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ARTICLE

Near-Infrared Reflectance Spectroscopy Predicts Protein, Starch, and Seed Weight in Intact Seeds of Common Bean (*Phaseolus vulgaris* L.)

Gokhan Hacisalihoglu,[†] Bismark Larbi,[†] and A. Mark Settles*[,‡]

[†]Biology Department, Florida A&M University, Tallahassee, Florida 32307 and [‡]Horticultural Sciences Department, University of Florida, Gainesville, Florida 32611

The objective of this study was to explore the potential of near-infrared reflectance (NIR) spectroscopy to determine individual seed composition in common bean (*Phaseolus vulgaris* L.). NIR spectra and analytical measurements of seed weight, protein, and starch were collected from 267 individual bean seeds representing 91 diverse genotypes. Partial least-squares (PLS) regression models were developed with 61 bean accessions randomly assigned to a calibration data set and 30 accessions assigned to an external validation set. Protein gave the most accurate PLS regression, with the external validation set having a standard error of prediction (SEP) = 1.6%. PLS regressions for seed weight and starch had sufficient accuracy for seed sorting applications, with SEP = 41.2 mg and 4.9%, respectively. Seed color had a clear effect on the NIR spectra, with black beans having a distinct spectral type. Seed coat color did not impact the accuracy of PLS predictions. This research demonstrates that NIR is a promising technique for simultaneous sorting of multiple seed traits in single bean seeds with no sample preparation.

KEYWORDS: *Phaseolus vulgaris*; common bean; seed composition; NIR; starch; protein

INTRODUCTION

Common beans are a good source of protein, starch, and fiber as well as mineral nutrients for a large segment of the world. There is a coordinated "phaseomics" effort to improve bean yield as well as seed quality and composition (*1*, *2*). Bean seed composition is influenced by environmental factors but also has a strong genetic component (*3*−*5*). Consequently, seed nutritional quality traits are targets for improvement through breeding. Bean seed composition is currently measured with destructive analytical techniques that require a bulk sample of seeds (*3*−*7*). Efforts to improve seed composition would be aided with nondestructive technologies that allow breeders to select composition variants at a single-seed level.

Near-infrared reflectance (NIR) and transmittance (NIT) spectroscopy have been widely adopted for low-cost, nondestructive analysis of fruits, vegetables, and grains (*8*, *9*). Biological materials have multiple overlapping near-infrared absorption bands that are due to overtone and combination vibrations of C−H, N−H, O−H, and S−H functional groups (*10*). The complexity of organic constituents in foods and biological materials leads to broad NIR or NIT absorbance peaks, and multivariate regression approaches, also known as chemometrics, are required to calibrate near-infrared spectra to chemical composition (*8*). Predictions using NIR calibrations are highly reproducible and can approach the accuracy of reference analytical tests for individual constituents (*11*). The major advantages of NIR analysis are its low-cost, nondestructive nature, and ability to predict multiple constituent and quality traits simultaneously. However, the absorbance interactions between chemical constituents within complex samples necessitate calibration development for different crops and sample preparations.

NIR calibrations have been developed for the major bean seed storage molecules using fine ground powders (*12*). Sample grinding requires the seeds to be destroyed prior to NIR data collection and limits the applicability of NIR to breeding and other seed-sorting applications. Single-seed NIR data can be used to predict individual seed composition in both oil seeds and small grains (*13*−*18*). Single-seed NIR has been more challenging for plants with larger seed sizes (*19*−*22*). A key technical advance for predicting the composition of larger seeds, such as maize or soybeans, has been the development of spectral acquisition systems that allow NIR data to be collected from the whole surface of the seed (*23*, *24*).

The spectral acquisition system developed by Armstrong (*23*) has recently been shown to predict multiple maize kernel traits, including starch, protein, oil, and seed weight (*25*). This seed analyzer collects a spectrum as a seed falls through an illuminated glass tube and uses a relatively inexpensive InGaAs diode array spectrometer. It has the capacity to collect NIR spectra at a rate of 10 seeds per second, which would allow for high-throughput seed sorting. The objective of this study was to determine whether the seed analyzer could be applied to other crops with large seed sizes. Common beans are an important part of the human diet in developing countries, but the crop has not been a historic focus of nutritional improvement breeding efforts (*1*). Armstrong's (*23*)

*To whom correspondence should be addressed. Telephone: 352-392-7571. Fax: 352-392-5653. E-mail: settles@ufl.edu.

