JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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# Starch Waxiness in Hexaploid Wheat (*Triticum aestivum L.*) by NIR Reflectance Spectroscopy

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**ABSTRACT:** Wheat (*Triticum aestivum* L.) breeding programs are currently developing varieties that are free of amylose (waxy wheat), as well as genetically intermediate (partial waxy) types. Successful introduction of waxy wheat varieties into commerce is predicated on a rapid methodology at the commodity point of sale that can test for the waxy condition. Near-infrared (NIR) reflectance spectroscopy, one such technology, was applied to a diverse set of hard winter (hexaploid) wheat breeders' lines representing all eight genotypic combinations of alleles at the *wx-A1*, *wx-B1*, and *wx-D1* loci. These loci encode granule-bound starch synthase, the enzyme responsible for amylose synthesis. Linear discriminant analysis of principal components scores 1−4 was successful in identifying the fully waxy samples at typically greater than 90% accuracy; however, accuracy was reduced for partial and wild-type genotypes. It is suggested that the spectral sensitivity to waxiness is due to (1) the lipid−amylose complex which diminishes with waxiness, (2) physical differences in endosperm that affect light scatter, or (3) changes in starch crystallinity.

**KEYWORDS:** Wheat, amylose, near-Infrared, breeding, classification, waxy starch

## INTRODUCTION

Cereal starch consists of two macromolecules, amylose and amylopectin. While each is composed of α-linked D-glucopyranosyl units, amylose is essentially a straight chain molecule by nature of the (1→4) linkage of these units. Amylopectin, on the other hand, is a branched molecule consisting of the same linear chains but also with branch points at (1→6) linkage sites.[1] The relative proportion of amylose and amylopectin, typically about 1:3 in wheat (*Triticum aestivum* L.), accounts for much of the cooking (pasting) properties associated with wheat. The synthesis of amylose, which occurs in the amyloplasts, is catalyzed by granule-bound starch synthase, or GBSS,[2] also called the 'waxy' protein. In the evolutionary development of hexaploid wheat, each of the three genomes (A, B, and D) possessed the gene responsible for encoding the production of GBSS. This became altered during evolutionary development by a reciprocal translocation of a section of chromosome 7B that contained the waxy gene loci to chromosome 4A. The fully waxy condition is realized when a nonfunctioning (null) allele occurs at each of the three chromosomal loci (*wx-A1*, *wx-B1*, and *wx-D1*). Likewise, an intermediate condition, known as 'partial waxy', occurs when one or two null alleles are present. The partial waxy condition results in a level of amylose intermediate between that observed in the fully waxy condition and the wild-type condition.[3,4] Since the development of waxy wheat genotypes in Japan in the mid-1990s,[2] breeding programs have been underway in the United States[5] and elsewhere in an effort to utilize the special properties of amylose-free or reduced amylose starches for processing and baking.[6] Applications include being a substitute for waxy maize in modified starch production,[7] a shelf life extender by nature of an ability to retain greater moisture and thereby retard staling,[8,9] and a more efficient ingredient in ethanol production than conventional wheat starch.[3,10,11]

One of the hindrances in development of waxy wheat cultivars has been the means to identify the waxy and partial waxy conditions. Measurement of amylose itself is restricted to complex and time-consuming wet chemical techniques, such as iodine binding complex methods accomplished by potentiometric measurement,[12] amperometric measurement,[13] or colorimetry.[14,15] Genotyping is accomplished by polymerase chain reaction (PCR)[16] or by protein analysis of GBSS isoforms using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[17] or enzyme-linked immunosorbent assay (ELISA)[18] procedures; however, these methods are also complex and slow. Rapid methods for waxy seed identification have been developed that are based on iodine staining[19] or near-infrared (NIR) reflectance.[20−22] These cases, however, were limited by either an incomplete set of hexaploid wheat of all eight waxiness genotypes (lines possessing a *wx-D1* null allele were absent)[20] or deliberate choice of a genetically less complex set involving tetraploid (durum) wheat,[21] thus preventing the study of all genetic combinations of the hexaploid waxy condition. The deficiency in genotypes with the *wx-D1* null allele has been rectified in the current study, with all partial waxy genotypes now represented. The objective of this study has been to determine the capabilities and limitations of NIR spectroscopy for identification of the waxy and partial waxy condition in hexaploid wheat. Further, the effect of sample format (bulk kernels, ground meal, and single kernel) is examined.

