

# Whole Grain Amylose Analysis in Maize Using Near-Infrared Transmittance Spectroscopy<sup>1</sup>

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## ABSTRACT

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The development of genetically modified starches has relied on the use of maize (*Zea mays* L.) endosperm mutant alleles that alter starch structural and physical properties. A rapid method for predicting amylose content would benefit breeders and commercial handlers of specialty starch corn. For this reason, a study was conducted to investigate the use of near-infrared transmittance spectroscopy (NITS) as a rapid and nondestructive technique for predicting grain amylose content (GAC) in maize. Many single- and double-mutant inbreds and hybrids were used to create a calibration set for the development of a predictive model using partial least squares analysis. A validation set composed of similar genetic mate-

rial was used to test the prediction model. A coefficient of correlation ( $r$ ) of 0.94 was observed between GAC values determined colorimetrically and those predicted by NITS; however, the predicted values were associated with a large standard error of prediction (SEP = 3.5). Overall, NITS discriminated well among high amylose and waxy genotypes. The NITS calibration was used to determine levels of contamination by normal kernels in waxy and high-amylose (Amy VII) grain samples intended for wet milling. In both cases, a 5% contaminated sample could be detected from pure samples according to predicted NITS values.

Starch from maize (*Zea mays* L.) endosperm mutant kernels varies widely with respect to structural and functional properties. Specialty starch hybrids containing endosperm mutant alleles such as waxy (*wx*), amylose-extender (*ae*) and, more recently, double-mutant combinations, are of importance to the wet-milling industry (Katz 1991). Additionally, use of waxy maize hybrids as an animal feed has been associated with small but consistent improvements in animal productivity (Watson 1988).

A rapid method to confirm the levels of amylose in maize kernels could be beneficial in plant breeding and commercial trade situations. Backcross conversion of inbred lines to mutant endosperm types usually requires visual identification of phenotypes that are not always expressed strongly in certain genetic backgrounds (Haunold and Lindsey 1964, Campbell et al 1995). Analysis of amylose in breeding material could serve to confirm the presence or absence of a particular mutant allele. Evans et al (1994) states that quality assurance in the shipment of identity-preserved crops will likely assume a more prominent role in agriculture. Clearly, the need for ensuring identity preservation at elevators and processing facilities will be necessary as specialty starch hybrids are increasingly produced.

Near-infrared reflectance spectroscopy (NIRS) has been used to identify differences in amylose content of several crop species including rice (Iwamoto 1987), pea (Letzelter and Wilson 1995), barley (Czuchajowska et al 1992), and maize (Poneleit et al 1994). Villareal et al (1994) examined the use of near-infrared transmittance spectroscopy (NITS) as a nonintrusive means of predicting amylose levels in unground brown rice and found a correlation coefficient ( $r$ ) of 0.98 between actual and NITS predicted amylose values. These results indicated that NITS would be beneficial for breeders interested in screening for amylose in rice germ plasm.

Our objectives for this study were to develop a calibration for the prediction of grain amylose content (GAC) in unground maize

kernels using a set of mutant lines and hybrids varying widely in starch amylose content. The utility of such a calibration for monitoring the purity of specialty starch maize lots prior to milling was also examined.

## MATERIALS AND METHODS

### Genetic Material

Grain samples were collected from a breeding nursery near West Lafayette, IN, and a winter nursery in Puerto Vallarta, Mexico, between 1993 and 1995. All samples were dried at 100°F for ~10 days. Endosperm mutant alleles were used singly or in double-mutant combinations to obtain 15 genotypic classes ranging widely in GAC for the NITS calibration procedure. Grain samples used in this study were collected from many near-isogenic inbred lines originating from 14 publicly available normal yellow dent corn-belt inbred lines from a backcross conversion program at Purdue University. Thirty hybrids derived from the inbreds were also included (Table I). Single- and double-mutant genotypic