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glass-tube seed analyzer is an attractive analytical tool for common beans because of its low cost and high-throughput capacity.

MATERIALS AND METHODS

Seed Samples for Calibration. A total of 91 diverse germplasm accessions of common bean (*Phaseolus vulgaris* L.) were obtained from the United States Department of Agriculture (USDA) National Plant Germplasm System. These accessions were selected on the basis of diverse geographic locations of original collection in an effort to sample a broad range of bean seed composition. Each accession was propagated at the USDA−Agricultural Research Service (ARS) Western Regional Plant Introduction Station (WRPIS) (Pullman, WA) in greenhouses with defined soil media and fertilization regimes. The seeds were stored in defined humidity and temperature seed rooms prior to distribution (Welsh, personal communication). Three seeds were sampled from each accession to provide biological and analytical replicates for each accession for a total of 273 seeds. The limited sampling of each accession allowed for a larger diversity of germplasm to be included in the study.

Seed Weight and NIR Data Collection. Individual seed weights and NIR spectra were recorded with a custom seed analyzer as described (*23*, *25*). Briefly, seed weights were recorded with a microbalance consisting of a MK4 microbalance head and a Stabal control unit (CI Electronics, Salisbury, U.K.). A single NIR spectrum was recorded at 1 nm intervals between 907 and 1689 nm with an InGaAs array-based spectrometer (NIR-256-1.7T1, Control Development, South Bend, IN) as each bean fell through a glass tube illuminated by multiple halogen lamps. A dark background and a reference Spectralon (Labsphere, North Sutton, NH) spectrum were measured prior to recording spectra. Individual spectra

Table 1. Seed Composition Statistics of Beans in the Calibration and Validation Sets

	calibration set (n = 178)			validation set (n = 89)		
trait	mean	SD	range	mean	SD	range
seed weight (mg)	272.6	88.8	114.0−515.2	237.7	76.1	80.1−477.5
protein (%)	24.9	3.8	15−34.7	24.5	3.9	14.8−35
starch (%)	43.2	6.3	18.9−60.7	41.7	7.3	15.6−51.4