**Received:** November 24, 2010
**Accepted:** February 9, 2011
**Revised:** February 8, 2011
**Published:** March 14, 2011

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## ■ MATERIALS AND METHODS

**Plant Materials.** Four breeding populations were developed by crossing advanced hard winter wheat breeding lines or cultivars to experimental waxy wheats. The crosses were made in the greenhouse in the spring of 2000. Population identities and pedigrees were the following: G973 = 99Y1445/G97380; RDLD = Redland/99Y1436; S151 = 99Y1435/NW97S151; WX97 = WX97-3207/99Y1448. In each pedigree, the "99Y" parent was a fully waxy selection developed by the USDA-ARS wheat genetics program at Lincoln, NE. In the summer of 2003, single-head selections were made from $F_3$ bulk populations and used to derive breeding lines. $F_4$ and $F_5$ generations were grown in 2004 and 2005, respectively. Thirty breeding lines per population ($F_6$ and $F_7$ generations) were grown in randomized complete block experiments with three replications at the University of Nebraska Agricultural Research and Development Center near Mead, NE, in 2006−2009. The 2008 sample set was lost due to poor climatic conditions. Samples harvested from 2006 and 2007 crop years were used in NIR analyses. Samples from 2005 were used for DNA genotyping (below). Samples from 2009 were used for starch granule protein analysis to confirm the consistency of allelic conditions for waxiness (also below).

**Genotyping.** Two methods were used to determine allelic status of the three hexaploid wheat waxy loci. DNA polymerase chain reactions (PCR) were used to verify allelic status at the *wx-A1* and *wx-B1* loci. (Published primer sequences for alleles at the *wx-D1* locus failed to provide reproducible results; therefore, PCR analysis of this locus was not continued.) Seed samples from the $F_5$ generation (2005 harvest) were used. DNA was isolated from 12 single plant selections per line as per Liu et al.[23] Markers Waxy-A1-AFC-AR2[16] and Waxy-B1-CoDom(BDFL-BRC1-BFC-BRC2)[24] were used to identify alleles at the *wx-A1* and *wx-B1* loci, respectively. Forward primers were modified to include an 18 bp 5′ tail for use in fluorescent detection on an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA). PCR conditions were as published for each primer set.[16,24] For the second method, starch granules were purified from 10 single seeds as separate samples, per entry from the 2009 harvest. Starch granule proteins were extracted and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by one of the authors.[25] The purity of triple-null (full waxy) genotypes was verified by staining smashed kernels with an iodine solution.[19] At each locus, lines were scored as either wild type (normal) or mutant (null). Eight genotypes were identified, namely, wild type, fully waxy (triple null), three single-null genotypes (mutant allele at one of the three *wx* loci), and three double-null genotypes (mutant alleles at two of the three *wx* loci). Samples with fewer than 8 of the 10 kernels correct for the anticipated 2005 genotype were discarded as were samples with eight or more kernels being of the expected genotype but with this genotype in disagreement with the PCR determination. Altogether, 29 samples passed these criteria, yielding (with 3× field replication) 87 spectral samples for Year 1 (2006) and an equal number for Year 2 (2007).

**Equipment.** Two visible−near-IR spectrometers were used in this study. The first spectrometer, an NIRSystems model 6500 scanning monochromator (Foss-NIRSystems, Laurel, MD), collected diffuse reflectance (1100−2498 nm, 2 nm increments) of intact kernels (approximately 5 g) placed in a 25 mm diameter ×10 mm deep ring cell. The ring cell was loaded into the rotating pivot apparatus of the spectrometer. Spectral samples were scanned 32 times, with an equal number of reference scans from a ceramic tile, before being stored in $\log(1/R)$ format. This procedure was duplicated for each spectral sample, with cell emptying and refilling between duplicates. Diffuse reflectance scans of ground meal were performed with the same equipment and settings. A cyclone mill equipped with a 1 mm screen ground the intact kernels once the bulk kernel spectral collection was complete. Before grinding, 24 kernels in each bulk spectral sample were selected at random and set aside for single-kernel scans.

Single-kernel scans were performed using a 128-element indium gallium arsenide diode array spectrometer (Zeiss MCS511, Jena, Germany). Reflected energy was collected by a single optical fiber strand (600 $\mu$m diameter) located approximately 5 mm directly above the highest point of the kernel lying in a round-bottom slot of 2.5 mm depth and 9 mm length milled from a Bakelite plate. Twenty-four of the 49 (7 × 7) available slots in the plate were used for each sample. A computer-controlled $x-y$ stage moved the plate into alignment with respect to the stationary fiber strand. Kernel illumination was provided by a pair of 5 V, 150 mA tungsten filament lamps with gold-coated parabolic reflectors that have a focal axes at 45° with respect to the vertical and on opposite sides of the slot at midspan. The kernel axis was in alignment with the length of the slot; however, the rotational alignment within the slot (e.g., crease up, crease down, on side) was random. Previous research on the effect of rotational alignment on other classification issues of single kernels indicated that differences in model accuracies were not significant.[1] Kernel reflectance readings were referenced to reflectance values from a flat surface of polytetrafluoroethylene tile (Spectralon, Labsphere, North Sutton, NH). Because of the nonuniform wavelength increment between neighboring array elements (ranging from 5.85 to 6.23 nm), linear interpolation was applied to the $\log(1/R)$ readings from the spectrometer, with the result of each spectrum spanning a wavelength range from 942 to 1704 nm at 6 nm increments.

