# Effect of Near-Infrared Transmission-Based Selection on Maize Hardness and the Composition of Zeins[1]

GUILLERMO H. EYHERABIDE,[2,3] JOSE L. ROBUTTI,[2] and FRANCISCO S. BORRÁS[2]

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**ABSTRACT**

Near-infrared reflection (NIR) spectroscopy may be used to estimate cereal endosperm hardness, but it requires grinding samples. Near-infrared transmission (NIT) spectroscopy, however, does not require grinding, and may be used advantageously to select for kernel hardness during breeding. A divergent-selection experiment for endosperm hardness was conducted in a flint breeding population using NIT spectroscopy. Kernel samples from 200 half sib families were analyzed to determine the wavelength of maximum absorbance between 620 nm and 680 nm (NIT1H), and absorbance at 860 nm (NIT2H). Divergent selection for hardness based on NIT1H and NIT2H divided the original population into two statistically different groups for each selected trait. Only divergent selection for NIT2H, however, effectively divided the original population into two groups regarding near-infrared reflection hardness (NIRH). Differences between groups in content of peak 2 (27 kDa γ-zein) were detected by reversed-phase high-performance liquid chromatography. Results indicated the feasibility of selection for endosperm hardness by determining NIT absorbance at 860 nm, and also emphasized the involvement of specific zein proteins in maize endosperm hardness.

Postharvest movement of maize grain during drying, storage, and shipping can damage kernels. Genotypes with softer kernel endosperm suffer greater damage. Endosperm hardness is a trait, among others such as kernel size and breakage susceptibility, that influences the yield of flaking grits. Breeding for reduced maize kernel breakage was suggested by Troyer (1991), who also emphasized the favorable association between kernel flintiness and whole kernels. Johnson and Russell (1982) reported that selection progress for some physical kernel-quality associated traits (such as endosperm type, Stein breakage test, 300-kernel weight, and 300-kernel volume) could be feasible to attain since these traits are under mostly additive genetic control. Large positive correlations between the physical kernel-quality traits measured in hybrids and their parental inbred lines were also detected.

Recently, Lambert and Chung (1995) reported the response to five cycles of selection for increased endosperm hardness modification in two U.S. corn belt *opaque-2* synthetics. Selection was based on a visual estimation of the "percentage of total endosperm area with hard texture" of kernel samples from S1 ears. The selection was effective to increase the percentage of endosperm modification in both populations at a rate of 5–7.4% per cycle. Nevertheless, the modification increase throughout cycles was not even. The authors suggest that the larger response in certain cycles could be mediated by dominant endosperm modifier genes.

Robutti (1995) emphasized that near-infrared reflection (NIR) spectroscopy has been widely used to estimate cereal endosperm hardness, but requires grinding samples. Near-infrared transmission (NIT) spectroscopy, however, does not require grinding, making it potentially advantageous for selection during breeding. This author found two NIT parameters that seemed associated with kernel hardness. The first was the wavelength of maximum absorbance between 620 and 680 nm (NIT1H), and the second was absorbance at 860 nm (NIT2H). Peak absorbance displacement and differences in absorbance among genotypes could be due to similar, but chemically different, compounds that influence texture or to differing amounts of such compounds among genotypes.

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[2]Maize breeder and cereal chemists, respectively, INTA Estación Experimental Agropecuaria Pergamino. CC31, 2700 Pergamino, BA, Argentina.
[3]Corresponsidng author. E-mail: eyherabi@permaiz.inta.gov.ar

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Robutti (1995) analyzed five groups of samples from widely differing sources and studied associations among quality attributes. NIT1H was significantly ($P < 0.01$) correlated with NIRH in all sample groups, while NIT2H correlated significantly with NIRH in four of the five groups. Correlations between NIT1H and NIT2H were significant ($P < 0.001$) in all groups.

