Evaluation of Selected Baking Quality Factors of Hard Red Winter Wheat Flours by Two-Dimensional Electrophoresis[1]

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ABSTRACT

Cereal Chem. 67(6):564–569

A two-dimensional electrophoretic technique, utilizing nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension, was used to separate the proteins in nonfractionated extracts of wheat flour. Individual proteins were quantitated by digital image analysis after staining with Coomassie Blue R250. This procedure was applied to flours experimentally milled from 14 varieties of hard

red winter wheat that possessed widely variable mixing and baking characteristics. Three individual proteins were found to be highly correlated with mixograph peak time, and two individual proteins were significantly correlated with loaf volume. Using data from these proteins, multiterm linear regression equations were developed to relate loaf volume and mixing time to protein composition. High correlations ($R > 0.93$) were obtained in both cases.

The endosperm proteins of wheat are largely responsible for the unique breadmaking properties of this cereal grain. The formation of gluten, which is of primary importance to production of leavened bread, results from a complex interaction of many protein components. The technique of two-dimensional (2-D) electrophoresis, combined with image analysis, has the potential to separate and quantitate most endosperm proteins and may thus serve as a useful tool in determining those proteins most important to the breadmaking quality of wheat.

Several researchers (Burnouf and Bouriquet 1980; Payne et al 1981a, 1984a; Paradies and Ohms 1984; Branlard and Dardevet 1985b; Moonen and Zeven 1985; Campbell et al 1987; Lawrence et al 1987, 1988) have studied high molecular weight (HMW) glutenin subunits and their relation to the breadmaking potential of a flour. These studies have been reviewed by Pomeranz (1988) and by Wrigley and Bietz (1988). Several proteins, as separated by one-dimensional electrophoretic techniques, have been associated with good or poor breadmaking characteristics. Particular interest has centered around HMW glutenin subunits 5 + 10, and 2 + 12 (nomenclature of Payne et al 1980, 1981a, 1981b), which are associated with resistance to dough extension and dough weakness, respectively. Payne et al (1984a) also included subunits 1, 17, and 18 as being important to flour quality and designed a scoring system for evaluating the baking quality of English and Spanish wheats (Payne et al 1987, 1988) based on the presence or absence of certain protein bands as separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Individual gliadins have also been found to be associated with desirable and undesirable breadmaking characteristics (Sozinov and Poperelya 1980; Wrigley et al 1982; Branlard and Dardevet 1985a, 1985b; Peruffo et al 1985; Sasek et al 1986; Campbell et al 1987; Cressey et al 1987), although there is apparently less agreement on which specific gliadins are influential (Wrigley and Bietz 1988). Specific gliadins have been found that appear to be related to the cooking quality of pasta made from durum wheat (Damidaux et al 1980, Kosmolak et al 1980, Payne et al 1984b, DuCros 1987).

This article describes our investigations using 2-D electrophoresis, coupled with image analysis, to determine which polypeptides may serve as indicators of breadmaking quality. For this study, endosperm proteins from 14 hard red winter (HRW) wheat varieties were separated by 2-D electrophoresis. In addition,

the breadmaking qualities of the wheat flours were objectively evaluated and the results compared to the polypeptide profiles obtained from quantitative image analysis of the electrophoretic gels after staining with Coomassie Blue R250.

MATERIALS AND METHODS

Wheat Flour Samples

The wheat samples selected for analysis represented 14 varieties of HRW wheat with wide genetic variability, as evidenced by allelic differences at the Glu-B1 and Glu-D1 loci, and were chosen to represent a wide range of loaf volumes and mixing times (Table I). Wheat varieties Delta, Jasen, and MV14 were originally from Poland, Bulgaria, and Hungary, respectively, and were grown in Arizona. The other 11 wheats included both commercial and experimental varieties grown as part of the 1986–1987 Nebraska varietal tests. For each variety, samples were obtained from five different locations in Nebraska, representing the southeast (Gage County), south central (Clay County), central (Nance County), southwest (Lincoln County), and panhandle (Scottsbluff County) regions of the state. Both dryland and irrigated plots in Lincoln County were represented. Samples of a given variety obtained from the different Nebraska locations were combined to minimize environmental factors, so that quality variation was attributable to primarily genetic differences. Baking characteristics of the three samples grown in Arizona were likely influenced by both genetic and environmental factors. All varieties were milled on a Buhler mill to obtain a straight grade flour. Before milling, the wheats were tempered to 15.2% moisture, with the exception of MV14,

