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ScienceDirect
Animal Feed Science and Technology
142 (2008) 247–258
ANIMAL FEED SCIENCE AND TECHNOLOGY
www.elsevier.com/locate/anifeedsci

Relationships between kernel vitreousness and dry matter degradability for diverse corn germplasm I. Development of near-infrared reflectance spectroscopy calibrations

D. Ngonyamo-Majee[a,*], R.D. Shaver[a], J.G. Coors[b], D. Sapienza[c], C.E.S. Correa[d], J.G. Lauer[b], P. Berzaghi[e,f]

[a] *University of Wisconsin, Department of Dairy Science, 1675 Observatory Drive, Madison, WI 53706, United States*
[b] *University of Wisconsin, Department of Agronomy, 1575 Linden Drive, Madison, WI 53706, United States*
[c] *Sapienza Analytica, LLC, 5335 Merle Hay Road, Suite 3 PMB 112, Johnston, IA 50131, United States*
[d] *Universidade Federal de Lavras, Departamento de Zootecnia, Lavras 37200-000, Brazil*
[e] *United States Dairy Forage Research Center, 1925 Linden Drive West, Madison, WI 53706-1108, United States*
[f] *University of Podova, Dipartimento di Scienze Zootecniche, Agipolis - Viale Dell'Universita', 16, Legnaro, Italy*

Received 4 December 2006; received in revised form 13 August 2007; accepted 4 September 2007

Abstract

Negative correlations between corn vitreousness and ruminal dry matter and starch degradabilities have been widely reported. To measure corn vitreousness and density more rapidly, Correa et al. [Correa, C.E.S., Shaver, R.D., Pereira, M.N., Lauer, J.G., Kohn, K., 2002. Relationship between

Abbreviations: BL, black layer maturity stage; C/F ratio, height ratio of course to fine particles; CH, total column height; GEM, germplasm enhancement of maize; ML, half milk line maturity stage; MPLS, modified partial least squares; NIR, near-infrared reflectance; NIRS, near-infrared reflectance spectroscopy; T, time (s) to collect ground sample to a set receptacle height.

* Corresponding author. Present address: Monsanto Company, 3302 SE Convenience Boulevard, Ankeny, IA 50021, United States. Tel.: +1 608 217 6085; fax: +1 515 963 4242.
E-mail address: dianah.majee@monsanto.com (D. Ngonyamo-Majee).

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doi:10.1016/j.anifeedsci.2007.09.023

corn vitreousness and ruminal in-situ starch degradability. J. Dairy Sci. 85, 3008–3012] initiated the development of near-infrared reflectance spectroscopy (NIRS) calibrations from 47 samples derived from 14 US and five Brazilian commercial hybrids. In this study, we generated more data to add to these NIRS calibrations with the objective of making them more robust. We also evaluated the potential of using Stenvert hardness measurements for NIR calibrations. Thirty-three diverse corn germplasm sources were grown at University of Wisconsin West Madison Research Station. These included a wide range of endosperm characteristics from *opaque 2* (*o2*) types to densely packed flint types, and a number of intermediates. Harvest was at 1/2 milkline and black-layer maturity stages. Dried kernels from middle portions of ears from 12 selected inbreds, four each from low (0–30%), medium (30–70%), and high (70–100%) vitreousness classifications were used to determine vitreousness by manual dissection and density by water displacement using a pycnometer. Hardness was determined on all 33 inbreds on a 20 g sample using a Stenvert micro hammer-cutter mill with 2 mm screen size and 3600 rpm to measure time to collect ground sample to a set receptacle height (T); total column height (CH); and height ratio of coarse to fine (C/F) particles. The NIRS equations were selected on the basis of high R^2-values (0.90, 0.92, 0.85, and 0.85) and low SEC (4.85, 0.01, 1.39, and 0.19) and SECV (6.04, 0.02, 1.79, and 0.25), for vitroueness, density, T and CH factors, respectively. Calibrations for vitreousness and density were regarded as the best prediction models compared to stenvert hardness measurements as determined by their RPD values (3.73 and 2.50, respectively). These results show that NIRS can be used as a screening tool in large-scale breeding trials to develop corn hybrids of desired endosperm properties for improved ruminal degradabilities.
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Keywords: Corn starch; Density; Near-infrared reflectance spectroscopy; Vitreousness

1. Introduction

The impact of corn kernel vitreousness and related endosperm properties on ruminal starch degradability was reported by Correa et al. (2002) and Philippeau and Michalet-Doreau (1997). In both studies strong negative correlations between vitreousness and ruminal starch degradability were reported. Thus, it is important to find less laborious and more consistent procedures of assessing kernel vitreousness than the direct procedure of manual dissection for screening germplasm in large corn breeding programs.

