the NIRS calibration model was less accurate than MIRS calibration model for predicting the external validation sets (Table 4 and Figure 2). The lower R^2 value of the Ibadan validation set than of the Minjibir and Toumnia validation sets in the NIRS calibration model suggests that a specific environmental factor influenced the accuracy of prediction of N content by altering the chemical composition of the cowpea grains. The limited accuracy of the NIRS calibration models indicates that this model can be used only for grading of the samples into groups [10].

The NIRS calibration model for estimating grain Ncontent was obtained with NIR spectral range (4000 to 9000 cm^{-1}), whereas the best MIRS calibration model

was obtained with a combination of NIR and MIR regions. The difference in accuracy of the calibration models could be attributed to difference of signal-to-noise ratios level of the spectrometry methods used. However, the best models developed by using either the NIR or the MIR region of the NIR-MIR spectra (wave number ranged from 400 to 7000 cm^{-1}) showed lower accuracy only in the cross-validation (R^2 = 0.84–0.88) and validation (R^2 = 0.80–0.89) processes. And interestingly, the MIR-N calibration model showed larger variation in R^2 and RPD values for the three validation sets than those of the MIRS-M calibration model. Because MIR shows specific absorption of each functional group of molecules, its use may contribute to quantitative analysis of the major chemical components [12]. The researchers results suggest that using MIR information can contribute to improve the robustness of the models against the environmental effects by using specific characteristic absorption bands, such as Amide I band (peak at 1640cm^{-1}) and Amide II (peak at 1545 cm^{-1}) in the MIR region, as reported by Veselá *et al*. [14].

The researchers' results indicate that NIR-MIR spectral measurement can be used to estimate the N content of ground grain samples from cowpea. Obtained MIRS calibration model showed a reasonable balance between accuracy and applicability in predicting the N contents of grain samples collected from different agro-ecological zones of West Africa. The range of N contents (2.97% to 5.04%) of the samples used for the model development covers the greater part of genetic variation (2.80% to 5.19%) reported in the researchers previous study using 1541 germplasm accessions [2]. The researchers' findings also suggest that MIR information may be useful in increasing the robustness of calibration models for quantitative analysis regardless of the environmental effects. With recent improvement of computation power, the spectra of NIR-MIR region (400 to 7000 cm^{-1}) can be obtained within 2 min per sample, with adoption of broadband beam splitter on a FT-IR system. With cost and time effective characteristics of the method, these accurate and robust calibration models for predicting grain N contents should be useful tools for field agronomic studies and breeding in cowpea.

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