TABLE I
Protein Contents, Mixograph Peak Times, Corrected Loaf Volumes, and Total Scores of the 14 Hard Red Winter Wheat Flours Evaluated

Variety	Percent Protein[a]	Mixograph Peak Time (min)	Loaf Volume[b] (cm³)	Total Score[c]
Scout 66	12.2	3.25	811	8.7
7805	10.9	6.50	931	7.7
7833	11.3	2.75	967	9.9
Bennett	13.4	3.10	869	9.7
Cody	12.1	4.60	907	8.7
Arapahoe	12.6	4.25	829	8.7
Redland	10.8	4.75	1,000	10.7
Victory	11.6	2.50	826	7.7
Buckskin	10.9	4.25	968	9.0
Centurk 78	12.0	4.50	939	9.6
Delta	13.3	2.00	628	4.0
Jasen	12.4	3.50	811	8.3
MV14	13.2	1.80	633	3.0
Rodeo	10.9	2.30	837	7.0

[a]Kjeldahl nitrogen × 5.7 on 14% moisture basis.
[b]Expressed on a uniform protein basis (12%).
[c]Summation of external, internal, and texture scores.

which was tempered to 15.4% moisture, based on kernel hardness evaluation (Mattern 1988). Flour yields ranged from 69.3 to 75.3%.

Mixograph Mixing Time

A 10-g mixograph (National Manufacturing, Lincoln, NE) was used to evaluate flour mixing times. All flours were weighed on a 14% moisture basis and run at 60% absorption. The time in minutes to reach the mixograph peak was recorded as mixing time.

Baking and Protein Analysis

Bread was baked from the flour samples according to AACC Method 10-10A (1983), using optimum bromate, mixing time, and absorption levels. The loaf volumes (cm^3), as determined by rapeseed displacement, were adjusted to express loaf volume on a uniform protein basis by dividing by the flour protein content (Kjeldahl N × 5.7, AACC method 46-12), then multiplying by 12. This minimized the effect of protein variation on loaf volume.

Electrophoresis

A 2-D electrophoresis system consisting of nonequilibrium pH gradient electrophoresis (NEPHGE), followed by SDS-PAGE, was used in these separations. The method is described in detail by Dougherty et al (1989), but will be briefly summarized here. The NEPHGE step incorporated high-field strengths (300 V/cm) and small-diameter (1.0-mm diameter, 100-μl volume) capillary tubes to achieve high resolution with reduced separation time. Samples were prepared from unfractionated wheat flour by overnight extraction in 9.0M urea containing 1.0 mM dithiothreitol and 2% (w/v) 3-[(3-cholamidopropyl)dimethyl-ammonio] 1-propanesulfonate (CHAPS), then stored at −70°C. Before electrophoresis, each sample was thawed and an ampholyte mixture, containing 50% pH 5.0–8.0 and 50% pH 8.0–10.5 (Pharmalyte brand, Sigma Chemical Co., St. Louis MO), was added to 100 μl of extract so that the final ampholyte concentration was 2%. NEPHGE was performed with gels containing 4% acrylamide (ratio of bisacrylamide to acrylamide = 1:38), 9.0M urea, 2% CHAPS, and 2% ampholytes, with the same ampholyte composition as in the sample. The gels were loaded with 10 μl of sample (containing about 45 μg of protein), then overlayed with 10 μl of 9.0M urea containing 2.5% ampholytes. The lower chamber (cathode) was filled with freshly degassed 0.01M NaOH, and the upper chamber (anode) contained 0.01M H$_3$PO$_4$. The NEPHGE separation was performed with a programmable power supply (ISCO, Lincoln, NE), which gradually increased the applied voltage to 3,000 V and continued application until a total of 4,800 V-hr was reached. Prefocusing was not found to be necessary. Immediately upon termination of the NEPHGE separation, the gel tubes were removed from the chamber and stored at −20°C.

