

# Effect of Nitrogen Fertilization on Quantity of Flour Protein Components, Dough Properties, and Breadmaking Quality of Wheat

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## ABSTRACT

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Three winter wheat varieties with differing breadmaking quality were grown at two locations in two years at 0 or 3 × 60 kg of nitrogen application. The effect of nitrogen on amount of different components of gluten proteins was determined by reverse-phase HPLC. A high amount of nitrogen led generally to a significant increase of total protein content. However, this increase was obvious only for the gluten proteins; albumins and globulins remained nearly unaffected. The effect of increased protein content on gliadin to glutenin (gli-glu) ratio was inconsistent. While increased protein content increased the gli-glu ratio in the variety Capo, the opposite was true for the variety Renan. Gli-glu ratio of the variety Lindos showed no discernible tendency. As total protein content increased, the ratio of low molecular weight (LMW) to high molecular

weight (HMW) glutenins decreased consistently, i.e., in all varieties, in both years and locations. Change of LMW to HMW ratio showed a significant negative correlation to sedimentation value and bread volume. There was no consistent change in the ratio between *x*- and *y*-type HMW subunits due to fertilization, as could be shown by densitometric measurements on SDS-PAGE gels. This ratio appeared to be dependent on the genotype and has decreased with decreasing quality. The amount of *x*-type subunits correlated closely with sedimentation value and bread volume. These results suggest that ratio of HMW glutenins, especially *x*-type subunits, to total protein content could be the best early detectable parameter with high predictive value for breadmaking quality.

Breadmaking quality of wheat depends on the quality and quantity of proteins in the endosperm. While the first parameter is genetically determined, the latter is highly influenced by the environment, especially by nitrogen fertilization. Considerable effort has been made to elucidate whether gliadins or glutenins are responsible for the quality differences induced by nitrogen fertilization. But a survey of literature reveals that fertilization trials yielded conflicting results.

Levi et al (1985), using densitometric measurements of proteins separated by SDS-PAGE, observed an increase of gliadins but no change of glutenins after nitrogen fertilization. Doekes et al (1982) came to similar conclusions using pure fractions, after extraction and precipitation. They found an increase in gliadins but no change of amount of glutenins upon nitrogen fertilization. As a consequence, gliadin to glutenin (gli-glu) ratio increased with protein content, resulting in a higher loaf volume. These results were further confirmed by Gupta et al (1992) with fractions separated by size exclusion HPLC (SE-HPLC). These authors also found a correlation between increasing gli-glu ratio and bread volume. These findings, however, contradict results obtained by Scheromm et al (1992), who studied two French wheat varieties in three locations at different levels of nitrogen supply. SE-HPLC revealed a varietal-dependent increase of both glutenins and gliadins. A varietal-dependent effect of nitrogen fertilization also was observed by Prieto et al (1992). The influence of environment on protein chromatograms was studied by Marchylo et al (1990). The authors found no qualitative but some statistically significant quantitative changes. The relative proportion of gliadin and glutenin, separated by HPLC, was more influenced by the environment at higher protein contents than at lower ones. Kolster et al (1991) measured an increase of HMW subunits if protein-content increased. Their amount was influenced by environmental conditions as well as by the genotype.

We used reversed-phase HPLC (RP-HPLC) as well as scanning densitometry on SDS-PAGE gels to study the effect of nitrogen fertilization on protein content and on changes of different protein fractions in relation to their effect on breadmaking quality.

## MATERIALS AND METHODS

### Wheat Samples

Three winter wheat varieties, Capo, Renan, and Lindos, were grown in two locations with different climatic conditions, Großenzersdorf (GE) and Gießhübel (GH), in two years (1993-1994 and 1994-1995). Either 0 or 60 kg of ammonium nitrate containing 27% nitrogen was applied in randomized 10-m<sup>2</sup> plots in two experiments and three replicates before tillering, at shooting, and shortly before heading.