The maize endosperm protein soluble in 70% ethanol plus reducing agent is called zein-2 (Z2) (Wilson 1987). The term Z2 will be used from now on to designate the sum of reversed-phase high-performance liquid chromatography (RP-HPLC) areas of peaks 1, 2 and 3, which are equivalent to β- and γ-zeins (Dombrink-Kurtzmann and Bietz, 1993). Zein-1 (Wilson 1987) is the maize protein fraction extracted with 70% ethanol and equivalent to RP-HPLC peaks 4 or α-zein (Dombrink-Kurtzman and Bietz 1993). Z2 is functionally associated with hardness in pellets made from mixtures of different ratios of Z2 and starch (Abdelrahman and Hoseney 1984, Robutti 1992). These authors also reported that increasing the amount of α-zeins in such pellets did not increase hardness, indicating that α-zeins do not interact chemically or physically with starch. These results support the concept that Z2 influences maize endosperm hardness.

Within Z2, the 27 kDa γ-zein (as defined from electrophoresis [Wallace et al 1990]), which is equivalent to RP-HPLC peak 2 (P2) (Paulis et al 1992), is closely associated with kernel texture. These studies showed that, in the same genetic background, modified *opaque-2* kernels have more γ-zein than do their normal or standard *opaque-2* counterparts. Kernels of modified *opaque-2* maize, also called quality protein maize or QPM, have regions of endosperm that have reverted to a flinty appearance, as opposed to the completely floury texture of standard *opaque-2* endosperm. In normal genotypes, the relation of γ-zein (Moro et al 1995) or P2 (Pratt et al 1995) to kernel texture is not so obvious, however. One recent report (Robutti et al 1994) does show a high association between the 27 kDa γ-zein (P2) and certain hardness parameters when the amount of P2 is expressed as percent of Z2 rather than percent of total zeins (TZ), that is the sum of Z2 plus α-zeins.

Robutti et al (1994) suggested that both P2 and α-zeins may jointly influence endosperm texture. Considering the location of γ-zein on the zein body periphery (Geetha et al 1991) and the fact that this protein requires a reducing agent for extraction, Paiva et al (1991) postulated the possibility that γ-zein is involved in disulfide bond interactions that influence kernel hardness. α-Zeins are located in the inner part of the zein body and have a lower sulfur-amino acid content, which would prevent disulfide bond interactions.

α-Zeins thus would act by merely filling voids that would otherwise be empty, giving higher mechanical stability to the endosperm structure. A similar hypothesis has been put forward for sorghum (Mazhar and Chandrashekar 1995); the kafirin mortar and bricks affecting sorghum endosperm hardness may be equivalent to P2 (mortar) and α-zeins (bricks) in maize.

One objective of the present study was to compare the feasibility of using NIT traits (Robutti 1995) to select for endosperm hardness, using NIR as a fundamental hardness reference. We also examined the effect of NIT-based selection on the composition of zeins.

## MATERIALS AND METHODS

### Plant Materials and Selection Procedures

Selection was applied in SR7691(HS)C3. This is a flint breeding population that derives from selection in Composite I, a broad genetic base population. Composite I was derived by intermating orange flint landraces and lines from Argentina with Caribbean germplasm. Four cycles of ear-to-row and full-sib selection for grain yield and standability in Composite I were conducted by INTA (National Institute of Agricultural Research) and CIMMYT (International Wheat and Maize Improvement Center). In the fourth cycle, the top 5% full-sib families were intermated, and the resulting population was named SR7691C0. Three cycles of half-sib selection for grain yield and standability using a single cross as a tester were performed. Selected families were intermated in the nursery using the bulk entry method, followed by another recombination in an isolation plot. Two hundred ears (half-sib families) were collected in the recombination isolation plot corresponding to SR7691(HS)C2 during the 1991–92 cropping season. The families were evaluated by NIT. Two groups, each composed of 10 families whose NIT1H values were at both ends of the frequency distribution, were selected. Similarly two other groups of 10 families were selected based upon NIT2H values. Balanced bulks of seed for each selected group (+NIT1, –NIT1, +NIT2 and –NIT2; plus and minus symbols indicate populations selected on the basis of highest and lowest values of NIT traits) were planted in the breeding nursery at the Experimental Research Station of Pergamino, and plants from each group were intermated. For doing so, two adjacent 10-row (21 plants each) plots were planted. Plants in one plot were detasseled before anthesis and used as female individuals. Bulks of pollen from male plants in the other plot were used to pollinate female plants. Tassels of male plants were removed after being used once. Cross-pollinated ears from each group (30–40) were harvested and hand-shelled. Seeds from each ear (half-sib family) were kept separate and analyzed for NIT1H, NIT2H, and NIRH. Seed from ears from groups selected by NIT2H were analyzed by RP-HPLC.