The second-dimension separation (SDS-PAGE) was performed in a minigel format (70 × 80 × 1.0 mm) (Biorad Laboratories Richmond, CA). Using steady air pressure from a syringe, a thawed gel was ejected from the tube into a small trough containing 5.0 ml of SDS equilibration buffer (2.3% SDS, 5% mercaptoethanol, 10% glycerol, and 0.0625M Tris-Cl at pH 6.8), and allowed to equilibrate for 15 min. The SDS-PAGE system was essentially that described by Laemmli (1970) and consisted of 4% stacking and 12% separating gels using a 1:50 bisacrylamide to acrylamide ratio. The second-dimension separation was performed at 200 V (constant voltage) until a prestained molecular weight marker (lactic dehydrogenase, Sigma Chemical Co., St. Louis, MO) reached 1.6 cm from the bottom of the gel, approximately 55 min. Gels were stained with 0.1% Coomassie Blue R250 (Sigma Chemical Co., St. Louis, MO) in 10% acetic acid/5% methanol for 1 hr, then destained overnight with 5% acetic acid/7.5% methanol.

Image Analysis

Image analysis was performed as described by Dougherty et al (1989) using a BioImage (Kodak, Ann Arbor, MI) Visage 110 machine vision analyzer. The image was acquired through a 512

× 512, 8-bit video camera. Analysis was performed using the BioImage EQ 2-D electrophoresis pattern analysis software in a Sun 110/3 supermicro computer. The software captured the gel image and constructed a data base referred to as the spot list, in which the x/y location, area, and shape of each spot was reported. In addition, the integrated intensity (absorbance × mm^2) was calculated for each spot. To reduce variation introduced by inconsistencies in sample loading and/or staining, integrated intensities were summed and the percent integrated intensity (percent II) of each spot was calculated. The computer-generated image was also recorded using a Gould 6320 colorwriter, with each spot plotted as a regular ellipse.

Three gels from each variety were prepared. All varieties were compared to Scout 66, which was selected as the reference since it has been widely grown in the central United States for several years and its baking characteristics and genetic background are well characterized. Spatial differences between a study and reference gel were mathematically corrected by a computer algorithm after manually identifying a limited number of spots that appeared on both gels. A computer program then identified all spots on a study gel that had an equivalent spot on the reference gel, based on the x/y location of spot centers. The available software allowed us to evaluate the percent II of any spot on a study gel that corresponded to one of the 99 spots present on the Scout 66 reference.

Data Analysis

For each variety, mean percent II values were calculated for each polypeptide spot present on the three replicate gels. Correlations of mean percent II values with loaf volume and peak mixing time were then calculated for each of the 99 polypeptides identified. The correlations were tested for significance at the 1 and 5% levels.

The practicality of using electrophoretic data to estimate functional qualities was further evaluated by multiple linear regression (MLR) analysis. Spots with correlations significant at the 1% level were included in MLR models relating percent II values to loaf volume and peak mixing time. The utility of the regression equations was assessed by evaluating the F of regression values for significance at the 1% level and by observing the multiple correlation coefficients (R) and standard errors of estimate (SEE). Contributions of individual polypeptides to the MLR models were evaluated by use of partial F-tests. Spots with F-values significant at the 10% level were retained in the regression equations.
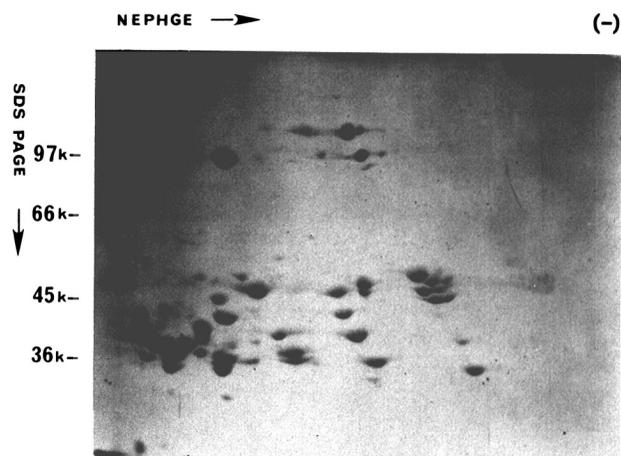