### Chemicals

Sequanal-grade trifluoroacetic acid, acetonitrile (HPLC gradient grade), dithioerythritol, propanol-1 (pa grade), and all other chemicals were supplied by Merck. Water used for HPLC was prepared by ion-exchange separation, followed by preparation in an Elgastat (ion-exchange and charcoal treatment).

### Quality Assessments

Quality parameters were determined according to ICC standards (ICC 1995). Protein content was first measured separately for all replicates using NIT technology. Since variation between replicates was negligible, they were mixed and a sample was taken for analysis with Kjeldahl (ICC standard 105/2; N × 5.7 % Dm); gluten quality (ICC standard 116/1; Zeleny sedimentation volume); wet gluten content (ICC standard 137/1); and farinograph properties (ICC standard 115/1). For baking tests, a method standardized for Austrian quality requirements was used (1,000 g of flour, 5% yeast, 1.8% salt, 1% cooking oil, 2% baking improver) (Oberforster et al 1993).

### Sample Preparation and Extraction Procedures for HPLC

Wheat was milled to flour with an ash content of 0.7% using a Brabender Quadrumat Jr. mill. Flour was defatted by cold petroleum ether treatment.

Extraction was made according to Seilmeier et al (1991) and Wieser et al (1994). Albumins and globulins were extracted from

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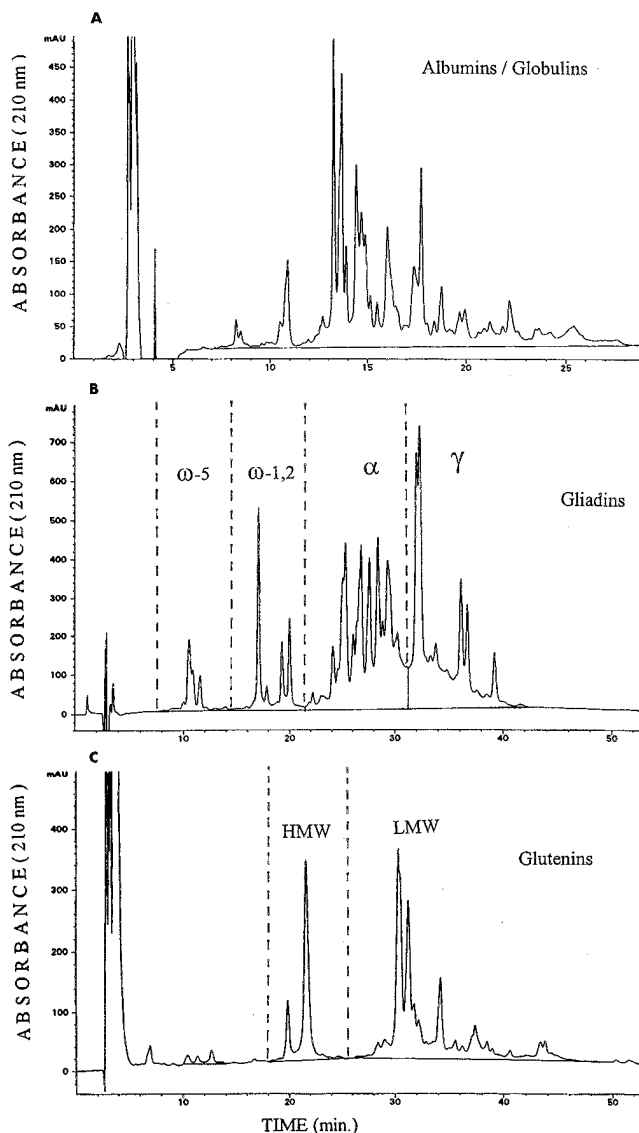
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100 mg of flour in three steps using 0.5 mL of salt solution (0.4M NaCl, 0.067M NaK-phosphate, pH 7.6) for each step under magnetic stirring at room temperature for 15 min. The three extracts were combined and filled up with the extraction solution to 2 mL. In a similar three-step procedure, gliadins were extracted using 0.5 mL of 60% (v/v) aqueous ethanol. From the final residue, glutenins were extracted twice with 1 mL of a solution containing propan-1 (50%, v/v), urea (2 mol/L), dithioerythritol (1%, w/v) and Tris/HCl (0.05 mol/L, pH 7.5) for 60 min using a 60°C waterbath. Glutenins were extracted under nitrogen. All extracts were centrifuged for 20 min at  $1,900 \times g$ . Aliquots of the 2-mL extracts were centrifuged at  $14,500 \times g$  and the supernatants were used for separation by HPLC. All extractions were duplicated and analyzed by HPLC. If the two measurements did not fit together at  $\pm 5\%$ , HPLC was repeated. If still there was no agreement between the two measurements, the extraction was repeated.