**Amylose and Protein Contents.** Starch amylose fraction, reported as the percentage of apparent amylose in isolated starch, was performed by an iodine binding dual-wavelength assay.[15] Briefly, a 100 ± 0.1 mg dry weight sample of the isolated starch was transferred to a 100 mL volumetric flask, whereupon 1 mL of 100% ethanol was added and the flask shaken to suspend the starch. Ten milliliters of 1 N sodium hydroxide solution was added, and the flask was swirled to disperse the sample. The dispersion was allowed to stand for approximately 1 h until the starch had gelatinized. The granule-free mixture was diluted to flask volume with distilled water. Two milliliters of this solution was transferred to a 100 mL volumetric flask, and 50 mL of purified water and 2 drops of phenolphthalein indicator (0.1% in ethanol) were added. This solution was titrated with 0.1 N HCl until the pink color just disappeared. Afterward, 2.0 mL of 0.2% iodine−potassium iodide solution was added to the flask and the solution filled to flask volume with distilled water, mixed, and allowed to stand for 30 min to fully develop the greenish-blue color. Absorbance was measured at two wavelengths (510 and 620 nm) and referenced to a blank consisting of 2.0 mL of 0.2% iodine−potassium iodide solution added to 100 mL of purified water. Starch amylose fraction was based on the difference in absorption at the two wavelengths referenced to a calibration curve developed from amylose standards prepared at the same time.

For protein content, combustion nitrogen analysis was performed on portions (150 mg) of the ground meal by a combustion instrument (Elementar, model vario MAX CN, Hanau, Germany) calibrated with nicotinic acid and supplied with reagents from the manufacturer. Percent nitrogen was converted to protein content using a factor of 5.7.[27] Repeatability precision, defined by the relative standard deviation ($RSD_r$) of a reference sample of dried wheat flour evaluated 30 times over the 1 month period of sample set testing, was 1.92%. Determination of protein content was conducted for the purpose of establishing whether protein differences among waxy genotypic groups were significant and, if so, whether these differences were responsible for pseudo classification of the waxy trait. Ideally, protein content differences would not be significant and thus allow spectral differences between waxy classes to be attributed to amylose and amylopectin themselves. Analyses of variance for the effect of waxiness class on protein content and starch amylose fraction and on single-kernel mass were performed in the GLM procedure in SAS (version 9.1.3, SAS Institute, Cary, NC).[28]

Table 1. Summary of Single-Kernel Masses and Sample Protein Content and Starch Amylose Fraction Values for Year 1

| | single-kernel mass (mg) | | | ground meal sample | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | protein content (%) | | amylose fraction in isolated starch (%) | |
| group[a] | $n$ | mean[b] | SD | $n$ | mean[a] | SD | mean[b] | SD |
| WT | 216 | 36.6 a | 6.8 | 9 | 13.9 a | 1.2 | 25.5 a | 0.83 |
| 1X | 504 | 37.0 a | 6.6 | 21 | 13.6 a | 1.2 | 23.7 b | 1.3 |
| 2X | 720 | 36.8 a | 6.3 | 30 | 14.2 a | 1.6 | 18.3 c | 3.0 |
| 3X | 648 | 36.6 a | 5.9 | 27 | 14.2 a | 1.0 | 0.80 d | 2.4 |

[a] Group refers to the number of null alleles for the gene that encodes the waxy protein, granule bound starch synthase. WT = wild type, 1X = single-null allele, 2X = double-null allele, 3X = triple-null or fully waxy allele. [b] Within a column of means, different letters adjacent to means indicate significant differences from Waller−Duncan test ($P = 0.05$).

**Spectral Analysis.** Spectra from the three sample formats (bulk, ground, and single kernel) were separately evaluated for their ability to naturally separate according to waxy genotype, number of waxy null alleles, or whether the sample possessed the fully waxy trait or not. Within each sample format and year, mean-centered spectra were resolved into their principal components using principal component analysis (PCA).[29] Spectral preprocessing was limited to first or second derivatives based on a Savitzky−Golay convolution algorithm.[30] For bulk and ground spectra, four convolution widths (5, 9, 15, and 25 points) were investigated using a quadratic polynomial for both first and second derivatives. For single-kernel spectra, the convolution widths were set to 5, 9, 11, and 15 points. PCA scores from these transformed and mean-centered spectra were evaluated for cluster formation according to the condition of waxiness. All preprocessing steps were programmed in SAS, as was PCA using the procedure 'PRINCOMP'.