There are other endosperm characteristics related to kernel vitreousness that may also need to be considered in evaluation programs. Bergquist and Thompson (1992) reported that hardness was the major factor determining grain density and density appears to be a good indirect measure of vitreousness (Philippeau et al., 1999; Correa et al., 2002). Kernel composition, structure, and density are known to affect hardness characteristics (Martin et al., 1987), while kernel density is in turn affected by the relative proportions of the major nutrient components (e.g. starch and its ratio of amylose to amylopectin, protein, and oil) and their packing within the kernel (Paulsen et al., 2003). Kernel hardness can be determined by grinding resistance test using a Stenvert hardness tester (Pomeranz et al., 1985). Density can be determined using a laboratory pycnometer method involving water (Correa et al., 2002). Near-infrared reflectance spectroscopy (NIRS) may provide a more rapid and non-destructive screening tool for corn germplasm of varying starch degradability in large-scale breeding trials. The NIRS procedure does not destroy samples or require the use of any

reagents that are costly and could potentially pollute the environment. Its predictions of nutritive value have been reported to have lower error values than data from chemical analyses (Adesogan, 2001). However, the NIRS procedure is heavily dependent on the reference laboratory method and sample set used in calibration development (Beever and Mould, 2000).

Preliminary NIRS calibration equations for kernel vitreousness and density were developed in our laboratory by Correa et al. (2002) using 47 samples from 14 US commercial corn hybrids harvested at three maturity stages and five Brazilian commercial corn hybrids. Although strong calibrations ($R^2 = 0.865$ for vitreousness, and 0.774 for density) were produced, they covered a limited range of hardness characteristics. In this study, our objectives were to (1) generate additional laboratory data to improve the robustness of the preliminary kernel vitreousness and density NIRS calibration equations, and (2) develop an NIRS calibration equation for kernel hardness measurements.

2. Materials and methods

This article is the first of two articles in a series from one study that evaluated the influence of corn vitreousness on dry matter degradability in 33 germplasm sources. This article discusses the development of NIRS calibrations as a more rapid and non-destructive procedure for estimating corn vitreousness, density, and other hardness measures. The companion article covers the evaluation of ruminal and post-ruminal dry matter degradabilities of the 33 germplasm materials and their correlations with endosperm characteristics (Ngonyamo-Majee et al., 2008).

2.1. Field production

Inbred corn lines were used in this study, because their endosperm properties can be directly traced to their genetic background. Thirty-three inbred lines were selected based on starch and endosperm characteristics that are likely to be related to starch granule packing and ruminal starch degradation. The range of endosperm characteristics extended from the *opaque 2* (*o2*) types with very loosely packed starch granules to densely packed flint types, and a number of intermediates. These included 17 lines from the germplasm enhancement of maize (GEM) project at Iowa State University; six flint lines, five from North Carolina State University (NCSU) and one *o2* modified line CML 176 from International Maize and Wheat Improvement Center (CIMMYT); six near-isogenic variants of Oh43 carrying *opaque-2* (*o2*), *floury-2* (*fl2*), *sugary-2* (*su2*), *amylose-extender-1* (*ae1*), *soft endosperm* (*h1*), and *waxy-1 sugary-2* (*wx1su2*) alleles that affect endosperm composition; an experimental breeding population from University of Wisconsin-Agronomy Department, developed for improved silage quality (WQS C2); and three check inbreds; B73 (Iowa Stiff Stalk Synthetic); Oh43 (Ohio State University), and W64A (University of Wisconsin). Germplasm selection for check lines carrying the allelic mutations (*o2*, *fl2*, *su2*, *wx1*, and *ae1*) was limited to the older material that had these mutant genes successfully introduced. Hence, the current popular inbreds from Lines of Holden germplasm background (e.g. LH 185 and LH198) were

not included, as they have not had any of these mutations introduced (Coors, personal communication).