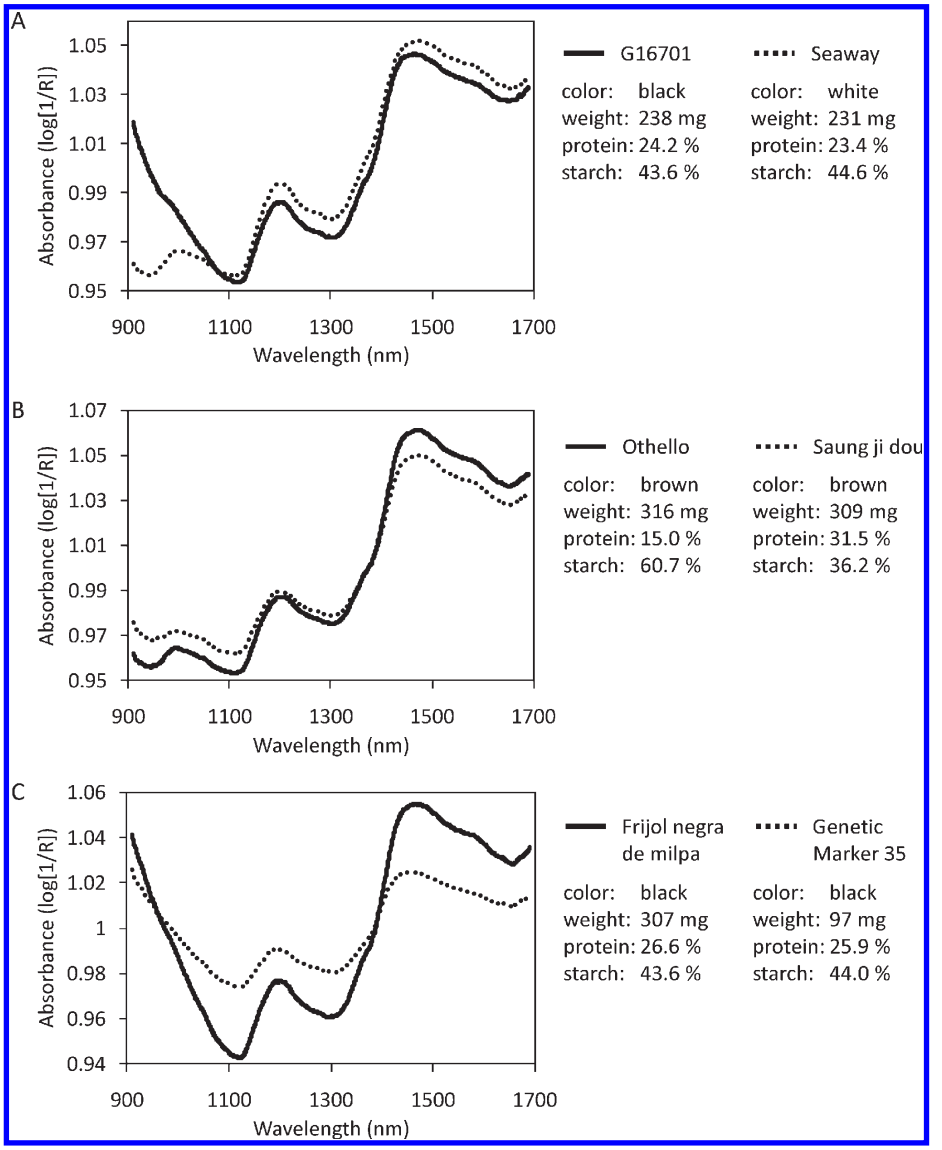

Figure 1. Examples of NIR spectra from single bean seeds. Each line represents the average of three NIR spectra from a single seed. The analytically determined values for seed weight, protein, and starch are given. (A) Comparison of two genotypes with similar weight and composition but different seed color. (B) Comparison of two genotypes with similar seed weight and color but different relative amounts of protein and starch. (C) Comparison of two genotypes with similar composition and seed color but different seed weights. The Othello and Frijol negra de milpa accessions are variegated for seed color. The seeds were scored according to the darker color sectors.

were recorded with a 40 ms integration time, and absorbance values were calculated as log(1/*R*). A custom Microsoft Visual Basic 6.0 program centered each spectrum to an arbitrary mean of 1. Three replicate seed weights and NIR spectra were collected for each seed, and the average of the data were used for partial least-squares (PLS) regression.

Protein and Starch Analysis. The individual bean seeds were transferred to 2 mL tubes with two steel beads (7.9 mm) and ground for 5 min with a MiniBeadBeater-96 (BioSpec Products, Bartlesville, OK). Total protein ($N \times 6.25$) was measured indirectly from total *N* concentration with a CN analyzer (Carlo Erba-NCS 2500, CE Instruments, Milan, Italy). Approximately 10 mg of dried bean flour (70 °C for 72 h) was used for each analysis. The total starch was determined by an enzymatic hydrolysis with thermostable α-amylase and amylogucosidase followed by a colorimetric determination of glucose with a glucose oxidase−peroxidase (GOP) system (*26*), as described by Spielbauer et al. (*25*).