**TABLE I**  
Genotypic Classes of Converted Public Inbreds and Hybrids Used to Develop Calibration and Validation Sets for Partial Least Squares (PLS) Modeling and Average Grain Amylose Content (GAC) Values Determined from Ground Samples

| Alleles <sup>a</sup> | Inbreds | Hybrids | Total | Avg. GAC |
|----------------------|---------|---------|-------|----------|
| <i>du wx</i>         | 4       | 7       | 11    | -4.8     |
| <i>wx</i>            | 8       | ...     | 8     | -0.4     |
| <i>su1</i>           | 4       | ...     | 4     | -0.4     |
| <i>wx S5</i>         | 1       | ...     | 1     | 1.0      |
| <i>ae wx</i>         | 4       | ...     | 4     | 3.8      |
| <i>sh1</i>           | 12      | ...     | 12    | 9.9      |
| <i>Wx wx wx</i>      | 1       | ...     | 1     | 10.8     |
| +                    | 8       | 7       | 15    | 13.4     |
| <i>du</i>            | 8       | ...     | 8     | 14.4     |
| <i>su2</i>           | 8       | ...     | 8     | 16.3     |
| <i>ae du</i>         | 8       | 7       | 15    | 21.4     |
| <i>ae su1</i>        | 4       | ...     | 4     | 21.5     |
| <i>du su2</i>        | 8       | 7       | 15    | 22.4     |
| <i>ae</i>            | 8       | 7       | 15    | 25.6     |
| <i>ae su2</i>        | 8       | 7       | 15    | 26.8     |
| Total                | 94      | 42      | 136   | ...      |

<sup>a</sup> Dull (*du*), waxy (*wx*), waxy S-5 (*wx S5*), dosage intermediate with two doses of *wx* (*Wx wx wx*), sugary-1 (*su1*), amylose-extender (*ae*), shrunken-1 (*sh1*), sugary-2 (*su2*) and normal (+).

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classes of inbreds and hybrids were developed using the following alleles: dull (*du*), waxy (*wx*), waxy S-5 (*wx* S-5), sugary-1 (*su1*), amylose-extender (*ae*), shrunken-1 (*sh1*), and sugary-2 (*su2*). GAC was determined on an as-is moisture basis for samples stored in ordinary laboratory conditions (temperature 20–25°C, ~40% rh).

### NITS Spectroscopy

An Infracore 1225 NITS grain analyzer (TecatorAB, Hoganas, Sweden) equipped with a sample transport module was used to collect spectra from unground maize kernels. A sample cell with a pathlength of 30 mm was used for scanning spectra composed of 100 data points across a wavelength range of 850–1,048 nm. Spectra used for partial least squares (PLS) modeling were averaged from 15 scanned subsamples. Between 15 and 100 g of unground grain was scanned for each genotype. When grain samples were limited so that 15 independent subsamples could not be achieved, kernels were repacked in the cuvettes and rescanned until the desired number of subsamples were collected.

To evaluate the final calibration for use in detecting contamination in grain lots of specialty starch maize, bulk samples of normal, waxy, and high amylose (Amy VII) grain were retrieved from a wet mill before processing. Samples (50 g) of normal-waxy and normal-Amy VII mixtures were prepared to achieve 0, 5, 10, 20,

40, 60, 80, and 100% contamination of waxy and Amy VII grain samples by weight. Scans of the various contaminated lots were the average of 20 subsamples. Waxy, Amy VII, and normal samples were provided by Cerestar, USA, Hammond, IN.

### Colorimetric Amylose Analysis

At least 10 kernels were milled using a UDI cyclone mill to pass a 1-mm mesh screen. GAC of ground maize kernels was determined in triplicate using a modified method described by Williams et al (1958). The standard curve used in the calculation of GAC was based on starch samples varying in amylose content. A standard error of 1.4% was observed for the replicated GAC values in this study.

### Model Development

Samples were placed into calibration or validation sets by sorting samples according to GAC. Every third sample was included in the validation set, while all remaining samples made up the calibration set. Distribution of GAC values for genotypes used in the calibration and validation sets are shown in Fig. 1. PLS analysis was performed using calibration set spectra with the software package Grams/386, Galactic Industries, Salem, NH. Delwiche et al (1995) described use of this software in developing calibrations for predicting amylose and protein content from milled rice by NIRS. PLS-1 was used as a spectral decomposition technique to reduce the data to a number of factors. This is based upon using chemical component concentration information (GAC in this case) to cause sample spectra containing higher concentrations to be weighted more heavily than those with low concentrations. The