The inbred lines were grown at the University of Wisconsin West Madison Research Station during the summer of 2002 in rows of 3.04 m × 0.76 m with 10 plants per row in a randomized complete block design with three replications. At flowering, each plant was self-pollinated by hand to preserve the endosperm characteristics specific to each inbred. Each inbred row was split into two for harvest at two maturity stages: one-half milk line (ML) and black layer (BL). After harvest by hand, ears were immediately frozen within 15 min in the field using liquid nitrogen and then stored in a freezer at −75 °C to maintain sample quality until shelling.

2.2. Laboratory procedures

Kernels from middle portions of ears (2–3 cm from the edges) were shelled when frozen, thawed, and oven dried at 40 °C to avoid disruption of kernel cell structure during processing (Philippeau and Michalet-Doreau, 1997). Grain density was determined by the water displacement method using a pycnometer with a removable cap that allows whole kernel inclusion (Correa et al., 2002). The cap on the pycnometer was adjusted to maintain a constant internal volume. A known weight pycnometer was filled with distilled water and weighed. Approximately 30 g of dried kernels were used, and density (g/cm^3) was calculated by dividing the weight of corn kernels by the volume of displaced water assuming a water density of 1.0. Hardness was determined on a 20 g sample using a Stenvert hardness grinder (Stenvert micro hammer-cutter mill model V from Glen Mills Inc. 395 Allwood Road, Clifton, New Jersey 07012) using a 2 mm screen size and 3600 rpm. The Stenvert hardness grinder measures the time taken to collect the ground sample to a set receptacle height (T); total column height (CH); and height ratio of coarse to fine (C/F) particles (Pomeranz et al., 1985).

Vitreousness was measured by a manual dissection method (Correa et al., 2002). From each sample, 100 kernels were randomly selected and divided into 10 visually homogeneous groups based on kernel size and shape. This was done to minimize the effect of kernel position within the ear on the vitreousness measurement. Vitreousness was then determined on one kernel randomly selected from each group. Kernels were soaked in distilled water for 3 min, dried with a paper towel, the pericarp and germ removed with a scalpel, and the total endosperm weighed. The floury endosperm was then manually removed using a scalpel, and the weight of the remaining vitreous endosperm was expressed as a percentage of the total endosperm.

2.3. NIRS calibrations

For vitreousness and density equations, 24 samples with three replicates were derived from a subset of 12 inbreds with two harvest stages (ML and BL) to provide laboratory data to add to the preliminary vitreousness and density NIRS equations developed by Correa et al. (2002). The selected samples provided data covering gaps in the two calibration equations: 0–40% and 80–100% vitreousness (Fig. 1) and <1.10 g/cm^3, 1.25–1.28 g/cm^3, >1.30 g/cm^3 density (Fig. 2). A few (eight) samples of intermediate vitreousness were also

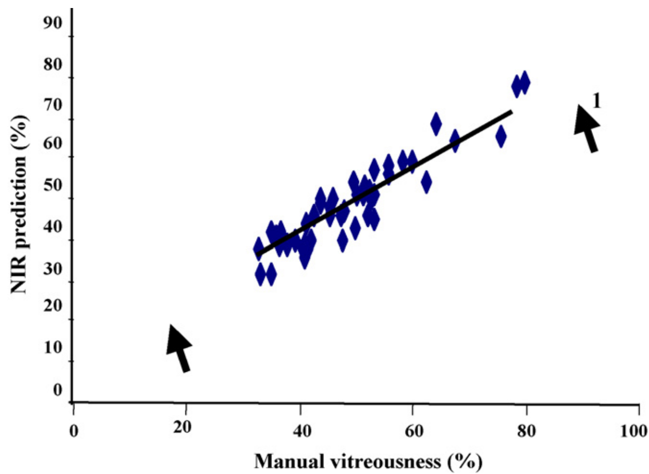

Fig. 1. Regression of near-infrared reflectance predicted vitreousness (NIRV) on vitreousness measured manually (LV) using data of Correa et al. (2002). NIRV = 0.858LV + 6.9048; R^2 = 0.865; P<0.0001 (1: arrows indicate the gaps in (Correa et al., 2002) calibration data).