Fig. 1. Two-dimensional nonequilibrium pH gradient electrophoresis/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (NEPHGE/SDS-PAGE) separation of Scout 66 endosperm proteins. Electrophoretic separations were conducted as described in Materials and Methods. Positions of molecular weight markers are indicated at the left and are: phosphorylase B, 97,000; bovine serum albumin, 66,000; ovalbumin, 45,000; and lactic dehydrogenase, 36,000. (Reprinted from Dougherty et al 1989; published with permission of Elsevier Publishing)

RESULTS AND DISCUSSION

The 2-D electrophoretic separation of endosperm proteins from the variety Scout 66 is shown in Fig. 1 and illustrates the resolution achieved with the methods described above. The use of high field strengths in the NEPHGE step enhanced resolution while reducing the separation time to 2.3 hr. Some polypeptides that migrate as a single band in one-dimensional electrophoresis appear to separate into multiple subunits based on charge difference. Equivalent separations were obtained for the other varieties, but those are not shown here. NEPHGE separated a majority of the endosperm proteins and was therefore used as the sole first-dimension separation mechanism, to simplify the image analysis. Polypeptides were stained with Coomassie Blue R250. Although silver staining has been shown to visualize more of the minor protein components than does Coomassie Blue (Dunbar et al 1985, Tkachuk and Mellish 1987), the numerous colors obtained with silver-stained wheat proteins make quantitation by image analysis difficult (Tkachuk and Mellish 1987). Coomassie Blue was selected for this study, as it visualized most of the major protein components that were most likely to be related to mixing and baking characteristics and provided reasonably good reproducibility of quantitation (14.8% relative standard deviation of replicate measurements). Image analysis was used to record and compare the separation patterns of all varieties; the computer-generated image of Scout 66 is shown in Fig. 2. In this study, endosperm proteins were not fractionated by differential solubility before electrophoretic separation. However, the protein separation patterns reported here are similar to those previously observed by Payne et al (1985), who did make classifications into various solubility fractions. Therefore references to protein classes in the following discussion are based upon visual comparison of our separations to those results, and classifications should be considered as tentative assignments pending confirmation.

Loaf Volume

Polypeptides correlated to loaf volume are shown with vertical cross-hatching in Fig. 2. The percent II values of spots 10 and 39 were significantly correlated to adjusted loaf volume at the 1% level ($r = -0.917$ and $r = 0.745$, respectively) (Figs. 3 and 4). When the intensity values of these two spots were combined in a two-term MLR equation, an R of 0.941, with a SEE of 41.85 cm^3, resulted. The following regression equation was obtained:

$$\text{Adjusted loaf volume (cm}^3) = 915.76 + 17.25 \, (\% \, \text{II}_{\text{spot 39}}) - 554.29 \, (\% \, \text{II}_{\text{spot 10}}).$$