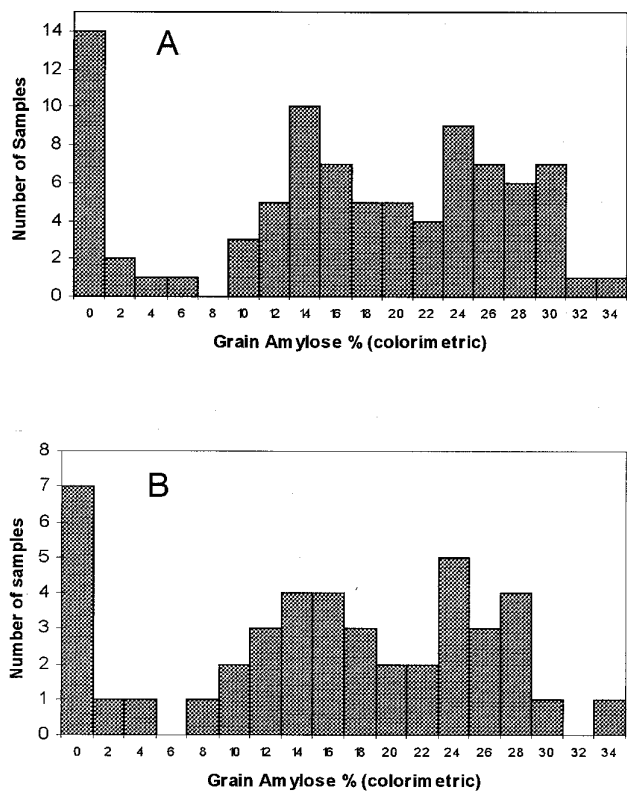


Fig. 1. Distribution of grain amylose content values for maize genotypes included in the near-infrared transmittance spectroscopy calibration (A) and validation (B) sets.

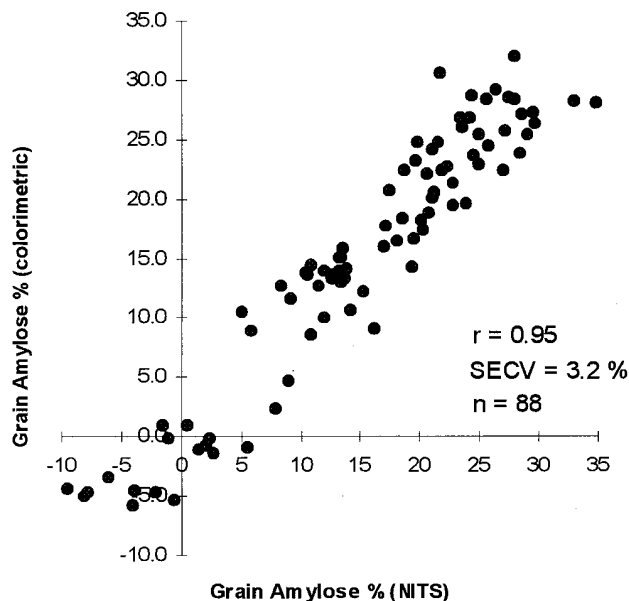


Fig. 2. Comparison of grain amylose content values determined colorimetrically and predicted by near-infrared transmittance spectroscopy (NITS) for maize genotypes included in the calibration set. SECV = standard error of cross-validation.

TABLE II  
Performance Statistics for Calibration Models and Validation Sets for Prediction of Grain Amylose Content in Unground Maize Kernels

| Samples Removed from Calibration Set |                   | Calibration Set |          |          |                   | Validation Set |          |                  |                  |      |
|--------------------------------------|-------------------|-----------------|----------|----------|-------------------|----------------|----------|------------------|------------------|------|
| Concentration Outliers               | Spectral Outliers | Factors         | <i>n</i> | <i>r</i> | SECV <sup>a</sup> | <i>n</i>       | <i>r</i> | SEP <sup>b</sup> | RPD <sup>c</sup> | Bias |
| 0                                    | 0                 | 18              | 92       | 0.92     | 4.1               |                |          |                  |                  |      |
| 3                                    | 1                 | 18              | 88       | 0.95     | 3.2               | 44             | 0.95     | 3.5              | 3.0              | 0.7  |

<sup>a</sup> Standard error of cross-validation.

<sup>b</sup> Standard error of precision (SEP).

<sup>c</sup> Ratio of the SEP to the standard deviation of actual grain amylose content values.