included. Calibrations for Stenvert hardness parameters included all 33 inbreds with two harvest stages (ML and BL) giving a total of 66 samples with three replicates. Dried kernels were ground to pass a screen size of 1 mm using the Stenvert micro hammer-cutter mill. Approximately 2 g were used to fill a cuvette fitted with a quartz window before sealing with a paper stopper. These were scanned in duplicate using near-infrared reflectance (NIR) spectrophotometer (FOSS NIRSystems model 6500, Silver Springs, MD, USA)) with a spectral range between 400–2498 nm. Spectral data were collected at wavelength intervals of 2 nm and the data points obtained for each sample were stored in the computer as their

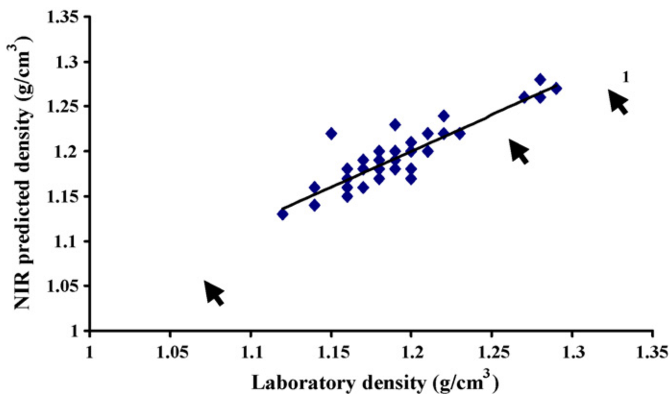

Fig. 2. Regression of near-infrared reflectance predicted density (NIRD) on laboratory measured density (LD) using data of Correa et al. (2002). NIRD = 0.8069LD + 0.232; R^2 = 0.774; P<0.0001 (1: arrows indicate the gaps in (Correa et al., 2002) calibration data).

absorbance, i.e. logarithm of the reciprocal reflectance values [$\log_{10}(1/R)$], where R is the percentage reflectance. Each spectrum was recorded as $\log_{10}(1/R)$ resulting from averaging 16 scans of the instrument internal reference and 32 scans of the sample. Spectra of duplicate samples were averaged before calibration development.

2.4. Data analysis

NIRS calibration equations were developed for vitreousness, density, and Stenvert hardness parameters (T and CH) using the Infrasoft International (ISI, Port Matilda, PA) NIRS version 3.0 software program "Calibrate" with the modified partial least squares (MPLS) regression option. The standard mathematical treatment of 1,4,4,1 (1st derivative, gap over which derivative was calculated, number of data points used in smoothing, and no 2nd smoothing) was used for all four equations with no scatter correction to allow any effects of particle size included in the calibration development process. Correlation analysis was done on laboratory data and NIRS derived spectral data across inbred means and 20% of samples for each equation were randomly selected for cross validation. Cross-validation is recommended when developing MPLS equations, in order to select the optimal number of factors and avoid overfitting (Shenk and Westerhaus, 1995). The equations were evaluated based on their standard error of calibration (SEC), standard error of cross validation (SECV), the residual predictive value determined as the ratio of the S.D. of the reference data to SECV (RPD), one minus the variance ratio calculated in cross validation ($1 - \mathrm{VR}$), coefficient of variability (CV) defined as $\mathrm{SEC} \times 100$ divided by mean of reference values, and the calibration coefficient of determination (R^2) achieved in modified partial least squares regression of spectra against laboratory derived data. Procedures from SAS (2001) were used for statistical analysis of laboratory data.

3. Results and discussion

3.1. Laboratory data

Summary statistics of laboratory data from vitreousness, density, Stenvert T and CH measurements are shown in Table 1. Laboratory vitreousness data had greater variation compared to the other measurements. The manual dissection of the floury portion of endosperm from the horny portion was difficult for some germplasm that had no clear separation of floury and horny endosperm such as *h1* (Oh43), B73 and FS8B(T):N1802-35-1-B, particularly for the immature kernels (ML). This might have caused the higher variability for our manual vitreousness data. Stenvert hardness measurements appeared to have a better correlation with NIR vitreousness than NIR density. Although both corn hardness and density are a function of kernel structural integrity, Simmonds et al. (1973) found hardness in wheat grain endosperm to be largely determined by the adhesive strength between starch granules and the surrounding protein matrix, which has also been recently found to determine corn vitreousness (Zhang et al., 2003). This may explain the better correlation that we found between Stenvert hardness parameters and vitreousness compared with density.