The calculated $F = 42.63$ for regression exceeded the tabulated value $F(2, 11, 0.99) = 7.21$, indicating that the equation is a good predictor. Partial F-values for spots 10 and 39 were calculated ($F = 80.86$ and 4.40, respectively). The F-value for spot 39 exceeded the tabulated value $F(1, 11, 0.90) = 3.23$, indicating that inclusion of spot 39 was useful to the model. A plot of actual loaf volumes compared to values estimated from the regression equation, as presented in Fig. 5, shows no obvious
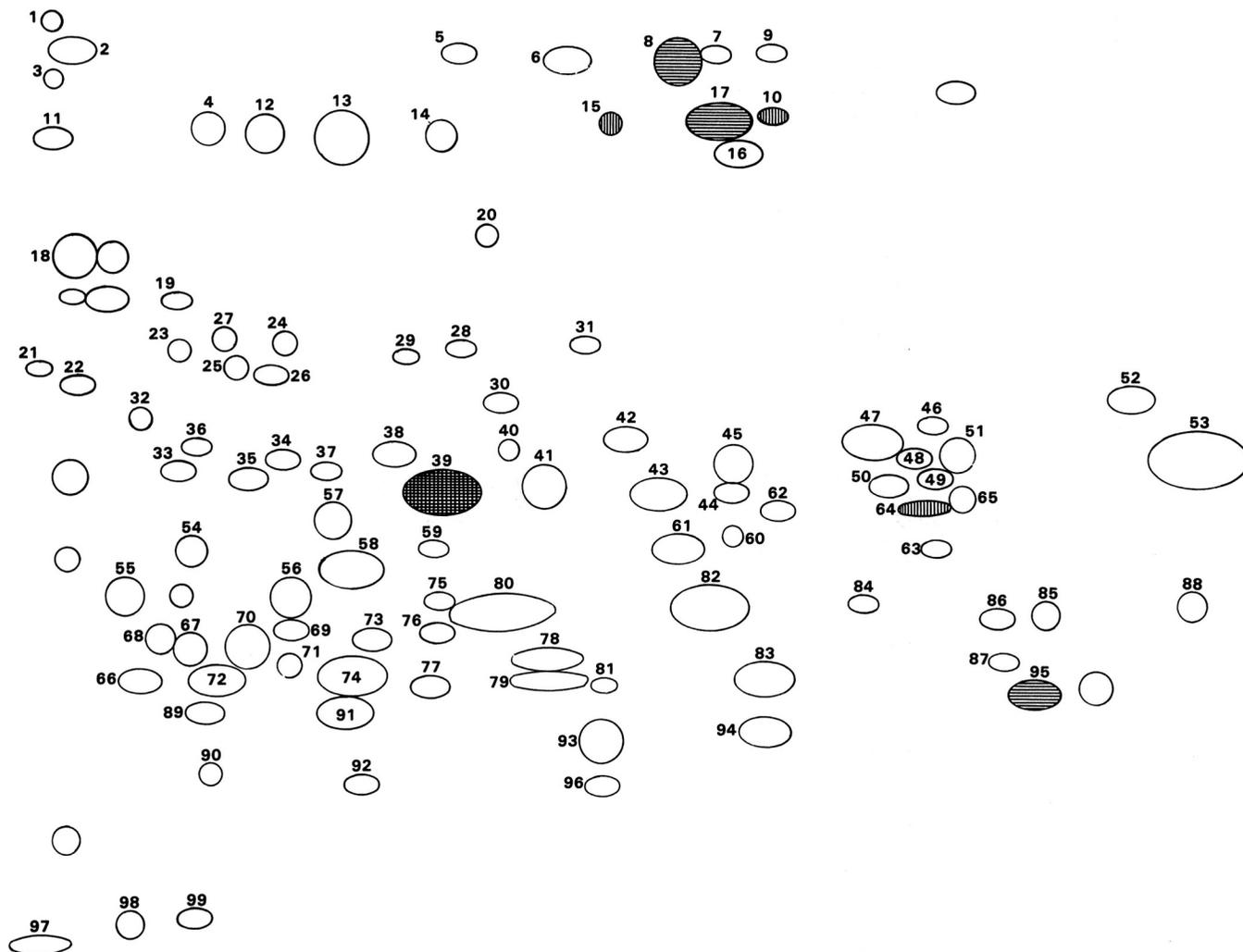

Fig. 2. Computer-generated plot from image analysis of nonequilibrium pH gradient electrophoresis/sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel containing Scout 66 endosperm proteins. The image analysis program assigned numbers (1–99) to each of the spots as indicated. Polypeptide spots shown with vertical cross-hatch are correlated with loaf volume. Polypeptide spots shown with horizontal cross-hatch are correlated with mixing time.

outliers.

Other polypeptides with correlations to adjusted loaf volume significant at the 5% level were spot 15 ($r = -0.633$) and spot 64 ($r = 0.631$).

Spot 10 is tentatively identified as a HMW glutenin coded by chromosome 1B. Its percent II ranged from 0 to 0.55%, and the spot was small in area when present (Figs. 1 and 2). Spot 39 was located in the region assigned to α-, β-, and γ-gliadins and had a percent II ranging from 0 to 6.29. Spot 15, tentatively a HMW glutenin coded by chromosome 1B, had a 0–0.26% II range, and spot 64, tentatively a low molecular weight (LMW) B glutenin (Jackson et al 1983), ranged from 0 to 2.54% II. There was a significant correlation at the 1% level between the percent IIs of spots 10 and 15 ($r = 0.775$).