Statistical Analysis. PLS regression was performed using JMP 8 (SAS Institute, Inc., Cary, NC). Prior to regression, six seeds were removed from the data set because of indexing errors within the weight and spectra replicates. These six seeds had either one weight replicate that was an obvious outlier based on the standard deviation of repeatability of the microbalance or an obvious outlier spectrum in the three replicates. PLS regressions were evaluated using leave-one-out cross-validation, and an optimal model was selected using default software settings. A subset of the data was partitioned for external validation. Multiple methods of partitioning the calibration and validation data sets were assessed including: (a) randomly assigning 30 bean accessions to the validation set, (b) randomly assigning 89 seeds to the validation set, and (c) sorting the analytical data and selecting every third sample for the validation set. The data were also partitioned on the basis of the black seed color, and separate PLS models were developed for black seeds and all other seeds with colors scored as brown, purple, red, green, and white. Variegated striped and pinto beans were scored according to the darkest color on the seed. In addition, spectral pretreatments were evaluated. First and second derivatives were calculated as given by Spielbauer et al. (*25*); multiplicative scatter correction (MSC) was calculated as described by Geladi et al. (*27*); and the standard normal variate (SNV) transformation was applied. Principal components analysis (PCA) of the spectra was completed with SAS 9.2 (SAS Institute, Inc., Cary, NC) using PROC PRINCOMP.

RESULTS AND DISCUSSION

Table 1 shows the range of seed composition for the bean accessions in this study. The calibration and external validation sets were partitioned according to bean accessions with 61 and 30 accessions within the calibration and validation sets, respectively. These data sets have comparable ranges, means, and standard deviations for all traits measured, indicating that the validation set is expected to report the quality of the PLS calibrations. The protein and starch levels showed a slightly larger range than the bean accessions used for a ground sample calibration (*12*). Thus, the bean accessions in this study are expected to provide a comparable range of composition to the NIR calibration with ground samples and to provide an accurate evaluation of the glass-tube NIR acquisition system.

Figure 1 shows examples of contrasting pairs of individual bean spectra. We noted that black bean varieties had a distinct region of absorbance between approximately 910 and 1100 nm that was not found in beans with other colors. **Figure 1A** shows this spectral difference between two beans that have approximately the same composition and seed weight. The high absorbance was found in most black beans and can be visualized with a scatter plot of principal component 1 versus 2 from a PCA of the NIR spectra (**Figure 2**). Black beans are rich in antioxidant phenolic compounds, especially anthocyanins (*28*, *29*). Potentially, anthocyanins or other derivative secondary metabolites cause the spectral difference. Maize kernels also produce anthocyanins that contribute to kernel color, but we have not noticed similar absorbance differences in the 910−1100 nm range based on maize kernel color (Settles, unpublished results).

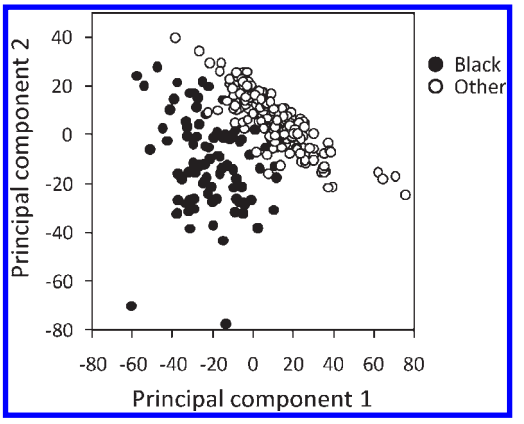

Figure 2. Scatter plot of principal components 1 and 2 of the average bean spectra. Filled circles are seeds with a black seed color, and open circles are beans of all other colors (white, brown, red, green, and purple).

Table 2. PLS Regression Statistics for Protein (%) Using Multiple Spectral Pretreatments

	calibration			validation		
data pretreatment	PLS factors	R^2	SEC	r^2	SEP	SD/SEP
none	12	0.84	1.5	0.82	1.6	2.4
MSC	11	0.83	1.6	0.81	1.7	2.3
SNV	11	0.83	1.5	0.80	1.8	2.2
1 Der	8	0.80	1.7	0.88	1.4	2.9
1 Der and MSC	8	0.81	1.6	0.82	1.7	2.3
1 Der and SNV	7	0.79	1.7	0.83	1.7	2.4
2 Der	7	0.78	1.8	0.82	1.7	2.3
2 Der and MSC	6	0.79	1.7	0.81	1.7	2.3
2 Der and SNV	5	0.77	1.8	0.82	1.7	2.4

The bean spectra also show overall differences when protein and starch composition or seed weight varies. For these comparisons, spectra were selected from beans with similar seed color and weight to illustrate the effects of seed composition (**Figure 1B**), while spectra were selected with similar seed color and composition to illustrate the effects of seed weight (**Figure 1C**). Prior work with maize kernels indicates that single-seed NIR spectra can be calibrated to both seed composition and individual weight (*19*, *25*). We evaluated PLS regressions for seed weight, percent protein, and percent starch.