**Classification Modeling.** Linear discriminant analysis (LDA) [31] was performed on the PCA scores, as executed by the SAS procedure 'DISCRIM', to determine the relative discrimination power of the PCs.[32] Preliminary trials (in SAS 'STEPDISC') that examined the relative contributions of the scores toward identifying fully waxy, partial waxy (single and double), and wild-type conditions (hereafter called groups) indicated that the importance of the score toward classification was generally consistent with the latent variable order (i.e., the size of the eigenvalue), namely, that scores 1−3 were usually selected as most helpful toward classification. A one-sample-out cross-validation was applied to the Year 1 samples for the purpose of selecting the number of PCs to use in all subsequent analyses.

## RESULTS AND DISCUSSION

A summary of single-kernel weights, sample starch amylose fraction, and sample protein content for Year 1 is shown in Table 1. Protein content differences among the four GBSS null allele groups (0X = wild type, 1X = single null, 2X = double null, 3X = triple null or waxy) were not significant nor were there group differences in single-kernel mass. Similar findings on the lack of protein concentration and protein quality differences between waxy lines and nonwaxy cultivars were reported by Graybosch et al.[33] For the Year 2 samples of the current study, protein content differences among the GBSS null allele groups were also not observed; however, the mean kernel mass of the double-null class was significantly different ($P < 0.01$) than that of the fully waxy class. The greatest group effect occurred with starch amylose fraction, in which differences were highly significant ($P < 0.01$) and the mean of each null allele group was significantly different from each of the three other groups.

Mean spectra of the wheat samples within a GBSS null allele group are shown for ground meal, bulk samples, and single kernels

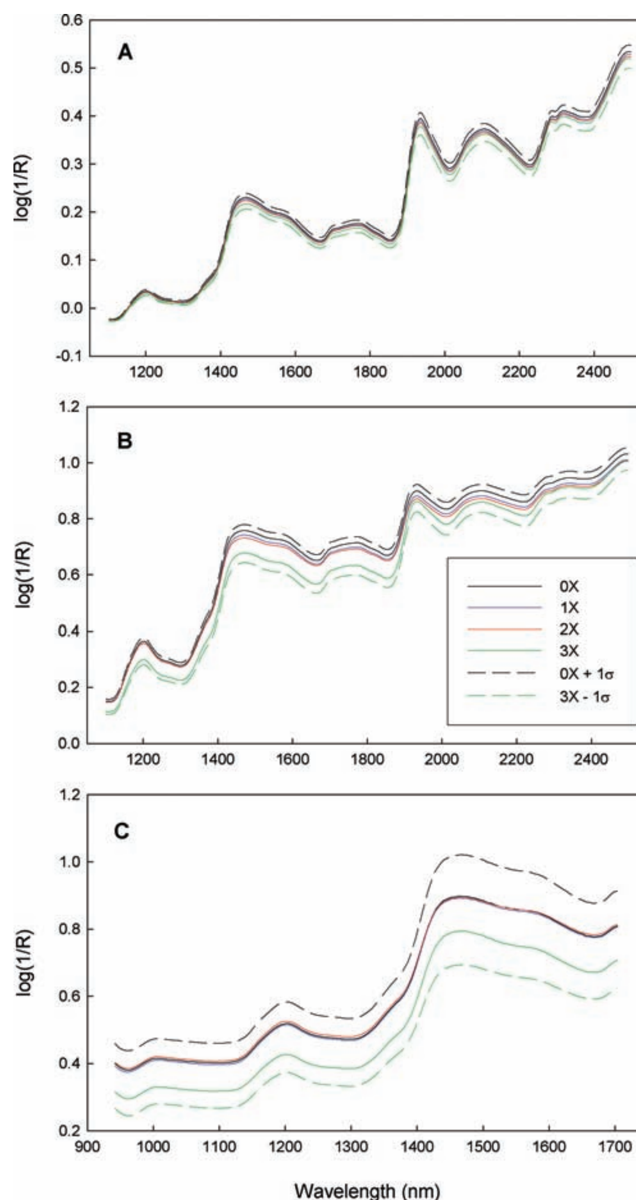


Figure 1. Mean spectra of the individual waxy null allele groups for all samples from one harvest (Year 1). Also included are the +1 standard deviation half-envelope for the wild-type group (0X) and the −1 standard deviation half-envelope for the fully waxy group (3X). A = ground meal, B = bulk kernels, C = single kernels.