Before analysis, samples were placed in shallow trays for 20 days. After this period, the extreme values in moisture content were 13.7 and 12.3%. The range in moisture content was considered sufficiently narrow so that corrections for moisture were not necessary.

**TABLE I**
**Means and Confidence Intervals of Hardness of Maize Populations After One Cycle of Divergent Selection Based on Near-Infrared Transmission (NIT) Spectral Characteristics**

| Population | NIT2H[a] | NIT1H[b] | NIRH[c] |
|---|---|---|---|
| NIT2 | | | |
| +NIT2[d] | 2.28a ± 0.03[e] | 631.48a ± 3.19 | 483.19a ± 6.70 |
| –NIT2[d] | 2.24b ± 0.03 | 627.34b ± 3.87 | 493.53b ± 6.47 |
| NIT1 | | | |
| +NIT1[f] | 2.25a ± 0.03 | 631.26a ± 3.20 | 484.06a ± 7.16 |
| –NIT1[f] | 2.22a ± 0.02 | 624.48a ± 2.57 | 489.00a ± 6.08 |

[a] Hardness estimated from absorbance at 860 nm; expressed in absorbance units.
[b] Hardness estimated from wavelength of absorbance peak between 620 and 680 nm, expressed in nm.
[c] Hardness estimated by near-infrared reflection (NIR), expressed in reflectance units (first three significant digits).
[d] Populations selected on the basis of absorbance at 860 nm.
[e] Means followed by the same letter within a column are not significantly different at $P < 0.10$.
[f] Populations selected on the basis of wavelength at which an absorbance peak occurs between 620 and 680 nm.

**TABLE II**
**Analysis of Variance for Hardness Estimates of Maize Populations After One Cycle of Divergent Selection Based on Near-Infrared Transmission (NIT) Spectral Characteristics for NIT2H**

| | | NIT2H[b] | | NIT1H[c] | | NIRH[d] | |
|---|---|---|---|---|---|---|---|
| Source of Variation | DF[a] | Mean Square | $P > F$ | Mean Square | $P > F$ | Mean Square | $P > F$ |
| +NIT2 vs. –NIT2[e] | 1 | 0.0346 | 0.052 | 310.16 | 0.097 | 1,942.11 | 0.031 |
| Within populations | 72 | 0.0088 | ... | 109.33 | ... | 402.12 | ... |
| Total | 73 | ... | ... | ... | ... | ... | ... |

[a] Degrees of freedom.
[b] Hardness estimated from absorbance at 860 nm.
[c] Hardness estimated from wavelength of maximum peak absorbance between 620 and 680 nm.
[d] Near-infrared reflectance hardness.
[e] Populations selected after one cycle of divergent selection for NIT2H.

### NIR Spectroscopy

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, and values of NIT1H and NIT2H were determined. NIRH was determined according to Pomeranz et al (1984) for samples (≈50 g) ground in a model 3600 Falling Number laboratory mill. The tightest (No. 0) setting was used since it gave better differentiation than the No. 3 setting. The Student's *t*-test values for the difference between the means of the 10 highest and the 10 lowest NIRH values were significant at $P = 0.008$ when setting No. 3 was used and at $P = 0.0000$ when setting No. 0 was used. Reflectance was then measured at 1,680 nm in a Trebor 7700s instrument, taking the first three significant, nonzero digits of the instrument reading as a measure of hardness. Higher readings indicated harder samples. Correlation analysis performed on data from the maize sample sets reported elsewhere (Robutti 1995) gave correlation coefficients between NIRH and percent floaters and coarse-to-fines ratio that were significant at $P = 0.005$ and $P < 0.001$, respectively, for set A; $P < 0.001$ and $P < 0.001$, respectively, for set B; $P = 0.005$ and $P = 0.006$, respectively, for set C; $P < 0.001$ and $P < 0.001$, respectively, for set D; and $P = 0.122$ and $P = 0.069$ for set E.

