

## Maize Kernel Hardness Estimation in Breeding by Near-Infrared Transmission Analysis

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**ABSTRACT**

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Near-infrared transmission (NIT) spectra between 600 and 1,100 nm were recorded for whole maize kernels of genotypes, including hybrids grown under experimental and commercial conditions, crosses between hybrids, populations of different races, and samples from storage facilities. Hardness was estimated from test weight, percent floaters, coarse-fine ratio, and near-infrared reflectance. Protein and oil contents were also determined, and spectra of hexane-soluble constituents were recorded from 600 to 1,100 nm. Two NIT measurements were highly cor-

related with hardness estimates. The first was absorbance at 860 nm (Abs): softer dent kernels absorbed more than did flintier (harder) kernels. The second was shift of a 620–680 nm absorbance peak (Shf): flintier hybrids had maximum peaks at lower wavelengths than did dent types. These NIT parameters were not related to protein, oil, or hexane soluble constituents. ANOVA showed that Abs and Shf were less sensitive to the block effect than actual hardness measurements. Thus, both NIT parameters may be useful for selection during breeding.

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Near-infrared reflectance (NIR) has been widely used as a primary method to estimate hardness of various cereal grains, including maize (Williams 1979, Pomeranz et al 1984, Pomeranz et al 1986). Reflectance at 1,680 nm appears to relate to grain hardness because it is greatly influenced by particle size but little by chemical composition. However, these NIR methods necessitate grinding of samples, which has certain drawbacks: first, time and effort are required for grinding; second, loss of grain might be unacceptable to breeders during early selection; third, "true hardness" may not be properly estimated by NIR, because particle size is also a function of breakage susceptibility (Pomeranz et al 1984).

In contrast, near-infrared transmission (NIT) measurements can be done on whole kernel samples, circumventing the problems of grinding (Rosenthal 1986). Williams (1991) used NIT to predict hardness of whole wheat kernels, calibrating the instrument against particle size index (PSI) as the reference method; NIT was a better predictor of PSI than was NIR. Delwiche (1993) measured hardness of single wheat kernels by NIT. The instrument was calibrated against a diffuse reflectance method (AACC 39-70A).

We attempted to find an NIT method that can be used to estimate maize kernel hardness. Such a method could be valuable in plant breeding and could be used as a primary method, requiring no previous calibration. Our results are described in this article.

### MATERIALS AND METHODS

Five sample sets were used. Set A consisted of eight maize hybrids differing widely in texture, as subjectively judged by visual inspection. Morgan 400 was classified as very flint; Morgan 505, Morgan 251, and Cargill R157 were classified as flint; Cargill SD 4 was classified as semi-dent; Asgrow 606 and Pioneer 3183 were classified as dent; and Pioneer 3901 was classified very dent. These hybrids were from a demonstrative yield trial at the Pergamino Agricultural Experiment Station (EEA Pergamino) during the 1990–1991 growing season. Because this was an exhibition trial, there was only one replicate per hybrid. Sample set B consisted of 16 genotypes, grown in three replicates (48 samples). These 16 genotypes were hybrids and crosses from the Maize Breeding Section (EEA Pergamino) grown during 1990–1991.

Textures of these maize lines ranged from semi-dent to flint. Samples of sets A and B were formed by shelling five selected ears from each plot and merging the grain from the five ears. Sample set C consisted of 33 commercial hybrids grown under production (as opposed to experimental) conditions within 100 km of EEA Pergamino in 1993–1994. Shelled samples of ≈1 kg were submitted by the companies that produced the seed. Sample set D contained 15 populations of different maize races from the germ plasm bank at EEA Pergamino. Sample set E consisted of 43 unidentified maize samples collected while unloading trucks at nine storage facilities within 70 km of EEA Pergamino. Sampling was done at different times during the 1992–1993 harvest. The plant operator took twin samples from each truckload for analysis, one of which was for our use. A composite sample was prepared from samples taken each week. Approximately 2 kg from these composite samples were bagged and sent to our laboratory. Samples from all sets were left at room conditions in shallow trays for 20 days. After this period, the range in moisture content was 1.4% (data not reported). This range was considered narrow enough to preclude corrections for moisture in determinations where such corrections would apply.