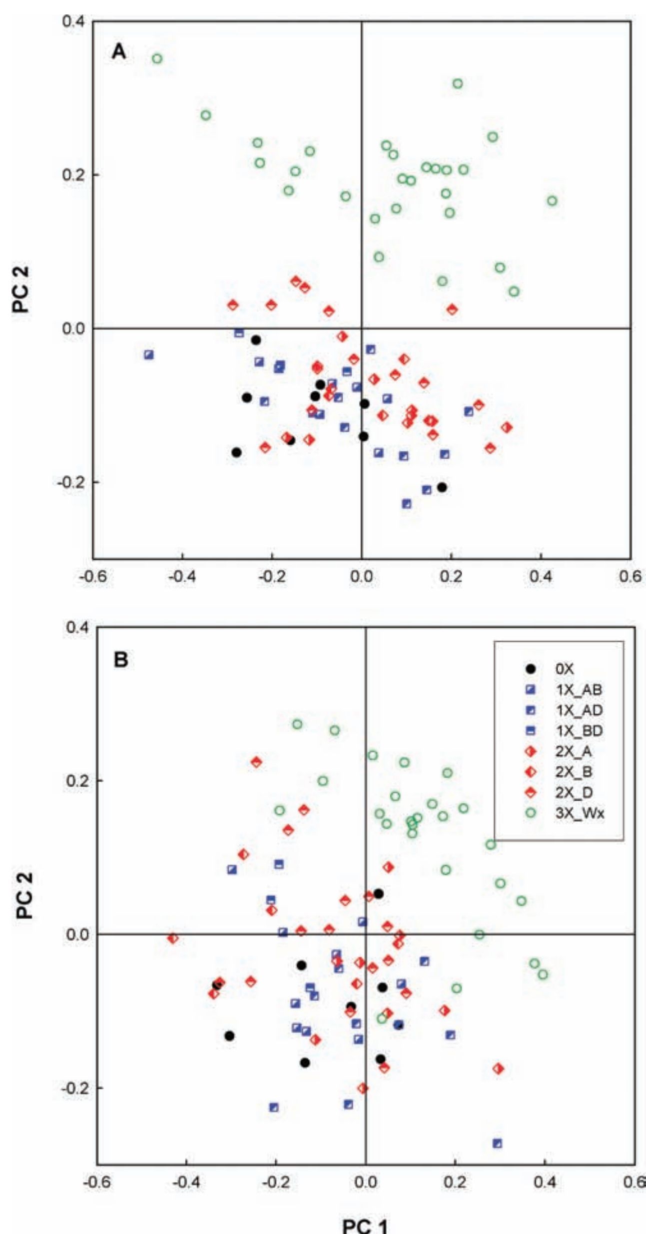


**Figure 2.** Principal component scores of ground meal spectra. Spectral preprocessing was a second derivative using a 9-point Savitzky–Golay quadratic function convolution. Symbol abbreviations for the waxy gene are as follows: 0X = wild type; 1X_AB, _AD, and _BD = single-null allele on D, B, and A genomes, respectively; 2X_A, _B, _D = double-null allele s on B and D, A and D, and A and B genomes, respectively; 3X_Wx = triple-null alleles. A = Year 1, B = Year 2.

in Figure 1. For ground meal (Figure 1A), spectral differences among the groups were very slight and nonspecific with respect to local absorption bands. Instead, the differences were systematic (additive and multiplicative) across the entire wavelength region, suggestive of grain hardness differences that are primarily manifested as differences in particle size from grinding. The strong similarities among groups is also confirmed by the standard deviation half-envelopes plotted for the systematically highest ($0X + 1\sigma$) and lowest ($3X - 1\sigma$) group mean spectra. These envelopes reveal that the natural spectral variation within a group is larger than the group effect. Similar trends were observed but with fewer noticeable differences when the mean

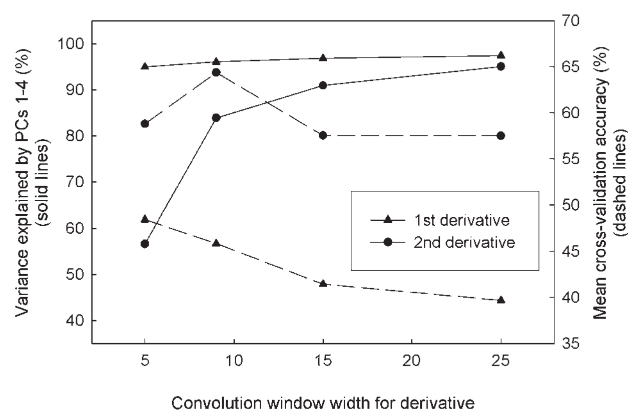


**Figure 3.** Plots of cumulative variance explained by principal components (PCs) 1–4 as a function of the convolution window width for Savitzky–Golay first and second derivatives of Year 1 ground meal spectral data. Also included are the mean (over four waxy allele classes) cross-validation accuracies from linear discriminant analyses based on the scores from PCs 1–4.

derivative traces of these spectra were plotted (not shown). Prior research reveals that milling yield is affected by starch waxiness, with speculation that this is caused by the increase in starch granule crystallinity as the amylose fraction decreases.[33] The order of the null allele group mean values for bulk spectra (Figure 1B) is the same as that for ground meal spectra. However, in the bulk case, the waxy group is noticeably offset (toward lower absorbance) compared to the double-null, single-null and wild-type groups, especially in the region between 1100 and 1900 nm. This suggests that the waxy trait is associated with a change to the physical structure of the kernel endosperm such that the effect becomes smaller when the kernel is ground into meal. Similarly, for the single-kernel system, the mean spectra (940–1700 nm) upon visual inspection are imperceptibly different among the wild-type and partial waxy groups, but these three groups are noticeably offset from the waxy group (Figure 1C).