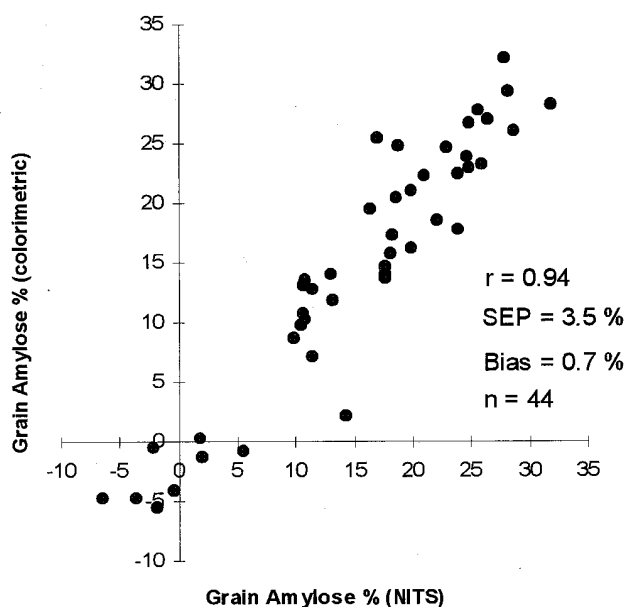
number of factors selected to optimize the model is accomplished by calculating the PRESS (Prediction Residual Error Sum of Squares) for all factors through a cross-validation procedure. Upon completion of the PLS analysis, a diagnostic function of the programs determined whether calibration samples were either concentration or spectral outliers. Spectral outliers may be the result of a problem due to measurement errors by the instrument or may be due to unique impurities in the sample not found in other samples. A concentration outlier occurs when the known value differs from the predicted by at least  $3 \times \text{SECV}$  (standard error of cross-validation). Outliers were subsequently removed from the calibration set to optimize the model.

The calibration model and the validation set to which the model was applied for predicting GAC was evaluated using several statistics described by Williams (1987). These include the coefficient of correlation ( $r$ ) between actual and predicted GAC values, the standard error of cross-validation from the calibration set (SECV), and standard error of prediction from the validation set (SEP), the bias or mean difference between actual and predicted GAC values and the ratio of the SEP to the standard deviation of actual GAC values (RPD).

### RESULTS AND DISCUSSION

GAC values averaged from each of the 15 genotypic classes are shown in Table I. Negative GAC values were observed for *wx*, *du wx*, and *su1* genotypes with *du wx* being, on average, the lowest. The negative values reported were not converted to zero since any difference between *wx* and *du wx* classes might be accounted for in the final calibration. Evidence for structural differences between *wx* and *du wx* starches has been demonstrated by Yuan et al (1993) from high-performance size-exclusion chromatography studies.

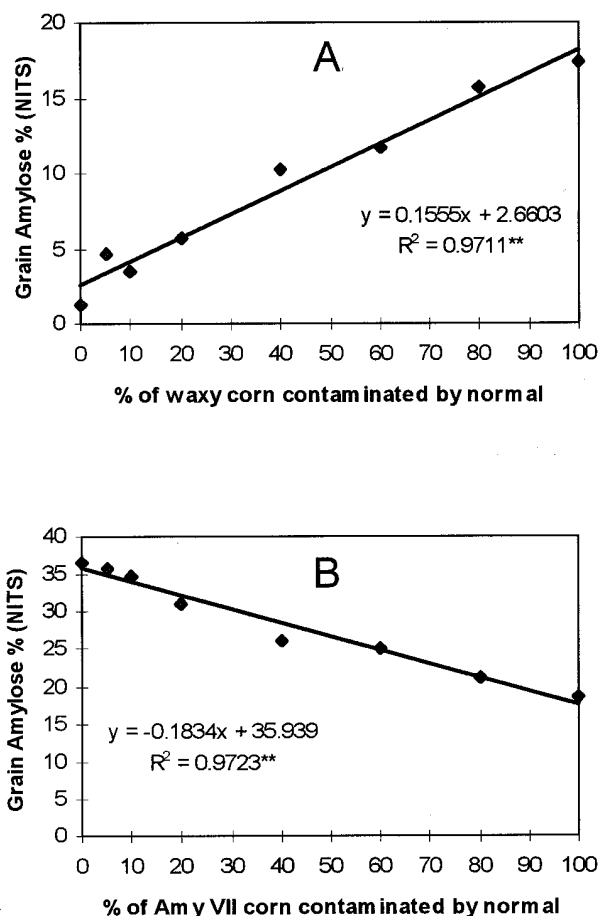
Distribution of grain GAC values for samples used to create the calibration and validation sets are shown in Fig. 1. A fairly good distribution of GAC contents was achieved for both calibration and validation sets, with the exception of the 1–10% region. The genotypes *wx*-S5, *ae wx*, and *Wx wx wx* were included in the study because their starch amylose contents are intermediate to waxy and normal maize and, therefore, served to increase representation in this region.