Table 1
The performance of near-infrared reflectance (NIR) calibration models and their correlations with laboratory data

	Constituents	*N*	Mean	SEC	R^2	SECV	1 − VR	RPD
NIR data	Vitreousness[a] (%)	65	49.6	4.85	0.90	6.04	0.84	3.73
	Density[a] (g/cm^3)	69	1.2	0.01	0.92	0.02	0.81	2.50
	Stenvert time (s)	63	20.1	1.39	0.85	1.79	0.76	2.16
	Stenvert total column height (cm)	63	7.3	0.19	0.85	0.25	0.79	2.08

	Constituents	*N*	Mean	Range	S.D.	*r*-Values	
						NIRV (%)	NIRD (g/cm^3)
Laboratory data	Vitreousness (%) from Correa et al. (2002) and new data	71	45.5	0–80.1	22.5	0.95	0.77
	Vitreousness (%) from Correa et al. (2002) and new data	47	48.6	32.8–79.9	11.4	0.93	0.91
	Density (g/cm^3) from Correa et al. (2002) and new data	71	1.187	1.040–1.290	0.050	0.72	0.92
	Density (g/cm^3) from Correa et al. (2002)	47	1.193	1.120–1.290	0.040	0.85	0.88
	Stenvert time (s)	66	20.2	9.5–31.5	3.9	0.61	0.34
	Stenvert total column height (cm)	66	7.27	6.3–8.7	0.52	−0.66	−0.33

N = data points or samples used to develop equation (a few outlier samples (4 max) were dropped by calibration process); SEC = standard error of calibration calculated in modified partial least squares regression; R^2 = the calibration coefficient of determination achieved in modified partial least squares regression of spectra against laboratory derived data; SECV = standard error of cross validation in modified partial least squares regression; 1 − VR = 1 minus the variance ratio calculated in cross validation; RPD = residual predictive value (i.e. S.D./SECV). This determines the strength of the calibration, with values >2.5 acceptable; NIRV = near-infrared predicted vitreousness (%); NIRD = near-infrared predicted density; *r*-Values = correlation coefficients of the constituents all significant at (P<0.0001).

[a] Equations are from combined data from Correa et al. (2002) and 24 new data.

3.2. Absorbance spectra for vitreousness and density calibration samples

3.2.1. The visible range (400–700 nm)

The visible range contains color or pigmentation information (Dowell, 2000). We observed more spectral variation between the samples at 400–550 nm range. For yellow corn, the vitreous (horny) portion shows yellow pigmentation while the floury portion is white. The concentration of the yellow pigmentation that shows absorbance in the blue color wavelength corresponds to the 400–500 nm range. The separation of spectra observed in this region could be associated with vitreousness for germplasm carrying the yellow pigmentation genotype (YYY, YYy or Yyy endosperm color genotype) (Weber, 1987), with the vitreous and more dense germplasm (i.e. AR16035:S02-447-1-B, W64A and CUBA164:2008a-23-1-B) having higher ($P<0.05$) absorbance. The carotenoid pigments, carotenes, and xanthophylls give the yellow color of corn grain with up to 86% of the kernel carotenoid found in the horny endosperm and the balance distributed in the floury endosperm (9–23%), germ (2–4%), and bran (1%) (Weber, 1987).

Inbreds B73, *h1* (Oh43), *o2* (Oh43) and *fl2* (Oh43) had lower absorbance in the visual region and this is in agreement with their zero to low vitreousness and low density classifications. Since density is correlated with vitreousness, the ranking based on density was as expected. The variation observed in this region could be a result of the mixture of germplasm from different backgrounds, ranging from white endosperm to deep yellow pigmentation in this study. Most of the GEM material and NCSU flints had variations in the depth of the yellow pigmentation.

These results agree with the wheat grain study findings of Wang et al. (2002) in that the visible region of the reflectance spectra represented mostly the surface and physical properties of the samples, while the reflectance spectrum in the near-infrared region represented both surface and intrinsic properties like chemical composition.