Mixograph Mixing Time

Polypeptides significantly correlated to mixing time are identified by horizontal cross-hatching in Fig. 2. Spots 17 and 8 were negatively ($r = -0.893$ and -0.767, respectively) correlated to mixograph peak time, whereas spot 39 was positively correlated ($r = 0.828$) (Figs. 6–8). Correlations for these three polypeptides were significant at the 1% level. When the percent II data from these three spots were combined in a MLR model, the partial F-test for spot 8 resulted in a value of 0.11. This value does not exceed the tabulated value at the 10% level, $F(1, 10, 0.90) = 3.29$, indicating that spot 8 provided little useful information to the model. This is likely due to a high intercorrelation ($r = 0.882$) between spots 17 and 8. Use of a two-term MLR that included percent II values from only polypeptide spots 17 and 39 resulted in an R equal to 0.932 and a SEE = 0.513 min.

The regression equation was:

$$\text{Peak time (min)} = 4.935 + 0.276\,(\% \, \text{II}_{\text{spot 39}}) - 1.367\,(\% \, \text{II}_{\text{spot 17}}).$$

The calculated $F = 36.64$ for regression exceeded the tabulated value $F(2, 11, 0.99) = 7.21$, indicating that the model should be useful for prediction. Partial F-values for spots 17 and 39 (67.25 and 6.02, respectively) both exceeded the tabulated value $F(1, 11, 0.90) = 3.23$ and indicated that both spots provided useful information. A comparison of actual mixing times with
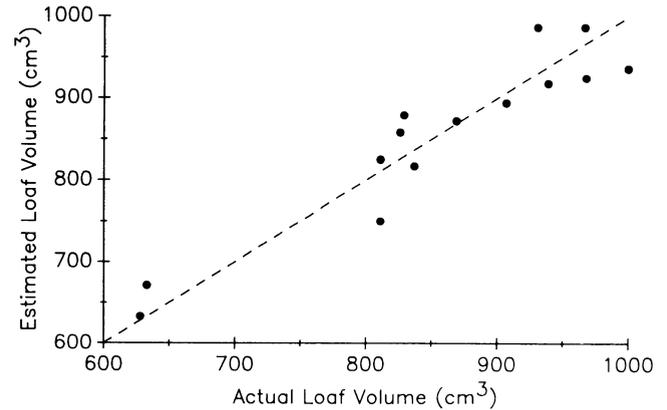

Fig. 5. Scatter diagram comparing the actual adjusted loaf volumes for 14 wheat varieties to the values estimated for those samples by a two-term multiple linear regression model.
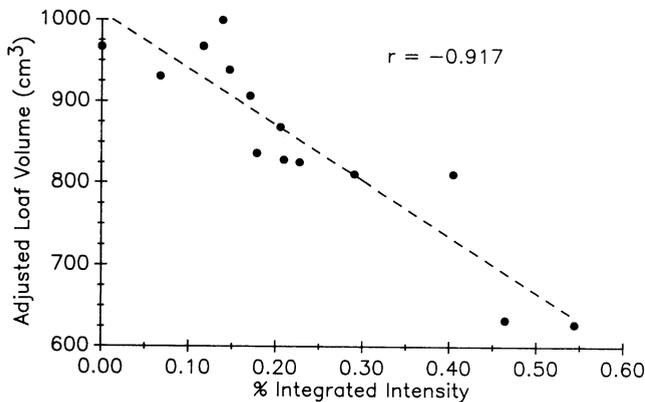

Fig. 3. Scatter diagram indicating the relationship between percent integrated intensity of the endosperm polypeptide identified as Spot 10 and adjusted loaf volume for 14 wheat varieties.
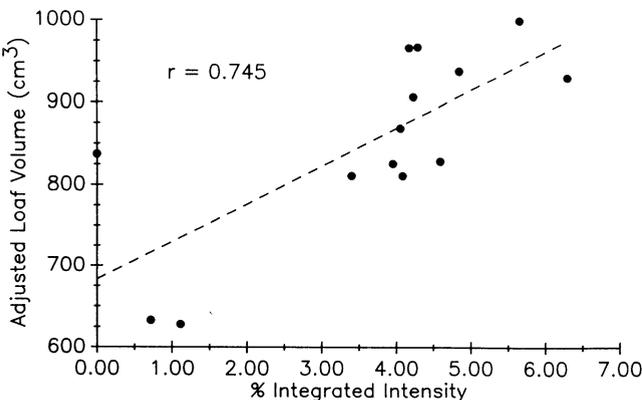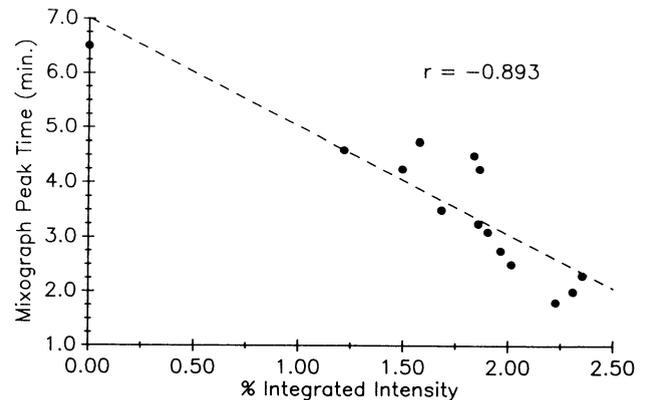