### Reversed-Phase HPLC

For quantitative analysis of the extracted storage proteins, a Hewlett Packard HPLC (type 1090) apparatus, with a DAD-detector in conjunction with an RP-HPLC Nucleosil 300-5C8 column ( $C_8$ , 300Å pore size, 5- $\mu$ m particle size, 25- $\times$  4-mm i.d.)



**Fig. 1.** Chromatograms of the variety Capo: albumins and globulins within 30 min (A),  $\omega$ -5,  $\omega$ -1,2-,  $\alpha$ - and  $\gamma$ -gliadins within 60 min (B), and high molecular weight (HMW) and low molecular weight (LMW) glutenins within 60 min (C).

(SRD, Vienna) and a 4-cm  $\times$  4-mm precolumn (same material) were used. Elution of proteins was done as described by Wieser et al (1994).

The differentiation of gliadins into  $\omega$ -1,2,  $\omega$ -5,  $\alpha$ -, and  $\gamma$ -gliadins, and differentiation of the glutenins into HMW and LMW subfractions was done by the method of Wieser et al (1987) based on their amino acid constituents. Examples of separations of albumins globulins, gliadins, and glutenins are given in Fig. 1A–C. The reproducibility was within  $\pm 5\%$ , except for the  $\omega$ -gliadins.

### SDS-PAGE

In addition to the quantification of HMW glutenins by HPLC, the subunits were separated by SDS-PAGE based on Galili and Feldman (1983). Scanning densitometry (ImageMaster software, Pharmacia) was used to measure protein content of bands (Fig. 2), ratios of  $x$ - and  $y$ -type subunits were calculated, and analysis of variance was done separately for the two locations on the basis of 16 measurements (replicates) per location and genotype.

## RESULTS AND DISCUSSION

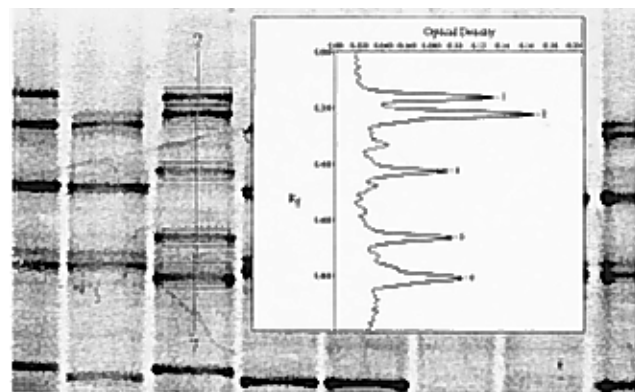
It is well established that increased protein quantity in wheat kernels leads to improved quality parameters, but it is still unclear whether one of the gluten protein components or the specific interaction of the components determines quality. In our study we were interested to see whether a change in amount of protein content induced by nitrogen fertilization causes a change in the proportion of any of the flour protein fractions, and whether this change can be related to changes in breadmaking quality.

### Protein Content

The two locations where the three varieties were grown differed in their climatic conditions. GH was characterized by cooler temperatures and higher precipitation. The three quality wheat varieties differed in their HMW-subunit composition, mainly in locus *Glu-1A* (Table I). In both years and locations, and for all varieties, fertilization caused a substantial increase in endosperm protein content. However, the increase varied according to variety, location, and year (Table II). With one exception (for the variety Lindos), in both years the increase of protein content was substantially higher at GE.