NIT spectra were measured from 600 to 1,100 nm in a Trebor 7700s instrument on whole kernels using the 25-mm path sample holder. Spectra were transferred to a personal computer by the supplied software. Percent protein of ground kernels was determined by NIR in a Trebor 7700s instrument, calibrated against Kjeldahl values. Oil content of whole kernels was determined by nuclear magnetic resonance using an Oxford 4000 instrument. Two points were used to calibrate this method: one was a pure maize germ oil and the other a maize sample of known oil content, as determined by a solvent extraction procedure.

Hardness-associated parameters were measured by four methods. 1) Test weight was determined on samples of ≈300 g using a Tripette & Renaud TR-400 instrument. Higher test weight values are generally associated with flintier maizes (Watson 1984a). 2) Percent of floaters was determined according to Lepes et al (1976). This method was a modification of Wichser (1961) in which 100 whole, sound kernels were placed into a 250-ml beaker containing ≈170 ml of a kerosene-carbon tetrachloride mixture having a density of 1.305 at 25°C. After being briefly stirred with a glass rod, floating kernels were counted. Higher numbers of floating kernels indicated a softer texture. 3) Coarse-to-fine ratio was determined in a manner similar to that described by Pomeranz et al (1986) for the weight coarse-fine (C-F) ratio.

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Samples (50 g) were ground for 15 sec in a Stein laboratory mill. Ground kernels were then sifted at full speed for 1 min in a Chopin Rotochoc mechanical sifter equipped with 0.5- and 1-mm sieves. Quantities of coarse material retained by the 1-mm sieve and of fine material passing through the 0.5-mm sieve were weighed with a precision of 0.1 g. The ratio of these two respective fractions was the C-F ratio, the ratio being higher for flintier maize. 4) NIR hardness was determined according to Pomeranz et al (1984) by grinding  $\approx 50$ -g samples in a model 3600 Falling Number laboratory mill. The no. 0 (tightest) setting was used instead of the no. 3 setting, because this gave better differentiation. Reflectance then was measured at 1,680 nm in a Trebor 7700s instrument, taking the first three significant, non-zero digits of the instrument reading as a measure of hardness. Higher readings indicated harder samples.

To record hexane-soluble constituent spectra, kernels were first peeled, degermed, and ground. Ground endosperm ( $50 \pm 0.01$  g) was extracted by refluxing with hexane for 4 hr. Extracts were diluted to 250 ml with hexane, and spectra were recorded from 600 to 1,100 nm with a Trebor 7700s instrument equipped with a sample holder for liquids. Results were transferred to a personal computer using software supplied by the manufacturer. The spectrum of pure hexane was subtracted from spectra of extracts.

All determinations were run in duplicate. Values reported and used in statistical analysis are averages of duplicates. Correlations were done using the CORR option of the MSTATC statistical analysis program and one-way analysis of variance with the ANOVA-1 option.

## RESULTS AND DISCUSSION

Absorbance ( $\log_{10}[1/\text{transmittance}]$ ) spectra (800–900 nm) for hybrid set A are shown in Figure 1. As kernels become more dent (softer), absorbance increases. As discussed below, differences among absorbances of hexane-soluble extracts of hybrids are nearly constant between  $\approx 800$  and 900 nm. This wavelength range, therefore, appears especially suitable for measuring hardness-associated differences in grain absorbance among hybrids. Absorbances at several wavelengths within this range were correlated with hardness tests; absorbance at 860 nm (designated Abs) gave, on the average, the highest correlation coefficients. Because of this, Abs was chosen in this study. The relation between Abs values (duplicate determinations) and hardness class for these hybrids are shown in Figure 2. To check reproducibility of measurements, a sample of Morgan 251 from another source was analyzed with measured Abs values of 2.523 and 2.549, which were very similar to those shown in Figure 2.