Fig. 6. Scatter diagram indicating the relationship between integrated intensity of the endosperm polypeptide identified as Spot 17 and mixograph peak time for 14 wheat varieties.
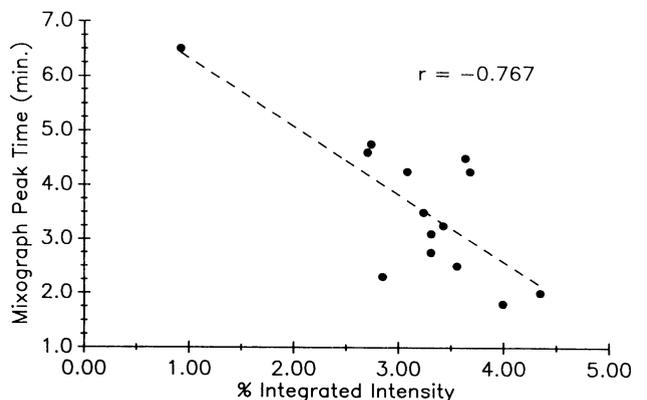

Fig. 4. Scatter diagram indicating the relationship between percent integrated intensity of the endosperm polypeptide identified as Spot 39 and adjusted loaf volume for 14 wheat varieties.

Fig. 7. Scatter diagram indicating the relationship between percent integrated intensity of the endosperm polypeptide identified as Spot 8 and mixograph peak time for 14 wheat varieties.

estimated mixing time values (Fig. 9) demonstrated a reasonable fit for all points.

Additionally, spot number 95 ($r = 0.630$) was positively correlated with mixing time at the 5% level.

Spots 8 and 17 are considered to be HMW glutenins coded by chromosome 1B. Spot number 17 ranged from 0 to 2.35% II, whereas spot 8 had a percent II in the 0.92–4.35 range. Spot 95 is tentatively identified as a LMW B glutenin, according to the terminology of Jackson et al (1983), with a percent II range of 0–3.32. The LMW B glutenins are a major group of very basic subunits.

The results of this initial work indicate that 2-D electrophoresis, coupled with image analysis, may be useful in identifying individual proteins associated with baking quality factors. As the mixing times and loaf volumes of a limited number of HRW wheat flour samples varied due to genetic and environmental factors, the quantities of certain polypeptides present were found to be highly correlated with these quality factors. The polypeptide identified as spot 39 appears to increase both loaf volume and mixing time, or to be associated with a factor that has this effect. In general, wheat flours with very short mixing times also tend to produce loaves of poor volume. The positive correlation of spot 39 to both mixing time and loaf volume may help to explain this relationship. When only the 11 Nebraska-grown samples are considered, thereby minimizing environmental differences, the correlation of percent II of spot 39 with mixing time remains significant at the 1% level ($r = 0.742$). However, the correlation with loaf volume is no longer significant when only these 11 samples are considered ($r = 0.482$). This indicates that environmental conditions may substantially affect both loaf volume and the quantity of this protein fraction. Previously, Huebner and Bietz (1986) showed that baking score, and the quantity of a
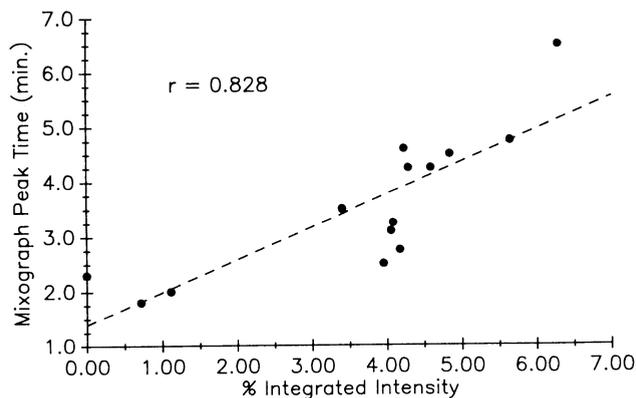
gliadin fraction isolated by high-performance liquid chromatography that was inversely related to baking score, were both influenced by environmental differences but not to the same degree.