3.2.2. The near-infrared region (700–2500 nm)

The absorption of radiation by O–H, C–H, N–H and C=O bonds of organic molecules of starch and protein occur in the near-infrared region. We observed seven peaks in this region, which may be related to protein differences at ≈950 nm (Delwiche, 1993; Delwiche and Massie, 1996), the C–H second overtone at ≈1 200 nm, protein, moisture and the C–H (starch) first overtone at ≈1400–1600 nm (Wang et al., 2002), moisture at ≈1900 nm, and protein and combination bands at ≈1950–2250 nm. Since vitreousness and density are correlated with protein content (Dowell, 2000), the pattern was similar to that observed on visible range, except for whitish or pale yellow corn germplasm which would be incorrectly classified in the visible range with low absorbance even when they might have largely horny endosperm. The enriched protein content in vitreous endosperm is associated with high contents of a specific group of prolamin storage proteins (i.e. the g-zeins) (Chandrashekar and Mazhar, 1999).

3.3. Absorbance spectra for hardness calibration samples

The differences in endosperm packing structure, as determined by density and vitreousness, were further confirmed with hardness determined by grinding characteristics.

Although samples for NIRS were ground to pass a 1-mm screen, the particles from the vitreous portion of the endosperm yield coarse and translucent particles compared to the fine and opaque particles from the floury portion. This was reported by Pasikatan et al. (2002), working with wheat grain, to be caused by the breakage properties which are influenced by the endosperm starch–protein matrix with absorbing bands for starch and protein occurring in the NIR region (1100–1700 nm) or the entire visible-NIR region (500–1700 nm). The particle size and chemical compositional differences combined to yield spectral differences for hardness. Absorbance increased at all wavelengths from 400–2498 nm with increasing kernel hardness.

The near-infrared region (700–2498 nm) varied little initially, but the 1450–2498 nm region showed clear variation between the spectra of the different samples. This variation could be related to particle size differences. NIRS has long been reported to be sensitive to particle size of ground or granular material (Wendlandt and Hecht, 1966; Wetzel, 1983; Pasikatan et al., 2001). Devaux et al. (1995) reported that particle size effects were expressed in the entire spectrum, while Pasikatan et al. (2001) found particle size effects to be more pronounced in the longer wavelength portion of the near-infrared region. Fine particles, which are more prevalent with the floury endosperm, will have low absorbance as more light is reflected, while coarse particles will have higher absorbance which vertically shifts the absorbance spectra. Corn hardness is determined by the strength of bonding between the cell contents, principally between the starch granules and matrix proteins, but probably also between the matrix proteins and cell walls (Zhang et al., 2003). In other studies (Wallace et al., 1990; Geetha et al., 1991; Lopes and Larkins, 1991) g-zeins were found to increase in concentration when soft *o2* mutant lines were converted to normal (i.e. hard, translucent) texture through the activity of genetic modifiers. These findings confirm the importance of protein content and type on corn hardness, which influenced spectral structure in this study.

3.4. NIRS calibrations

Table 1 shows data on calibration statistics. The equations shown were selected on the basis of high R^2-values (0.90, 0.92, 0.85, and 0.85) and low SEC (4.85, 0.01, 1.39, and 0.19) and SECV (6.04, 0.02, 1.79, and 0.25) for vitreousness, density, and Stenvert hardness T and CH factors, respectively. Calibrations for vitreousness and density were regarded as the best prediction models compared to Stenvert hardness measurements as determined by their RPD values. The RPD statistic should be as high as possible, preferably >2.5 (Hsu et al., 1998) in order to determine whether a calibration equation is acceptable for accurately predicting a constituent. Validations done on these equations with a random selection of 20% of samples on the calibration equations gave overall values of global H (GH) and neighborhood H (NH) within the recommended limits of 3.0 and 0.6, respectively. The GH value has practical significance in that it shows when a predicted value for a given sample is an outlier when GH >3.0. The NH value <0.6 indicates that the prediction sample has very close neighbors from the sample population used for calibration development. This means the calibration is robust enough to cover germplasm of diverse background. We found a slightly stronger correlation ($P<0.0001$) between laboratory and NIRS predicted vitreousness (Fig. 3) than between laboratory and NIRS predicted density ($P<0.0001$) (Fig. 4). The spectral data accounted for 90% and 85% of the variability in the laboratory reference data for vitreousness and density,