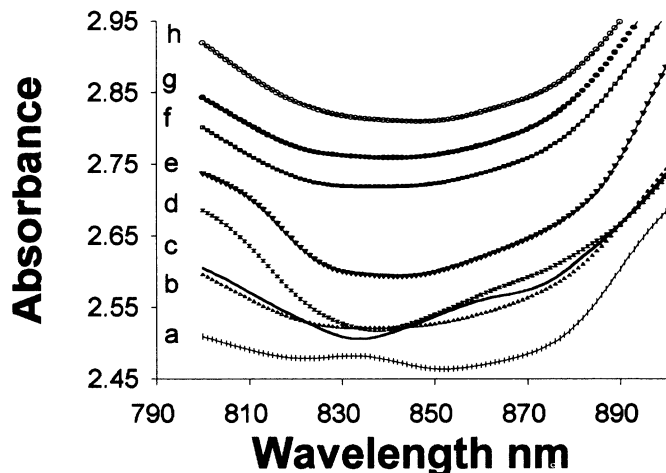


Fig. 1. Near-infrared transmission absorbance curves (800–900 nm) for eight maize hybrids (set A). Each curve is an average of two spectra. a = Very flint; b–d = flint; e = semident; f and g = dent; and h = very dent.

Another major difference among NIT spectra of maize hybrids varying in endosperm texture was revealed by comparing first derivative curves. Absorbance maxima in the 610–680 nm region are indicated by points where curves intersect the abscissa (Fig. 3). Morgan 400, the flintiest hybrid, has a peak maximum at the lowest wavelength, while the peak of Pioneer 3901, the most dent genotype, is at the highest wavelength. Peaks shift toward longer wavelengths as endosperm becomes softer. The wavelength maxima of these “shifting peaks” has been designated Shf. Values of Shf for the hybrids of Set A are related to hardness class in Figure 4. A sample of Morgan 251 from a different source had Shf values of 640 and 638 nm, comparable to those of Figure 2.

### Relationships Among NIT and Hardness Tests

To objectively test the associations visually revealed in Figures 1–4, regression and correlation analysis were performed. Correlation coefficients ( $r$ ) are shown in Table I. Abs and Shf are highly correlated with each other in set A, and there are good correlations between the NIT measurements and tests commonly used to estimate maize kernel hardness (test weight, percent floaters, C-F ratio, and NIR hardness). Similar correlation analyses were performed on sample sets B–E (Table I). In general, significance levels of correlation coefficients are similar to those for sample set A, but the correlation coefficients themselves are lower. Samples in set A were few and chosen specifically to enable detection of spectral differences in maize varying widely in hardness. Thus, differences in texture among samples in set A were large.

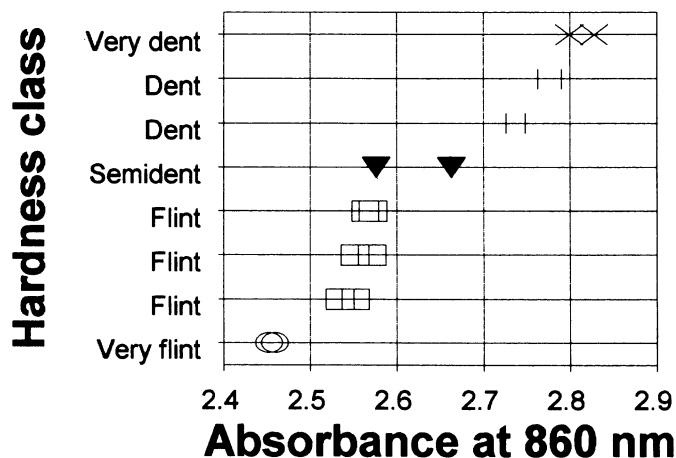


Fig. 2. Differentiation of hybrids (set A) of different hardness classes by their absorbance at 860 nm (duplicate values).