### Zein Extraction

Ten kernels of each half-sib family from +NIT2 and –NIT2 selected groups were soaked in water for 10 min, and endosperms were dissected from the seeds. Air-dried endosperms were ground in a Udy cyclone mill through a 0.5-mm screen. Alcohol-soluble proteins were quantitatively extracted at room temperature by vortex shaking for 2 hr with 70% (v/v) ethanol containing 0.5% (w/v) sodium acetate and 0.2% (w/v) dithiothreitol (DTT) using 0.2 g of meal per 3 ml of solvent. Suspensions were then centrifuged for 30 min at 3,000 × *g*, and supernatants filtered (0.2 μm). After 24 hr, TZ were analyzed by RP-HPLC.

### RP-HPLC

Amounts of α-zeins, Z2, TZ, and P2 were determined by RP-HPLC using a Hewlett-Packard 1050 HPLC system with a quaternary pump, autosampler, and UV detector. Chromatography was as described by Paulis and Bietz (1986) with minor changes.

The column used, at 60°C, was a Vydac C18, (4.6 × 250 mm, 5 µm particles, 300Å pores), preceded by a 22 × 3.5-mm precolumn. Samples (5 µl) were eluted at 1 ml/min with a linear 50 min 28% to 60.5% B gradient (solvent A, water + 0.1% [v/v] trifluoroacetic acid; solvent B, acetonitrile + 0.1% [v/v] trifluoroacetic acid), followed by 10-min isocratic elution at 60.5% B. Detection was by UV absorbance at 210 nm. Quantitative analysis was performed using HP ChemStation 3.0 software. Duplicate extracts were analyzed; determined retention times and peak areas were reproducible within limits found in earlier experiments. These earlier experiments, run on six replicates from a maize endosperm sample, indicated a maximum difference of 0.24 min for retention times and a coefficient of variation in all cases <2% for absolute peak areas. Peaks having relative areas >2% were taken for this analysis.

### Statistical Procedures

After a cycle of divergent selection for NIT1H and NIT2H, total variance for each pair of selected populations (+NIT1/–NIT1 and +NIT2/–NIT2), was divided into variance between and within selected populations. Analyses of variance were made for all evaluated traits. The within populations mean square was the denominator of *F*-tests to prove significance of differences between selected populations because this is a 1 DF contrast ($F = t^{0.5}$). *F* and *t* values both give the same probability level. Differences between selected populations were declared significant when $P < 0.10$. Confidence intervals ($P = 0.95$) were computed for each selected population. Phenotypic correlation coefficients among variables were also estimated.

## RESULTS AND DISCUSSION

Table I lists observed means and confidence intervals ($P = 0.95$) for hardness estimates (NIT1H, NIT2H, and NIRH) of maize populations selected on the basis of NIT spectral characteristics (+NIT1, –NIT1, +NIT2, –NIT2).

### Direct Responses to Selection

One cycle of divergent selection for NIT2H caused significant differences ($P < 0.052$) between +NIT2 and –NIT2 for NIT2H (Table II). Similarly, populations selected on the basis of NIT1H (+NIT1 and –NIT1) differed significantly ($P < 0.002$) for NIT1H (Table III). Direct selection among half-sib families was thus effective for modifying levels of NIT1H and NIT2H.

### Indirect Responses to Selection

Significant differences were detected between +NIT2 and –NIT2 for NIT1H ($P < 0.09$) (Table II), but no difference seemed to exist between +NIT1 and –NIT1 for NIT2H (Table III). Selection for NIT2H also caused +NIT2 and –NIT2 to differ in NIRH ($P < 0.031$) (Table II), while selection for NIT1H (+NIT1 and –NIT1) did not cause significant differences in NIRH (Table III).