### Quality Parameters

All measured quality parameters showed a definite increase after nitrogen fertilization (Table III). Although the varieties Capo and Renan belong officially to the same high-quality category in Austria, and Renan has a higher Payne score than Capo, the measured quality parameters place Capo slightly ahead of Renan (Table III).



**Fig. 2.** Scanning densitometry (ImageMaster software, Pharmacia) to measure protein content of high molecular weight subunits. Protein content of a single band is determined in arbitrary units as the area under the peak.

## Protein Fractions

Fractions were obtained by a quantitative extraction and separated by RP-HPLC. The areas under the curve of the UV-signal were calculated per milligram of defatted flour and used as a direct measure for amounts of protein in the specific fractions. Using the total area and the flour protein content of the varieties, the absolute amount of all fractions was calculated (Table IV).

Increase of protein content generally resulted in a negligible variation of the amount of albumins and globulins. This finding agrees well with available literature data (Doekes 1982, Prieto 1992, Wieser et al 1994).

Gliadins represent ≈60% of total gluten proteins. Therefore, this fraction, especially  $\alpha$ - and  $\gamma$ -gliadins, gained the most from the total increase. Increase of all gliadin subfractions, except  $\omega$ -5, showed a very high correlation to total protein content (Table V). The low correlation of the  $\omega$ -5 gliadins is remarkable, but it is probably because  $\omega$ -5 represents the smallest gliadin subfraction with only 5% of all the gliadins but with the largest standard deviation.

The change of total glutenins was proportional to the increase of total protein content, but HMW glutenins alone showed a much higher correlation to total protein content than the LMW glutenins (Table V).

## Ratio of Gliadins to Glutenins

No consistent change was seen regarding gli-glu ratio and nitrogen fertilizer application. Although a consistent increase was found for the variety Capo in both years and environments, the situation was the reverse for the variety Renan in most cases. The variety Lindos showed almost no variation in gli-glu ratio. It appears that in this experiment, the change in gli-glu ratio is probably influenced more by the genotype and by the environment than by the protein content (Table IV).

**TABLE I**  
Varieties Used in This Study, Their High Molecular Weight Glutenin Subunit (HMW-GS) Composition, Payne Scores, and Quality Score as Described in the Official List of Registered Varieties in Austria

Variety	HMW-GS			Payne Score	Quality Score <sup>a</sup>
	<i>Glu A-1</i>	<i>Glu B-1</i>	<i>Glu-D1</i>		
Capo	1	7+9	5+10	9	8
Renan	2*	7+8	5+10	10	8
Lindos	0	7+9	5+10	7	6

<sup>a</sup> Highest quality score is 9.

**TABLE II**  
Protein Content (%) of the Varieties and Their Differences at Two Locations, Years, and Levels of Nitrogen Fertilization

Variety	Year	Location	0 kg	180 kg	Difference in	
					Absolute	%
Capo	1993-94	GE	13.0	15.5	2.5	19.2
		GH	12.9	14.6	1.7	13.2
	1994-95	GE	9.2	15.2	6.0	65.2
		GH	13.1	14.9	1.8	13.7
Renan	1993-94	GE	12.8	15.0	2.2	17.2
		GH	13.5	14.9	1.4	10.4
	1994-95	GE	10.0	15.5	5.5	55.0
		GH	14.1	15.1	1.0	7.1
Lindos	1993-94	GE	11.1	14.2	3.1	27.9
		GH	10.2	13.1	2.9	28.4
	1994-95	GE	8.4	13.1	4.7	56.0
		GH	11.9	13.7	1.8	15.1

<sup>a</sup> GE = Großenzersdorf; GH = Gießhübel.

These observations support earlier results of Prieto et al (1992) and Scheromm et al (1992), but contradict data of several authors who found a constantly higher relative increase of gliadins in comparison with glutenins (Doekes and Wennekes 1982, Levy et al 1985, Janssen et al 1990, Shipper 1991, Gupta et al 1992). It should be mentioned that, in the present work, the fraction of gliadins may include oligomeric proteins (i.e., HMW gliadins or ethanol-soluble glutenins) as described by Huebner et al (1993). But because comparisons are being made only within varieties using the same technique, the error arising through the methodology is probably negligible.