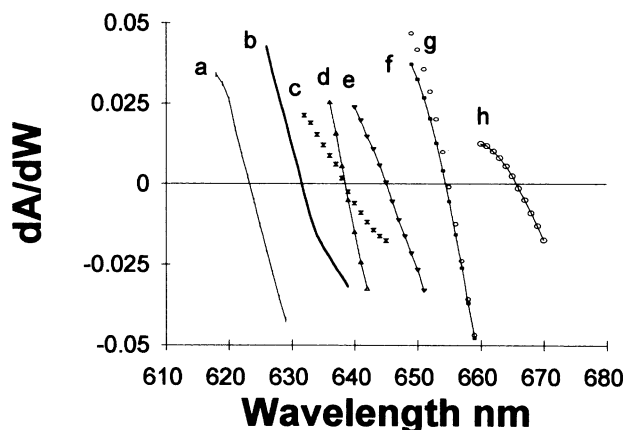


Fig. 3. First derivative near-infrared transmittance absorbance spectra of maize hybrids varying in endosperm texture. a = Very flint; b–d = flint; e = semident; f and g = dent; and h = very dent. dA/dW = Absorbance first derivative. Gap = 10 nm.

Values of measured parameters showed major discontinuous shifts among samples, and there was no overlapping. This appears to have contributed to the high correlation coefficients noted for set A. Sample sets B–E, however, contained more samples than did set A, and samples were randomly selected. In such sets, texture will likely vary more continuously, rather than in sharp steps. For samples of similar texture, measurements may not exactly reflect property differences, due to experimental errors. In fact, some samples similar in texture did exhibit a reversal in values. For example, one sample had values of Abs = 2.492 and C-F = 3.79, while another had Abs = 2.480 and C-F = 3.65, even though Abs and C-F were negatively correlated. If several such instances occur in a sample set (e.g., sets B–E), correlation coefficients will decrease. Nevertheless the correlation coefficients noted for sample set A are still valid for sets B–E at similar statistical probability levels, owing to the larger number of observations for these sample sets.

#### Relationship of NIT Tests to Kernel Protein and Oil Content

The NIT parameters found to be associated with hardness were absorbance at 860 nm and peak shift in the 620–680 nm range. Several kernel constituents absorb between 800 and 900 nm, including protein and oil. To test whether the differences in NIT spectral characteristics were due to protein or oil rather than to hardness, we examined correlations between Abs and maize kernel oil and protein contents.

For set A, the calculated correlation coefficient ( $r$ ) of percent protein against Abs was  $-0.521$  ( $P = 0.194$ ). For sets B–E, values of  $r$  and  $P$  were, respectively,  $-0.029$  and  $0.865$ ,  $0.088$  and  $0.626$ ,  $-0.299$  and  $0.278$ , and  $0.113$  and  $0.512$ . These significance levels indicate no close association between Abs and protein content.

The correlation of oil content with Abs was high and highly significant:  $-0.955$  ( $P < 0.001$ ) for set A. For set B, however, figures were substantially lower ( $r = -0.261$ ,  $P = 0.076$ ). Thus, the high correlation for set A appears anomalous. This might be ex-