Selecting the ground meal samples with a 9-point second-derivative pretreatment as a representative case, the scores of the first two principal components, PC 1 and PC 2, are shown for Year 1 in Figure 2A. Most apparent in this scores plot is the separation between the fully waxy samples and all other genotypes. Among the wild-type and partial waxy samples, a slight gradient is apparent, with double-null samples generally lying between the waxy and single-null samples and wild-type samples lying in a region most distant from the waxy samples. Some overlap exists between single- and double-null samples. Within the double-null samples, separation by waxy genotype (i.e., double-null alleles on B and D, A and D, or A and B genomes) was not evident. Similarly, graphical separation of the three single-null alleles was also not observed.

A scores plot for the Year 2 set reveals clustering behavior that is similar to Year 1, but with more overlap among the nonwaxy samples (Figure 2B). While the fully waxy samples are once again separate, a general gradient within the partial waxy classes and wild-type class is less evident. For either year, inclusion of the third and fourth principal components improved the discriminant function; however, additional components did not improve classification accuracy as determined by cross-validation, such that the model accuracies reported herein are limited to models based on four PCs.

The portion of variance cumulatively explained by the first four principal components of ground meal spectra is shown in Figure 3 for the first and second derivatives of varying convolution window

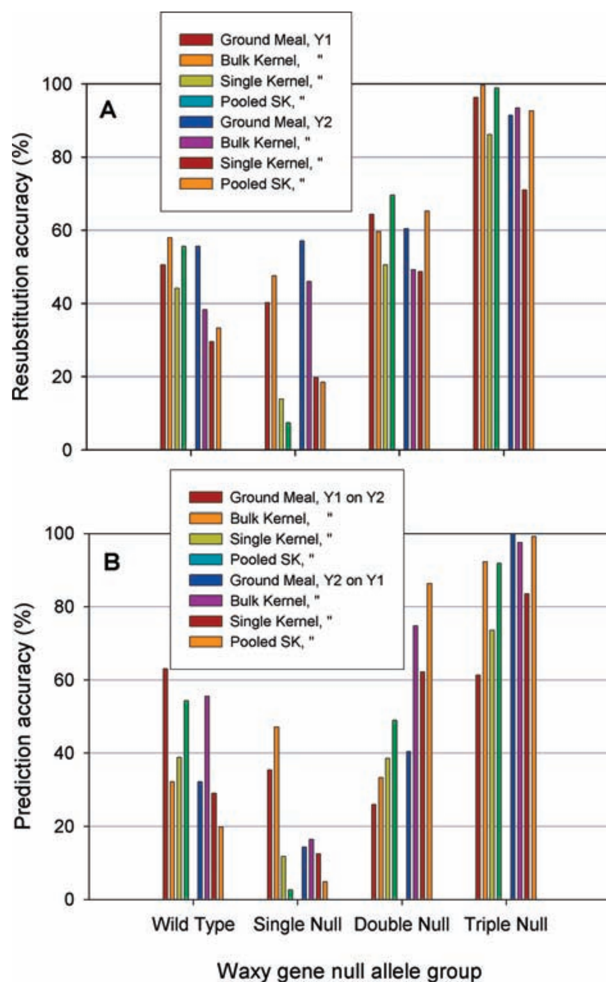


**Figure 4.** Accuracies of waxiness classification models. Each bar is the mean accuracy of nine linear discriminant analysis trials using principal component scores 1−4. The trials arose from nine spectral preprocessing (or lack of) transformations as follows: four convolution window widths for a Savitzky−Golay first derivative, a similar four widths for second derivative, and one involving no spectral preprocessing. A = calibration, performed separately on Year 1 (Y1) and Year 2 (Y2) spectra; B = validation on opposite year. Sample format shown in legends, with additional explanation that "Pooled SK" represents the case in which each sample was assigned a group according to the most numerous single-kernel assignments within the sample.