**TABLE III**
**Analysis of Variance for Hardness Estimates of Maize Populations After One Cycle of Divergent Selection Based on Near-Infrared Transmission (NIT) for NIT1H**

| | | Traits | | | | | |
|---|---|---|---|---|---|---|---|
| | | NIT2H[b] | | NIT2H[c] | | NIRH[d] | |
| Source of Variation | DF[a] | Mean Square | *P* > F | Mean Square | *P* > F | Mean Square | *P* > F |
| +NIT2 vs. –NIT2[e] | 1 | 0.0134 | 0.198 | 711.29 | 0.002 | 377.56 | 0.288 |
| Within populations | 60 | 0.0079 | ··· | 64.66 | ··· | 327.90 | ··· |
| Total | 61 | ··· | ··· | ··· | ··· | ··· | ··· |

[a] Degrees of freedom.
[b] Hardness estimated from absorbance at 860 nm.
[c] Hardness estimated from wavelength of maximum peak absorbance between 620 and 680 nm.
[d] Near-infrared reflectance hardness.
[e] Populations selected after one cycle of divergent selection for NIT1H.

### Correlation Analysis

Phenotypic correlation coefficients among NIT1H, NIT2H, and NIRH were significant ($P < 0.0001$) in all instances. The correlation coefficient of NIT1H with NIRH was –0.365, and the correlation coefficient of NIT2H with NIRH was –0.369. The correlation between NIT1H and NIT2H was 0.844.

Both divergent selection cycles for NIT hardness divided the original population into two statistically different groups for the selected traits. The relative magnitude of the within-groups component of variance reflects the level of variability exhibited by each group due to genetic and environmental confounded effects.

### Zein Profiles

Zein compositions were estimated from absolute integrated RP-HPLC areas. Since only selection for +NIT2H caused +NIT2 and –NIT2 to differ in NIRH, only the zein profiles of those populations were studied.

Figure 1 shows the zein RP-HPLC typical chromatograms of a sample from a +NIT2 and a sample from a –NIT2 half-sib family. Zein classes are indicated on the chromatogram. Table IV presents analyses of variance for contents of α-zeins, Z2, and P2, and for P2 expressed as percentage of TZ (P2/TZ) and of Z2 (P2/Z2). Differences between +NIT2 and –NIT2 for content of α-zeins and Z2 were not significant. A highly significant difference in Z2 composition was detected between populations selected on the basis of NIT2H. Thus, Z2 from the –NIT2 population had a greater percentage of P2 than did Z2 from +NIT2. Absolute or relatively greater contents of P2 were associated with increased endosperm hardness, as determined by NIRH. Observed means

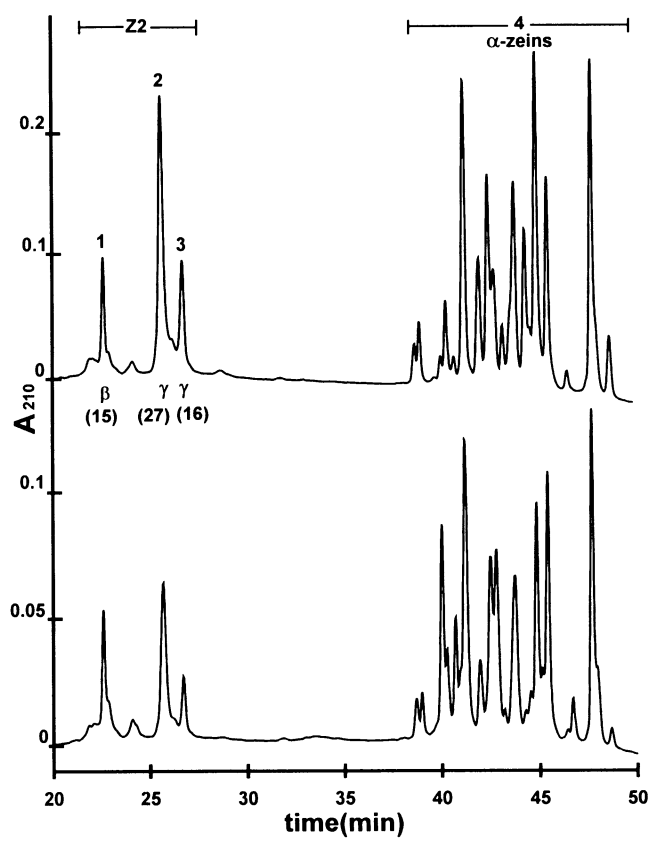


**Fig. 1.** Reversed-phase high-performance liquid chromatograms of alcohol-soluble proteins from a +NIT2 (bottom) and a –NIT2 (top) half-sib family.