plained by the following hypothesis. Set A samples varied widely in hardness and flintier kernels are rounder and smaller than more dent ones. The germ-endosperm ratio is a function of kernel size (Weber 1984), smaller kernels tend to have a higher ratio. In general, then, smaller kernels have higher oil contents than larger ones because ~85 % of kernel lipids are in the germ (Watson 1984b). This may explain the large variation in oil content of samples in set A (c.v. 15.67%). Because we postulate that Abs is an estimator of hardness, Abs would vary with kernel size. We thus hypothesize that the high correlation found between Abs and percent oil may be mediated by kernel size to which both Abs and percent oil are associated. Therefore, there would not be a direct relation between Abs and percent oil. In set B, though no attempt was made to quantify kernel size, it was visually evident that kernel type was much more uniform than in set A. It follows that oil content should be more uniform; this was actually found (c.v. 2.16%) and appears to explain the less significant correlations of Abs to percent oil in set B. Nevertheless, correlation coefficients between Abs and some hardness estimators (test weight, percent floaters, C-F ratio, and NIR hardness) had significance levels generally similar to those of set A. That is, in the more representative sample set B, Abs correlates significantly with hardness but not with oil content.

Differences in Abs among maize genotypes thus do not appear to be related to differences in protein or oil contents. This does not preclude the possibility, however, that specific kernel constituents may be associated with Abs or Shf. For example, the amount or distribution of the maize endosperm storage protein class zein-2 may influence endosperm hardness (Abdelrahman and Hosney 1984, Wallace et al 1990, Paulis et al 1992, Robutti 1992); similarly, in wheat, friabilins are associated with kernel softness (Greenwell and Schofield 1986). Studies are now in progress in our laboratory to relate Abs and Shf to quantity of zein-2, as revealed by high-performance liquid chromatography. Also, a close negative relationship exists between maize amylose-amylopectin ratio and certain hardness estimators, such as percentage of horny endosperm and test weight (Krisman and Curá 1991). This might indicate an association, perhaps functional, between maize starch composition and hardness. One might then speculate that differences in Abs and Shf among maizes of different hardnesses might be caused by differences in the composition of some kernel constituents, such as protein or starch. Such differences could, of course, cause kernels to differ in their spectral characteristics.

#### Relation of NIT Values to Maize Hexane-Soluble Constituents

The hexane-soluble constituents (including some of the pigments) of maize endosperm might also influence its Abs and Shf values. If they did, then observed Abs and Shf differences would not directly relate to hardness. To investigate this point, absorption characteristics (original and second derivative spectra) of hybrids and their hexane-soluble fractions were compared.

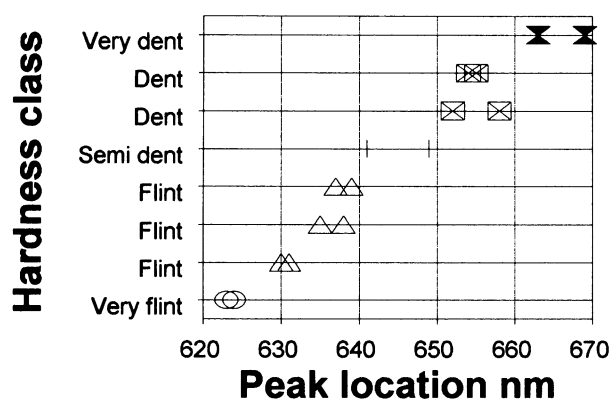


Fig. 4. Differentiation of hybrids (set A) of different hardness classes by shifting peak wavelength location (duplicate values).

TABLE I  
Correlation Coefficients and Significance Levels Among Near-Infrared Transmittance (NIT) Spectral Parameters and Estimates of Maize Kernel Hardness

Parameter <sup>a</sup>	Set A (n = 8)		Set B (n = 48)		Set C (n = 33)		Set D (n = 15)		Set E (n = 43)	
	Shf	Abs	Shf	Abs	Shf	Abs	Shf	Abs	Shf	Abs
Shf	1.000	0.970*** <sup>b</sup>	1.000	0.585***	1.000	0.804***	1.000	0.602*	1.000	0.767***
Abs	0.970***	1.000	0.585***	1.000	0.804***	1.000	0.602*	1.000	0.767***	1.000
TW	-0.615	-0.675	-0.348*	-0.358**	-0.455**	-0.652***	-0.479	-0.604*	-0.672***	-0.656***
NIR	-0.845**	-0.803*	-0.397**	-0.412**	-0.654***	-0.521**	-0.856***	-0.771***	-0.236	-0.335**
C-F	-0.903***	-0.840**	-0.223	-0.297*	-0.406	-0.318	-0.731**	-0.744***	-0.606***	-0.627***
FLO	0.947***	0.953***	0.481***	0.486***	0.644***	0.522**	0.890***	0.786***	0.766***	0.716***