Table 2 shows the PLS regression statistics for percent protein calibrations developed from the data sets given in **Table 1**. The regressions were assessed by comparing the coefficient of multiple determination (R^2) and the standard error of calibration (SEC) of the calibration data set to the r^2 and standard error of prediction (SEP) in the external validation set. A variety of spectral pretreatments were evaluated including first derivative (1 Der), second derivative (2 Der), MSC, and SNV as well as combinations. With the exception of the external validation set for the first derivative pretreatment, all of the calibrations in **Table 2** showed similar R^2 and r^2 values and similar SEC and SEP values, suggesting that the leave-one-out cross-validation fit the PLS regressions accurately. The PLS regression statistics for the external validation data using a first derivative pretreatment suggested that this model may be more accurate. However, the calibration statistics for the first derivative model were similar to all other models, and we interpret the external validation statistics to be anomalous and likely not to be a robust indicator of performance on future samples. Similar results were obtained for spectral pretreatments for seed weight and starch (not shown). We conclude from these results that spectral pretreatments in

Table 3. PLS Regression Statistics with No Spectral Pretreatments

	calibration			validation		
trait	PLS factors	R^2	SEC	r^2	SEP	SD/SEP
seed weight (mg)	10	0.85	34.5	0.74	41.2	1.9
protein (%)	12	0.84	1.5	0.82	1.6	2.4
starch (%)	9	0.51	4.4	0.56	4.9	1.5

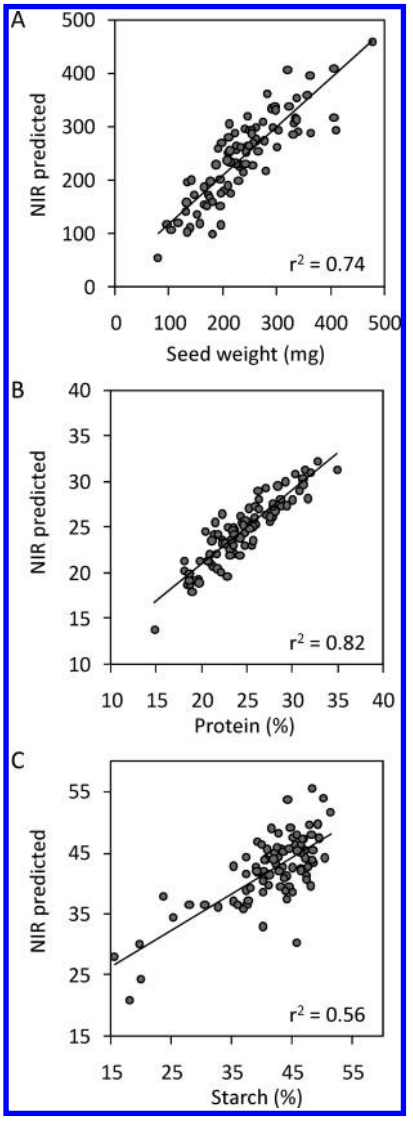

Figure 3. Scatter plots of the NIR-predicted and analytical reference values for (A) seed weight, (B) protein, and (C) starch. Each plot shows the values for the external validation set and a linear regression trend line. All NIR-predicted values used the PLS regressions given in **Table 2**.

addition to mean centering did not markedly improve the accuracy of the PLS regressions.