**Fig. 3.** Comparison of grain amylose content values determined colorimetrically and predicted by near-infrared transmittance spectroscopy (NITS) for maize genotypes included in the validation set. SEP = standard error of prediction.

Performance statistics for the calibration sets are shown in Table II. An initial PLS analysis of the entire calibration set ( $n = 92$ ) revealed a coefficient of correlation of  $r = 0.92$  and SECV of 4.1% using 18 factors in the analysis. Diagnostic functions in the software indicated that three of the samples were concentration outliers and one was a spectral outlier. The four outliers did not fall under any single genotypic class, but rather they were represented by several different classes including *su1*, *su2*, *ae su2*, and *sh1*. The spectral outlier, a *su1* inbred, was unique compared to other samples in that it had a relatively large presence of kernel rot.

Repeating the PLS analysis using the calibration set without the outliers ( $n = 88$ ) resulted in an improvement of the coefficient of correlation ( $r = 0.95$ ) and a lower SECV (3.2%). The plot of GAC values determined colorimetrically and predicted by NITS for the improved calibration model is shown in Fig. 2. The improved calibration model was then used to predict GAC for validation samples. The performance statistics are given in Table II. The coefficient of correlation was reduced slightly ( $r = 0.94$ ), while the SEP (3.5%) worsened when compared to the SECV (3.2%). According to Williams (1987), a good calibration should have an RPD of a least 10. In our case (RPD = 3.0), the calibration appears to be limited with respect to overall precision. The plot of actual vs. predicted values, however, indicates that the predicted values clearly discriminated among the high amylose and waxy starch types (Fig. 3). In addition, it is interesting to note that the difference in actual GAC between the *wx* and *du wx* genotypes is clearly reflected in the predicted values. From this study, the coefficient of correlation between NITS predicted and actual GAC



**Fig. 4.** Near-infrared transmittance spectroscopy (NITS) predicted grain amylose content values for waxy (A) and high-amylose (Amy VII) (B) samples at various levels of contamination (0, 5, 10, 20, 40, 60, 80, and 100%) by normal.

## LITERATURE CITED

values ( $r = 0.94$ ) was only slightly lower than that observed for rice ( $r = 0.96$ ) according to Villareal et al (1994). The SEP value observed in our study with maize (SEP = 3.5%), however, was substantially larger than that observed for rice (SEP = 1.06%). Several factors may confound the collection of accurate GAC values determined colorimetrically from ground grain samples which may adversely affect NITS prediction models. For example, the presence of lipids in the milled grain may interfere with the iodine affinity method for determining amylose. Also, colorimetric GAC determination methods may not accurately reflect differences in the fine structure of amylose and amylopectin. In addition, the single- and double-mutant genotypes used in this study vary dramatically in kernel phenotype, as documented by Garwood and Creech (1972). Variability in size, degree of collapse, and color may, in part, account for the relatively larger prediction errors. Although limited in precision, the NITS prediction models may provide maize breeders with an analytical method for monitoring the presence of mutant genotypes in breeding stock. Such a tool may be beneficial when identification of mutant kernels based on phenotype is difficult.

The final calibration was then used to determine whether NITS could detect contamination in specialty maize grain lots. It was observed that NITS was a good predictor of waxy and Amy VII grain (Fig. 4) when contaminated with normal kernels, according to the large coefficients of determination observed ( $R^2 > 0.97$ ) in both cases. These results demonstrate that the NITS calibration model based on mutant inbreds and hybrids can be used to identify composites of normal and mutant kernel types and could be a useful quality control tool for monitoring the purity of specialty starch hybrids. For *wx* maize, deviations from a predicted GAC value of zero would be a clear indication of contamination. In the case of Amy VII maize, however, variations in GAC due to environmental or genetic background effects must be considered. For this reason, a more comprehensive survey of commercially processed Amy VII sources will be required to establish an acceptable minimum value for Amy VII grain.

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