<sup>a</sup> Shf = absorbance wavelength (nm) of shifting peaks; Abs = absorbance, 860 nm; TW = test weight, kg/hl; C-F = coarses to fines ratio; FLO = percent floaters.  
<sup>b</sup> \*\*\* = Significant at 0.1% level; \*\* = significant at 1% level; \* = significant at 5% level.

Spectra (800–900 nm) of hexane-soluble extracts from set A hybrids are shown in Figure 5. In much of this range, there was little difference in absorbance among spectra, indicating that extract differences do not explain observed differences in Abs among maize kernels. Second derivative spectra (620–680 nm) of grain of Cargill SD 4 and its extract are shown in Figure 6. The shifting peak (for this hybrid at 645 nm) has no counterpart in the extract spectrum. This was true for all set A hybrids, indicating that differences in Shf among hybrids are not related to hexane-soluble constituents. Thus, amounts and types of hexane-soluble constituents (including some of the pigments) do not appear to relate to variation in Abs or Shf among maize hybrids.

#### Influence of Certain Environmental Factors on NIT Estimation of Maize Kernel Hardness

We have shown the potential of NIT methods for indicating hardness of maize kernels. In breeding, such measurements should relate to genotype and not to environment. We thus examined effects of environmental factors on NIT maize kernel hardness prediction.

Sample set B contained 16 genotypes, grown in three replicates (48 samples) within the experimental field. In field experiments, replicates indicate the ever-present micro-environmental variability among plots. If the environment were constant throughout a field, no replicate would be necessary in field trials. Because field plots on which triplicates of Set B were grown differed in soil quality, pest incidence, and other noncontrollable environmental factors, we considered each replicate to be a different microenvironment. To check whether NIT measurements were influenced by environmental effects on plots, ANOVA was run to test differences of means among replicates. A one-way comparison of means was performed. For each measured parameter (Abs, Shf, test weight, percent floaters, NIR, and C-F), means were computed over the 16 hybrids in each replicate. For each parameter, there were therefore three means (one per replicate) to be compared.

ANOVA results are presented in Table II. Differences of Abs and Shf were not significant among replications, but differences were more or less significant for test weight, percent floaters, C-F ratio, and NIR hardness. Therefore, the environmental factors that differed among plots appear to have less effect on Abs and Shf than on the other tests. From this preliminary experiment, it appears that NIT measurements are less sensitive to the block effect, and fewer replicates would be required in a field experiment if hardness were screened for by Abs or Shf. Thus, NIT-based selection could be advantageous during plant breeding. One must bear in mind, however, that this evidence for presumptive independence of Abs and Shf from certain environmental factors was drawn from an experiment in which differences in environment were only due to plot position in the experimental field. These preliminary results warrant further research. Conclusive evidence should be based on trials spanning different locations and years.

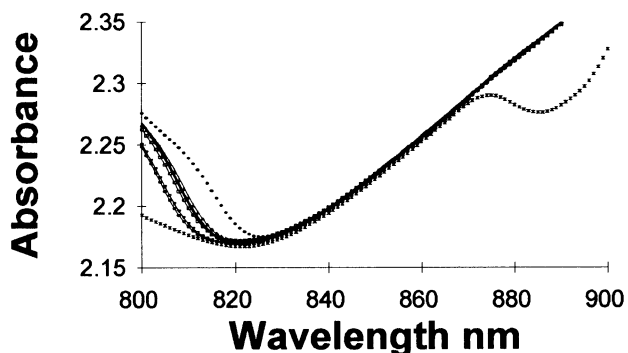


Fig. 5. Absorbance spectra (800–900 nm) of hexane-soluble extracts from maize hybrids (set A).