In contrast to spot 39, the presence of spots 10 and 17 may serve as markers for decreased loaf volume and shortened mixing times, respectively. Even when only the 11 Nebraska-grown samples are considered, the correlations involving these two spots are still significant at the 1% level ($r = -0.785$ for percent II of spot 10 and loaf volume; $r = -0.888$ for percent II of spot 17 and peak mixing time). Both spots 10 and 17 appear to be HMW glutenin subunits coded by chromosome 1B. Previous one-dimensional electrophoretic studies have shown a qualitative relationship between the presence of certain HMW subunits coded by this chromosome and baking quality of bread (Payne et al 1984a, 1987). Our results appear to indicate that a quantitative relationship may exist as well. Other workers have also found a qualitative relationship between HMW glutenin subunits coded by chromosome 1D (5 + 10 and 2 + 12 according to the nomenclature of Payne) and baking quality. In our study, spots 12 and 13, which appear to be HMW glutenins associated with the 1D chromosome, showed no relationship with either loaf volume or mixing time. However, additional subunits coded by this chromosome do not appear on the NEPHGE gel and are yet to be evaluated; therefore, a relationship may yet be observed.

Using only two polypeptides in each of the models, the MLRs based on 14 samples produced surprisingly high R values for both loaf volume and peak mixing time. For the 14 varieties evaluated, 88.6 and 86.9% of the variability in loaf volume and mixing time, respectively, were explained. Other researchers have postulated a quantitative relationship between various protein fractions and baking quality. In addition to identifying a gliadin fraction with an inverse relationship to baking score (1986), Huebner and Bietz (1985) have also reported that the ratio of chromatographically separated HMW to LMW glutenins was directly proportional to mixing time, loaf volume, and general score. More recently, Mosleth and Uhlen (1987) have used partial least squares regression to relate densitometric data obtained from one-dimensional electrophoretic separation of gliadins and glutenins to Zeleny sedimentation data. We believe that our results support the evidence for quantitative, in addition to qualitative, relationships.

Research is continuing and will focus on analyzing a larger number of wheat varieties to further test the validity of the relationships reported here. Additionally, since polypeptides not found in the Scout 66 reference may also be correlated with quality factors, we are developing procedures for quantitating those spots.

Fig. 8. Scatter diagram indicating the relationship between percent integrated intensity of the endosperm polypeptide identified as spot 39 and mixograph peak time for 14 wheat varieties.
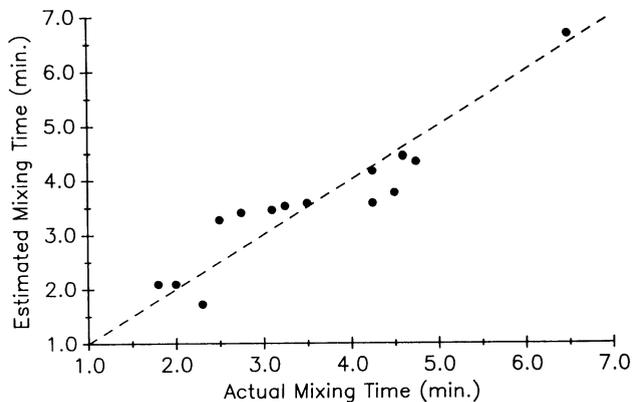

Fig. 9. Scatter diagram comparing the actual mixograph peak times for 14 wheat varieties to the values estimated for those samples by a two-term multiple linear regression.

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