TABLE IV
Analysis of Variance for Populations After One Cycle of Divergent Selection for NIT2H

| Source of Variation | DF[a] | Traits[b] α Mean Square ×10⁻⁵ | α P > F | Z2 Mean Square ×10⁻⁵ | Z2 P > F | P2 Mean Square ×10⁻⁵ | P2 P > F | P2/TZ Mean Square | P2/TZ P > F | P2/Z2 Mean Square | P2/Z2 P > F |
|---|---|---|---|---|---|---|---|---|---|---|---|
| +NIT2 vs –NIT2 | 1 | 25.5 | 0.68 | 17.4 | 0.17 | 20.1 | 0.01 | 45.4 | 0.01 | 574.5 | 0.00 |
| Within populations | 65 | 151.8 | ··· | 9.1 | ··· | 2.9 | ··· | 7.0 | ··· | 41.8 | ··· |
| Total | 66 | ··· | ··· | ··· | ··· | ··· | ··· | ··· | ··· | ··· | ··· |

[a] Degrees of freedom.
[b] Traits examined were absolute integrated RP-HPLC areas of α–zein (α), zein-2 (Z2), and peak 2 (P2), and the percent ratios P2/TZ and P2/Z2.

TABLE V
Means and Confidence Intervals for Absolute Integration Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) Areas Corresponding to α-Zein, Zein-2, and Peak 2 Areas Expressed as Percentages of Total Zein (%TZ) and of Zein-2 (%Z2) for Popularions After One Cycle of Divergent Selection for NIT2H[a]

| Traits | Selected Populations +NIT2 | Selected Populations –NIT2 |
|---|---|---|
| α-zein | 17,812a ± 4,072 | 17,416a ± 3,635 |
| Zein-2 | 4,271a ± 980 | 4,598a ± 910 |
| Peak 2 | 1,607a ± 588 | 1,959b ± 455 |
| Peak 2 (%TZ) | 7.45a ± 2.77 | 9.12b ± 2.45 |
| Peak 2 (%Z2) | 36.83a ± 6.07 | 42.76b ± 6.98 |

[a] Means followed by the same letter within a row are not significantly different at the probability levels shown in Table IV

for zein fractions of the NIT2H selected populations are listed in Table V.

### NIT2H Heritability

Levels of NIT1H and NIT2H for the original set of families during the 1991–92 season (cycle 0) were outside the range of values observed for their descendants during 1992–93 (data not shown). Therefore, it was not possible to determine realized heritability for unidirectional selection. Nevertheless, response to the first cycle of selection could be estimated from divergence within each pair of selected populations. Selection on the basis of NIT2H resulted in two subpopulations differing in NIRH. This suggests a high genetic correlation between NIT2H and NIRH. The ratio of divergence between +NIT2 and –NIT2, and the differential of divergent selection applied on the base population, gives an estimate of realized heritability of 0.33 on an individual plant basis. Since the selection experiment was conducted for one cycle, inbreeding effects should not have been important. Systematic changes due to environment could have occurred. Consequently, the estimate of realized heritability may not reflect the real heritability in the original population (Falconer 1981). However, the magnitude of the response to one selection cycle suggests that it is feasible to modify endosperm hardness using nondestructive NIT2 hardness scores as a selection criteria.

## CONCLUSIONS

This study indicates the feasibility of selecting for endosperm hardness by determining whole kernel absorbance at 860 nm in population SR7691. It also provides new evidence about the involvement of P2 (27 kDa γ-zein) in maize endosperm hardness.


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