## Ratio of LMW to HMW Glutenins

In our study, the proportion of glutenins varied between 22 and 28% of total protein content. The ratio of the LMW to HMW subfraction varied from 2:1 to 3:1. This ratio showed a consistent decrease as protein content increased following nitrogen fertilization (i.e., the relative amount of HMW proteins increased and the relative amount of LMW proteins decreased) (Table IV). The correlation between differences of protein (absolute) contents due to fertilizer application and the differences of LMW-HMW ratio is highly significant (0.8477). Singh et al (1990) and Seilmeier and Wieser (1994) found a similar change in LMW-HMW ratio upon change in protein content.

## Ratio of $x$ - and $y$ -Subunits

Using HPLC, Seilmeier and Wieser (1994) found a decrease of this ratio with increasing amounts of protein after fertilization, due to a higher increase of the  $x$ - than  $y$ -type subunits. In our study, these values could only be obtained only for the material harvested in 1995. Using SDS-PAGE and densitometry, we found a clear decrease of this ratio only for two varieties and only at one location. In all other cases the ratio slightly increased. Analysis of variance, calculated for the two locations separately showed a significance only for one location (Table VI). These results can not be connected to an increased nitrogen supply. Kolster et al (1991), applying scanning densitometry as in the present study, reported no effect of nitrogen fertilization on the ratio between total amount of HMW subunits and total amount of protein content.

It was interesting to see, that in our material,  $x$ - $y$  ratio decreased consistently with the quality rating of the varieties. The highest ratio of 2.3 was found for the best quality wheat (Capo) followed by 1.9 for the variety Renan and 1.8 for the variety Lindos. A fourth variety, feed quality Hai, which we studied but did not include in this analysis, had a ratio of 1.1. It appears that the  $x$ - $y$  ratio could have a certain predictive value for the quality of a wheat variety. This assumption is supported by the high correlation values of the  $x$ - and  $y$ -type subunits to bread volume (Table V). A more detailed experiment with a large sample of selected genotypes is in progress.

## Correlation Between Protein Fractions and Quality Parameters

With the exception of the  $\omega$ -5 gliadins, all fractions of proteins showed a high to very high correlation to total protein content, a somewhat lower correlation to sedimentation value, and much lower correlation to bread volume (Table V). The low correlation of the  $\omega$ -5 gliadins can probably be explained by their very low relative amounts accompanied by the highest standard deviation.

In absolute values, the change in the gli-glu ratio following the change of protein content was not correlated with any of the investigated quality parameters (Table V). In our study, the gli-glu ratio did not reflect the change of quality associated with increased protein content. This finding contradicts that of Wieser et al (1993), who found a strong influence of this ratio on rheological properties and baking volume.

The high correlation of both glutenins and gliadins to total protein content prevents the evaluation of the influence of any of these two components on wheat flour quality.

**TABLE III**  
Quality Parameters of the Varieties for Two Years, Locations,<sup>a</sup> and Nitrogen Fertilization Levels

	1993-1994				1994-1995			
	0 kg GE	180 kg GE	0 kg GH	180 kg GH	0 kg GE	180 kg GE	0 kg GH	180 kg GH
Capo								
Sedimentation volume	51	68	53	67	28	55	52	66
Amount gluten	29.4	36.6	31.9	37	19	41.4	30.5	35.3
Farinograph :								
Water absorption	61.7	61.0			58.5	63.9	60.0	60.5
Dough development	5				1	5	4	6.5
Stability	9.5	20			3	8.5	13.5	13
Degree of softening	60	20			130	80	50	70
Bread volume	640	702			522	705	632	701
Renan								
Sedimentation volume	41	56	45	57	29	48	51	60
Amount gluten	29.7	34.6	30.8	35.9	21.2	38.7	31.7	35.2
Farinograph :								
Water absorption	62.6	63.8			58.6	62.0	59.5	62.7
Dough development	6.5	8.5			2	5	5.5	5.5
Stability	8	22			6	12.5	10	10
Degree of softening	50	30			110	50	70	60
Bread volume	457	449			534	698	640	692
Lindos								
Sedimentation volume	44	64	37	50	23	38	42	49
Amount gluten	22.4	32.5	22	28.3	16.9	30.9	26.7	30
Farinograph :								
Water absorption	57.1	56.6			53.0	56.4	53.6	57.8
Dough development	2	5.5			1.5	2	2.5	2.5
Stability	6	22			2.5	10.5	4	6.5
Degree of softening	80	30			130	50	100	70
Bread volume	580	612			532	646	581	641