Table 3 reports the PLS regressions for seed weight, protein, and starch calculated from mean-centered spectra. Scatter plots of the analytical and NIR-predicted values for the external validation set indicate that the models have relatively little bias and a similar level of error across the full range of analytical values (**Figure 3**). The protein calibration gave the best statistics for both calibration and prediction. The standard deviation (SD)/SEP ratio for this regression suggests that this model can be used to predict protein levels (*30*). The seed weight and starch predictions had lower SD/SEP ratios, suggesting that these calibrations can be used to group seeds according to high and low values for the traits. To assess the repeatability of the NIR measurements, we predicted seed weight, protein, and starch for the single spectra from the three spectral replicates for each bean in the external validation set. The standard deviations of repeatability for the single spectra predictions were 44 mg for seed weight, 1.4% for protein, and 1.8% for starch. We then compared the SEC and SEP of these PLS models to the SEC and SEP reported for NIR predictions with ground bean samples (*12*). For both protein and starch, the individual seed predictions have approximately 3−4-fold greater error. These statistics suggest that much of the prediction error is due to the high-throughput nature of the single-seed NIR data collection. Alternatively, it is possible that the particular random assignment of 61 accessions to the calibration set and 30 accessions to the external validation set led to a suboptimal PLS model.

Table 4. Range of PLS Regression Prediction Error with Three Random Partitions of the Bean Data

	calibration (SEC)			validation (SEP)		
trait	Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
seed weight (mg)	35.4	29.4	36.2	38.9	49.5	32.1
protein (%)	1.5	1.4	1.5	1.8	1.8	1.4
starch (%)	4.4	4.4	4.4	4.5	4.1	4.2

Table 5. PLS Regression Prediction Error When the Bean Data Are Partitioned on the Basis of the Seed Color

	calibration (SEC)		validation (SEP)	
trait	black seeds (n = 71)	other colors (n = 116)	black seeds (n = 20)	other colors (n = 60)
seed weight (mg)	24.1	27.8	31.5	44.4
protein (%)	1.6	1.1	1.9	1.7
starch (%)	3.4	4.2	6.8	5.5

We evaluated the stability of the PLS regression using three additional methods for partitioning the bean data. First, we randomly assigned individual bean seeds instead of bean accessions to the calibration (178 seeds) and validation (89 seeds) sets. We completed three random assignments and calculated the SEC and SEP for these PLS regressions (**Table 4**). Random assignment of seeds resulted in regressions with a similar range of prediction error to the models given in **Table 3**. In addition, we ordered the bean analytical data and assigned every third value to the external validation set to ensure that the calibration and external validation sets had nearly identical ranges of analytical values. For these regressions, the SEC and SEP were also in the same range as given in **Tables 3** and **4** (SEC = 33.2 mg, 1.6%, and 4.2% and SEP = 34.2 mg, 1.9%, and 5.1% for weight, protein, and starch, respectively). These results suggest that the PLS models in **Table 3** and **Figure 3** are representative.

It is possible that the large differences between NIR spectra from black beans and other bean colors may have a confounding effect on PLS regression. We partitioned the bean data to test for effects because of seed color. **Table 5** shows SEC and SEP values for PLS regressions based on seed color. These models showed reduced SEC but similar SEP, suggesting that the 910−1100 nm absorbance within black bean seeds does not contribute to a confounding effect on the NIR predictions.

This study demonstrates that single-seed NIR spectra can be used to sort bean seeds according to individual weight and the major nutritional constituents of the seed. A generalized PLS regression can be developed for common beans, even though black beans have a distinct NIR absorbance from 900 to 1100 nm. Although the accuracy of single-seed NIR is not as good as with

fine-ground samples, there are significant advantages in avoiding grinding. Individual seeds require no sample preparation, and the spectra can be acquired at high throughput. The intact seeds are viable after NIR predictions, which would allow breeders to simultaneously enrich for improved composition along with acceptable seed size. It is important to note that common beans have a diverse range of protein and starch composition. Beans are commonly thought to be a "high-protein" food, but a significant number of bean varieties have similar protein and starch levels to maize. Incorporating a NIR step in bean breeding selections would enable more efficient crop improvement, particularly in maintaining high-protein levels while selecting for high yield and disease resistance.

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