#### Other Selection Criteria

Work is in progress at our lab testing NIT as a selection criterion. Abs and Shf were determined on kernels from 200 ears of a segregating maize population. One cycle of divergent selection for Abs and Shf split the original population into two groups statistically different for the traits for which they were selected. Groups differing in Abs also differed statistically in NIR hardness, a trait for which no selection was performed. These preliminary results suggest the feasibility of indirect selection for maize kernel hardness through NIT measurement of Abs. Research is continuing to see if, within both groups (high and low NIR hardness), selection for Abs in further cycles is still effective in selecting high and low hardness materials.

Breeders are often more interested in relative ranking of characters under selection than in absolute values. Because of this, when selection is based on NIT, no calibration of the instrument against hardness reference methods would be necessary. To monitor possible year-to-year changes in Abs and Shf values, however, standards of known hardness should be included in each batch of analyses.

#### CONCLUSIONS

Our results suggest that two NIT measurements, Abs (measured NIT absorbance at 860 nm) and Shf (shift of a 620–680 nm absorbance peak) are highly associated with maize kernel hardness, but not with protein, oil, or hexane-soluble materials. Because these NIT measurements are nondestructive, rapid, and only slightly affected by certain environmental factors, they may be applicable to maize breeding.

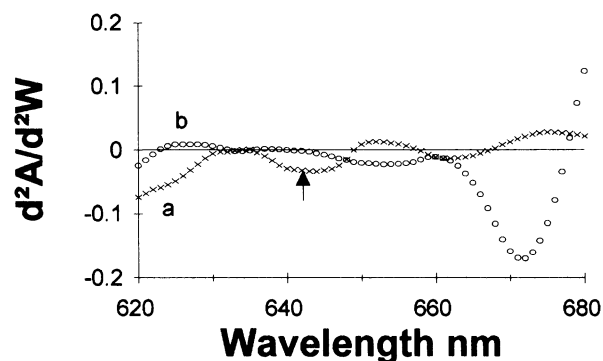


Fig. 6. Second derivative spectra (610–680 nm) of grain of Cargill SD 4 and its hexane-soluble extracts. Arrowhead shows position of shifting peak in grain. a = Grain; b = extract.  $d^2A/d^2W$  = Absorbance second derivative. Gap = 10 nm.

TABLE II  
One Way Comparison of Means Among Replicates for Measured Parameters from 48 Maize Grain Samples

Parameter <sup>a</sup>	Shf <sup>b</sup>	Abs <sup>c</sup>	TW <sup>d</sup>	NIR <sup>e</sup>	C-F <sup>f</sup>	FLO <sup>g</sup>
<i>F</i>	1.02	0.860	3.50	15.75	9.64	2.48
<i>P</i>	0.369	≈1.000	0.038	<0.001	<0.001	0.095
R1	636.6	2.57	76.6	464.8	6.01	16.25
R2	638.4	2.58	76.4	441.3	5.11	19.88
R3	640.2	2.60	75.6	427.3	4.57	26.27
CV	1.13	2.59	1.42	4.30	15.91	61.08

<sup>a</sup> *F* = *F* value for differences among replicates; *P* = significance level for *F*; R1 = first replicate mean; R2 = second replicate mean; R3 = third replicate mean; CV = coefficient of variation, %.

<sup>b</sup> Absorbance wavelength (nm) of shifting peaks.

<sup>c</sup> Absorbance, 860 nm.

<sup>d</sup> Test weight, kg/hl.

<sup>e</sup> Near-infrared hardness; reflectance 1,680 nm.

<sup>f</sup> Coarse-to-fine ratio.

<sup>g</sup> Percent floaters.

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