<sup>a</sup> GE = Großenzersdorf; GH = Gießhübel.

**TABLE IV**  
Fractions of Gluten Proteins (%) and Selected Ratios of Varieties for Two Years, Locations,<sup>a</sup> and Nitrogen Fertilization Levels as Determined by Reverse-Phase HPLC

Fractions <sup>b</sup>	1993-1994				1994-1995			
	0 kg GE	180 kg GE	0 kg GH	180 kg GH	0 kg GE	180 kg GE	0 kg GH	180 kg GH
Capo								
Σ A+G	1.24	1.20	1.12	1.09	1.48	1.64	1.38	1.55
Σ Gliadin	8.36	10.21	8.20	9.51	5.48	9.98	8.40	9.92
ω 5	0.31	0.40	0.25	0.34	0.13	0.42	0.31	0.45
ω 1,2	0.70	0.87	0.59	0.69	0.42	1.01	0.76	1.03
α	3.95	4.86	3.78	4.07	2.73	5.00	4.13	5.29
γ	3.39	4.09	3.58	4.41	2.20	3.62	3.20	3.15
Σ Glutenin	3.34	4.09	3.57	4.00	2.24	3.58	3.32	3.42
HMW	0.81	1.13	0.89	1.02	0.50	1.03	0.83	0.98
LMW	2.58	2.55	2.68	2.98	1.74	2.55	2.49	2.40
Gli-glu	2.46	2.50	2.30	2.38	2.45	2.80	2.53	2.90
LMW-HMW	3.19	2.61	3.01	2.92	3.40	2.47	3.00	2.47
Renan								
Σ A+G	1.07	1.16	1.11	1.18	1.46	1.29	1.69	1.92
Σ Gliadin	8.31	9.83	9.14	9.80	6.06	10.50	9.20	9.53
ω 5	0.26	0.40	0.27	0.32	0.15	0.45	0.32	0.35
ω 1,2	0.56	0.71	0.52	0.62	0.40	0.94	0.72	0.75
α	4.49	5.32	4.62	5.06	3.12	5.62	4.90	5.04
γ	3.00	3.39	3.73	3.80	2.47	3.49	3.25	3.39
Σ Glutenin	3.42	4.01	3.25	3.93	2.48	3.71	3.21	3.65
HMW	0.88	1.16	0.95	1.14	0.61	1.24	0.92	1.07
LMW	2.54	2.85	2.30	2.78	1.87	2.47	2.29	2.58
Gli-glu	2.40	2.45	2.81	2.50	2.90	2.50	2.87	2.61
LMW-HMW	2.98	2.46	2.42	2.44	3.04	2.00	2.50	2.42
Lindos								
Σ A+G	1.06	1.19	1.01	1.22	1.41	1.81	1.34	1.47
Σ Gliadin	6.87	8.80	5.92	7.67	4.79	7.86	7.71	8.99
ω 5	0.36	0.76	0.32	0.50	0.22	0.57	0.53	0.72
ω 1,2	0.46	0.66	0.35	0.48	0.32	0.68	0.67	0.81
α	3.31	4.22	2.88	3.72	2.32	3.78	3.79	4.19
γ	2.78	3.17	2.37	2.97	1.93	2.83	2.72	3.27
Σ Glutenin	3.12	4.20	3.26	4.20	2.18	3.43	2.86	3.23
HMW	0.69	1.04	0.84	1.14	0.52	0.94	0.78	0.95
LMW	2.45	3.16	2.42	3.07	1.65	2.49	2.07	2.28
Gli-glu	2.20	2.10	1.82	1.83	2.20	2.29	2.70	2.78
LMW-HMW	3.48	3.04	2.98	2.70	3.16	2.64	2.65	2.41

<sup>a</sup> GE = Großenzersdorf; GH = Gießhübel.