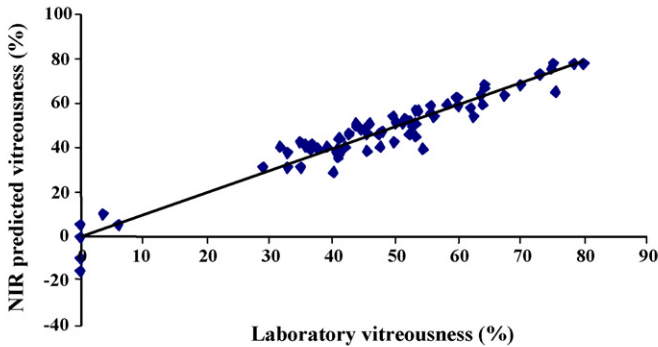

Fig. 3. Regression of near-infrared reflectance predicted vitreousness (NIRV) on laboratory vitreousness (LV) using data of Correa et al. (2002) and 24 new samples combined. NIRV = 1.002LV − 0.9635; R^2 = 0.903; P<0.0001.

respectively. The regression models relating the NIRS predictions to laboratory reference values were not forced to pass the origin, hence negative vitreousness rating was predicted for the non-vitreous corn germplasm like *o2* (Oh43). This may be corrected by rerunning the model and forcing the regressions through the origin.

Both measurements showed a small improvement in the correlations between laboratory and NIRS predicted values from Correa et al. (2002) calibrations (Figs. 1 and 2), and the added benefit of increased diversity with the inclusion of new genetic material. However, a data gap still exists between 10 and 30% vitreousness, which could further improve our calibration. Buxton and Mertens (1991) suggested caution in use of NIRS for detecting differences between samples, since significant bias can exist in NIRS data. We do agree with this observation, and therefore recommend the use of these NIRS calibrations as screening

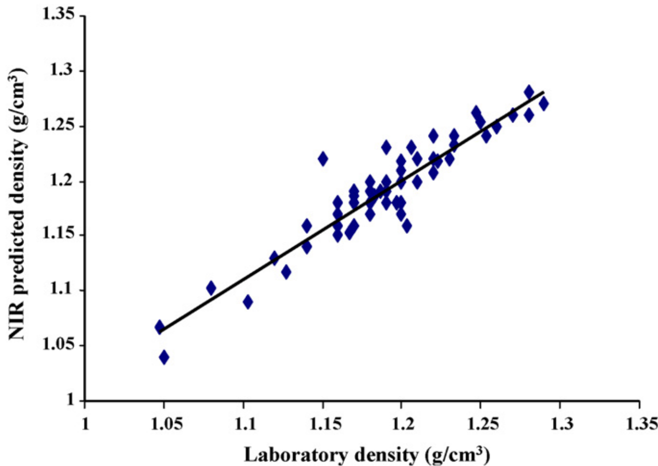

Fig. 4. Regression of near-infrared reflectance predicted density (NIRD) on laboratory measured density (LD) using data of Correa et al. (2002) and 24 new samples combined. NIRD = 0.8994LD + 0.1211; R^2 = 0.846; P<0.0001.

tools for corn vitreousness, density, and hardness in breeding programs where the selected genotypes could then be verified by conventional analysis.

4. Conclusions

These results demonstrate potential of NIRS to screen corn germplasm of the same color background for vitreousness. The basis of this screening technique appears to result from differences in light scattering from effects of endosperm color, protein and starch concentrations, particle size distribution, and density and hardness parameters on the absorption spectra. The visible region will be more effectively calibrated using samples originating from the same race or genetic background, particularly, of the same color pigmentation genotype. Since the near-infrared region was capable of discerning particle size (hardness and density) and compositional differences, it may be effectively used to screen corn germplasm irrespective of color effects. Overall, use of the full 400–2500 nm spectra reduces the influence of kernel color on NIRS prediction ability. Also, as more samples are continuously added to the calibration, the color effects will be further reduced. Hence, these results show potential for using NIRS as a screening tool for corn vitreousness, density, and hardness in large-scale corn breeding programs.

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