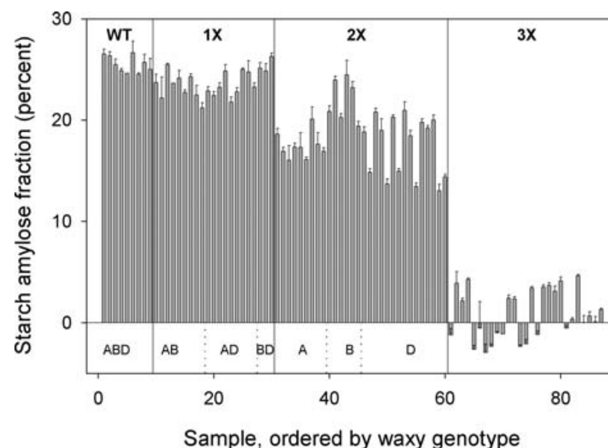


**Figure 5.** Starch amylose fractions of Year 1 samples, as determined by starch−iodine colorimetric two-wavelength assay. Each bar is the mean of 3 replicate assays, with a standard deviation bar positioned above the mean bar. WT = wild type, 1X = single null, 2X = double null, 3X = triple null (waxy). Letters below the bars, separated by solid or dotted vertical lines, identify the actual granule bound starch synthase genotype by listing the non-null alleles.

widths. Compared to the first derivative, the second derivative collapsed the spectra into a tighter family of curves, thus leaving a larger portion of the differences between curves attributable to chemical variation at the expense of physical effects.[35] Therefore, the fractional level (or percent) of the variance that was explained by the first four principal components was smaller for the second derivative, especially at the smaller convolution widths. Increasing the width of the Savitzky−Golay second-derivative convolution window had a noticeable effect of increasing the fraction of variance explained by PCs 1−4, whereas the effect was minor for the first derivative. Conversely, this inherent smoothing effect associated with wider convolution windows had a slight negative effect on overall classification accuracy during model calibration, as seen by the downward trending dashed lines in Figure 3. Anticipating the benefit of wide convolution windows during model validation, a decision was made to maintain all four convolutions for the first- and second-derivative preprocesses. Therefore, the classification accuracies of the 5-, 9-, 15-, and 25-point first and second derivative with mean centering preprocesses along with that of mean centering alone were averaged to produce the LDA classification accuracies for each waxy genotypic group within a crop year and physical state (ground, bulk, and single kernel), as shown in Figure 4.

Mean LDA calibration accuracies of the waxy samples were high, exceeding 95% for ground meal and bulk kernel formats in Year 1 and exceeding 90% for the same formats in Year 2 (Figure 4A). In contrast, the mean accuracies of these formats for the wild-type and partial waxy groups generally ranged between 40% and 60%, with a low of 38% (Year 2 bulk kernel) and a high of 64% (Year 1 ground meal). Misclassifications occurred as assignments within these three groups rather than assignment to the fully waxy group. Single-kernel calibration accuracies tended to be lower than the ground meal and bulk kernel calibrations, with this trend particularly apparent in the single-null group (14% and 20% accuracies for Years 1 and 2, respectively). Additionally, the 24 single-kernel classifications within each sample were pooled together, and the waxiness group for that sample was awarded to the group with the greatest number of single-kernel assignments. This resulted in accuracies for the fully waxy and double-null groups that were on par (99% and 93% fully waxy accuracies for Years 1 and 2, respectively; 70% and 65% double-null accuracies correspondingly) with those from ground meal and bulk kernel formats. For the wild-type and single-null allele groups, the effect pooling of single-kernel assignments was less consistent, with one year group (Year 1 single null) having a noticeable drop in accuracy from single-kernel (14%) to pooled single-kernel (7%) formats but both accuracies being uncharacteristically low.

Mean LDA validation (Year 1 LDA model applied to Year 2 samples and vice versa) accuracies, depicted in Figure 4B, were slightly lower in general than their corresponding calibration accuracies, though the fully waxy samples were still correctly identified at a substantially higher rate than the wild-type and partial waxy samples. For the bulk sample format, the validation accuracy for the fully waxy groups exceeded 90% whether it was the Year 1 model applied to Year 2 (92%) samples or the opposite year application

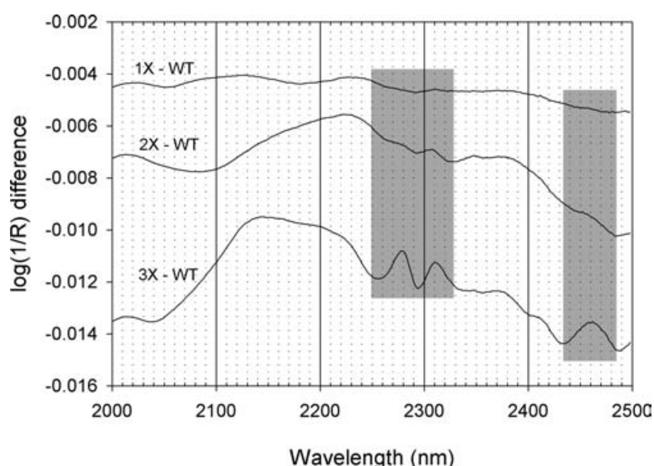


**Figure 6.** Difference spectra of Year 1 samples. Each trace is calculated as the spectral difference between the mean spectrum of the waxy (3X) or partial waxy (1X or 2X) samples and the mean spectrum of the wild-type (WT) samples. Regions of change are identified with light gray shading.