<sup>b</sup> HMW = high molecular weight; LMW = low molecular weight; gli-glu = gliadin to glutenin ratio.

**TABLE V**  
**Correlation Coefficients<sup>a</sup> Between Protein Fractions of Varieties for Two Locations, Two Years, and Two Nitrogen Fertilization Levels and Data Obtained from Flour, Gluten, and Bread**

Fraction <sup>b</sup>	Flour Protein (%)	Sedimentation Value	Dough Development	Bread Volume
Gluten	0.97***	0.83***	0.75***	0.61**
Σ Gliadin	0.99***	0.84***	0.83***	0.56**
ω 5	0.45*	0.42*	0.29 ns	0.38 ns
ω 1,2	0.83***	0.70***	0.69**	0.74***
α	0.93***	0.72***	0.83***	0.46*
γ	0.85***	0.83***	0.78***	0.58**
Σ Glutenin	0.82***	0.83***	0.60**	0.38 ns
HMW	0.91***	0.76***	0.64***	0.48*
x <sup>+</sup>	0.98***	0.85***	0.82***	0.97***
y <sup>+</sup>	0.87***	0.64*	0.69*	0.84***
LMW	0.68***	0.76***	0.45 ns	0.24 ns
Gli-glu	0.30 ns	0.13 ns	0.41 ns	0.31 ns
LMW-HMW	-0.73***	-0.41*	-0.55*	-0.50*
Bread volume	0.58**	0.57**	0.15 ns	

<sup>a</sup> \*, \*\*, \*\*\* = Significant at  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ , respectively; ns = not significant.

<sup>b</sup> HMW = high molecular weight; LMW = low molecular weight; gli-glu = gliadin to glutenin ratio; + = data from only 1994-1995.

**TABLE VI**  
**Ratio of x- and y-Type Subunits Measured by SDS-PAGE and Densitometry, for Three Varieties at Two Locations,<sup>a</sup> and Two Levels of Nitrogen Application**

	0 kg/ha	180 kg/ha	Bonferoni (5%)
GE			
Capo	2.63	2.27	*b
Renan	2.06	1.90	*
Lindos	1.77	1.81	ns
Mean	2.16	1.98	*
GH			
Capo	2.36	2.48	ns
Renan	1.94	1.93	ns
Lindos	1.79	1.93	ns
Mean	2.03	2.11	ns

<sup>a</sup> GE = Grobenzersdorf; GH = Gießhübel.

<sup>b</sup> \* = Significant at  $P < 0.05$ , ns = not significant.

The HMW proteins show a higher correlation to total protein content, bread volume, and farinograph parameters than do LMW proteins (Table V). This finding, in connection with the fact that increased protein content consistently led to a relative increase of HMW subunits the expense of LMW subunits, underlines the significance of HMW subunits for these quality parameters. This could be due to the higher aggregate-building properties of the HMW subunits compared with the LMW subunits.

## CONCLUSIONS

The strong correlation of both gliadins and glutenins, especially HMW glutenins, to total protein content prevents establishing which one of the two is more important for breadmaking quality. The ratio of gli to glu appears to be of no predictive value. Further experiments with selected genotypes are necessary to clearly separate the effect of these two gluten protein fractions on quality. HMW glutenins represent only a tenth of the total amount of gliadins. Therefore, based on the present results, we suggest that the ratio of HMW glutenins, especially the x-type subunits, to total protein content could be the best early detectable parameter with the best predictive value for breadmaking quality.

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