(98%). With corresponding validation accuracies of 92% and 99%, the pooled single-kernel models were also very successful at identifying the fully waxy condition. However, as with the calibration accuracies (Figure 4A), the validation accuracies for the fully waxy and wild-type groups were much lower, with 15 of the 24 average accuracies for these three groups being less than 40% and 7 of these actually less than 20%.

Starch amylose fraction values for Year 1 are shown in the bar graph of Figure 5. Negative values for some of the waxy samples are the result of the colorimetric procedure that indexes the light intensity responses of the starch−iodine complex at two wavelengths (i.e., absorption difference between 510 and 620 nm) to the amylose fraction, based on a linear calibration curve established using standard amylose/amylopectin preparations. While the mean values of starch amylose fraction of each null allele group were significantly different, the differences were evident on an individual sample level, with each waxy sample fully distinguished from the three other groups. Actual LDA calibration findings on the Year 1 samples based on starch amylose fraction alone (Year 2 not assayed) are as follows: wild type = 8 of 9, single null = 14 of 21, double null = 27 of 30, and waxy = 27 of 27 samples correctly identified. Given that these classification accuracies are similar to (in many cases better than) those from spectral analyses, it appears that the starch amylose fraction is strongly related to spectral identification of the null allele classes. An attempt is made to better understand the basis of classification. Considering the work of Srisuthep and co-workers,[36] who noted a difficulty to distinguish oligosaccharides $G_3$−$G_{12}$, a spectral sensitivity to molecular configuration differences between amylose and amylopectin alone is remote. More plausible explanations include the following: (1) physical structural differences within the starchy endosperm or on the seed coat that alter light scatter, thus inducing a systemic change across the entire wavelength region; (2) a higher degree of crystallinity for the fully waxy genotype, owing to a greater abundance of crystalline amylopectin, whereby changes to the C−H bands and hydrogen-bonded OH groups are spectrally sensed;[37] and (3) the presence or absence of nonstarch compounds that preferentially occur in either amylose or amylopectin (e.g., lipids in amylose) at levels sufficiently high to be spectrally sensed. While scattering differences attributed to the seed coat would favor models based on bulk kernel format, crystallinity differences and nonstarch compound differences would be favored by those based on sample grinding. To explore the latter, a mean ground meal spectrum of a partial waxy or waxy group has been subtracted from the mean spectrum of the wild-type group (Figure 6). From these difference spectra, two wavelength regions (shaded) demonstrate the greatest degree of change as the difference in null alleles increases. For example, local maxima at 2288 and 2312 nm are barely noticeable in the (1X − WT) difference spectrum but quite apparent in the (3X − WT) spectrum. The same trend, though less pronounced, occurs with the bulk kernel difference spectra (graph not shown). This wavelength region contains CH combination vibrations from $CH_2$ attributed to lipid groups,[38] which diminish as amylose becomes scarcer. Further study is needed to verify and ascertain the relative contributions of the three suggested explanations and their interaction.

In summary, this study examined the ability of NIR reflectance spectroscopy to differentiate the starch waxy genotypic groups in hexaploid wheat. Apart from our earlier studies that were either incomplete in representation of all genotypes associated with the granule-bound starch synthase (GBSS) waxy protein gene or reliant on genetically simpler tetraploid wheat, the current study utilized four breeders' populations of hard winter wheat that spanned all eight genotypes. On the basis of linear discriminant analysis of principal component scores from NIR spectra of ground meal, whole kernels in bulk, or single kernels (single or pooled within a field sample), the results indicated that fully waxy wheat is identifiable at typically 90−100% accuracy. However, the remaining genotypes are much less identifiable, even when combined into groups of common number of functioning waxy alleles (wild type, single null, and double null), whereby the accuracies of correct assignment into an allele group were typically between 40% and 60%. Spectral discernment of the waxy condition is speculated to be attributed to the lipid−amylose complex. Because results show that the fully waxy trait can be easily identified by NIR, regardless of the genetic background (population) within which it resides, breeders could easily use NIR to select waxy lines from early generation materials. Further, end users could also use NIR to differentiate waxy crops at harvest.

## SAFETY

As sample preparation for NIR reflectance procedures involve grinding at most, no special precautionary handling procedures were used in this study.

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## DISCLOSURE

Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply endorsement or recommendation by the USDA.

## ACKNOWLEDGMENT

The authors give thanks to E. Laraway (ARS, Beltsville) for spectral data collection and combustion nitrogen analysis and to V. Hansen (ARS, Lincoln) for genotyping by gel electrophoresis and starch amylose fraction determinations.

## ABBREVIATIONS USED

GBSS, granule bound starch synthase; NIR, near-infrared; PCA, principal component analysis; PCR, polymerase chain reaction; R, reflectance; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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