Influence of Nitrogen Fertilization on the Potential Bread-Baking Quality of Two Wheat Cultivars Differing in Their Responses to Increasing Nitrogen Supplies

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Cereal Chem. 69(6):664–670

ABSTRACT

Two French wheat cultivars were grown in three locations at different levels of nitrogen supply. The two cultivars showed different effects of increased nitrogen levels on baking strength: little variation in baking strength for cv. Camp Rémy, strong variation for cv. Fandango. Changes in protein content, glutenin subunits, and aggregates were monitored by nitrogen determination, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and size-exclusion high-performance liquid chromatography, respectively. Whereas the composition in glutenin subunits remained unchanged with increasing N fertilizer, the total amount of high molecular weight aggregates evolved differently according to cultivar. These results were discussed in connection with the regularity of baking quality expression in wheats.

The success of breeding for high technological quality depends on improvements in both the potential level of genotypes and the stability of quality expression. When the selection is performed on quality criteria, it is highly desirable to develop high-quality cultivars that remain stable in their response to variable environmental conditions rather than very high-quality, but unstable, cultivars. Several recent reports suggested that environmental conditions could quantitatively affect storage protein components (Kruger and Marchylo 1985, Huebner and Bietz 1988, Marchylo et al 1990). However, very few studies based on accurate experimental designs (including highly controlled nitrogen applications) were performed that took into account the effects on baking quality.

Several technological or biochemical tests for predicting the potential baking quality have been developed: Zeleny index, sodium dodecyl sulfate (SDS) sedimentation, Chopin alveograph (*W* index), farinograph, identification of protein markers by electrophoresis, size-exclusion high-performance liquid chromatography (SE-HPLC), etc. However, it is still extremely difficult to predict the behavior of a genotype with regard to the variation of environmental factors because the bases of phenotypic quality, which govern the stability or instability of quality expression, are not clearly understood.

It is largely accepted that nitrogen nutrition affects the protein content and composition and directly influences the technological quality of wheat samples. For instance, strong effects of nitrogen fertilization (along with those of climate, growing year, and growing location) have been observed on both protein content and baking quality (Séroux and Metayer 1990). While breadmaking quality (e.g., loaf volume) directly depends on protein content—at least in the range of protein contents encountered in commercial wheats flours (Finney and Barmore 1948, Bushuk et al 1969)—

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the slope of the regression line of this relation varies among cultivars. Furthermore, when typical southwestern European breadbaking varieties are considered, very high protein contents are detrimental to loaf volume, the optimum protein content being cultivar-dependent (Martin 1987). It is essential, therefore, to emphasize this concept of stability of quality in response to various levels of nitrogen fertilization or protein content.

Among hard wheats, baking quality is determined by the amounts or ratios of the main classes of proteins, gliadins and glutenins (Huebner and Wall 1976; Huebner and Bietz 1986, 1988). Allelic variation at loci encoding high molecular weight (HMW) glutenin subunits and at other loci encoding gliadins and low molecular weight (LMW) glutenin subunits determines the potential baking quality of bread wheat genotypes (Payne et al 1984, Branlard and Dardevet 1985, Autran 1987). Because the latter is also associated with the presence of large protein aggregates (Huebner 1970, Field et al 1983, Miflin et al 1983, Bushuk 1987), SE-HPLC has been introduced as a new tool for quantifying the native protein aggregates and assessing baking strength (Burnouf and Bietz 1987, Dachkevitch and Autran 1989).

The aim of the present work was to assess cultivar tolerance in the presence of various conditions of nitrogen fertilization. Changes in protein content and aggregate composition were investigated in relation to baking strength. This study was part of a larger experiment in which 20 cultivars were grown at four or five levels of nitrogen and tested for baking quality. Two of these 20 cultivars were selected for more specific investigations on the physicochemical basis of quality expression. Cultivars Camp Rémy and Fandango were considered because they have similar average values of the *W* index, yet they behave quite differently in response to the level of fertilization: little variation of baking quality with changes in protein content for Camp Rémy, stronger variation for Fandango. Using nitrogen determination, SDS-polyacrylamide gel electrophoresis, and SE-HPLC, we have investigated the variation in physicochemical parameters as the result of agronomic changes in the protein content of the wheat kernel.

MATERIALS AND METHODS

Wheat Samples

The wheat grains and flours from cultivars Camp Rémy and Fandango were provided by the Institut Technique des Céréales et des Fourrages. In 1989, Camp Rémy was harvested in four locations and Fandango in six locations in different areas in France. Only the correlation between flour protein content and *W* was calculated for these samples. In 1990, these cultivars were grown in small plots in different locations, Aube, Loir et Cher, and Oise, at four or five levels of nitrogen supply (split-plot design). According to the location, different amounts of nitrogen fertilizer (nitrate), definite from a median value calculated by the balance method (Remy and Hebert 1977), were applied: 70, 120, 180, and 250 kg/ha in Oise; 70, 110, 150, 190, and 230 kg/ha in Loir et Cher; 100, 150, 200, 250, and 300 kg/ha in Aube. The nitrate supply was divided in two applications: 50 kg/ha was applied at the tillering stage and the remainder at the beginning of the stem elongation. For the location Loir et Cher, the nitrate supply was divided in three applications, the third being added eight days after the second. After the harvest, the wheat samples were weighed and the grain yield was expressed on the basis of 15% humidity.

Protein Extraction

Each sample of Camp Rémy and Fandango flours (1 g) was extracted sequentially with the following: 0.5*M* NaCl (2 × 10 ml), 70% (v/v) ethanol (2 × 10 ml), and phosphate buffer, pH 6.8, containing 2% SDS and 5% 2-mercaptoethanol (2 × 10 ml). Extractions were performed at 20°C, except for salt extractions, which were performed at 4°C. Extraction time in all cases was 1 hr with continuous stirring followed by centrifugation for 10 min at 18,000 × *g*. First and second extracts were pooled before nitrogen and electrophoretic analyses. Each result is the average of two replications.

Damaged Starch

Damaged starch determination was performed in an SD4 apparatus (Tripette et Renaud, Villeneuve-la-Garenne, France) by amperometric measurement of the rate of iodine absorption by a flour suspension (Medcalf and Gilles 1965).

Nitrogen Determination

The nitrogen content of each extract and of the flour was determined by Kjeldahl analysis using a Cu-Se catalyst.

Electrophoresis

Proteins extracts (1 ml) were reduced with a solution containing 2-mercaptoethanol (0.1 ml) and 0.2*M* Tris-HCl buffer (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol, and 0.01% (w/v) pyronine G (0.4 ml). For ethanol extracts, the solution was saturated with glycerol. The samples were incubated at room temperature for 2 hr and at 100°C for 2 min and then centrifuged. Reduced proteins were then electrophoresed according to Laemmli (1970) in vertical SDS-polyacrylamide gel electrophoresis slabs at a gel concentration of 13% in a discontinuous, pH 6.8–8.8, Tris-HCl-SDS buffer system (Payne et al 1979). Gels were fixed in 12% trichloroacetic acid and stained overnight with Coomassie Blue.

SE-HPLC

Flour samples (80 mg) were stirred for 2 hr at 60°C in the presence of 0.1*M* sodium phosphate buffer (pH 6.9) containing 2% SDS. Extractions were followed by centrifugation at 37,500 × *g* for 30 min at 20°C. Supernatants were then submitted to SE-HPLC fractionation on a TSK 4000-SW size-exclusion analytical column (7.5 × 300 mm, Beckman, Carlsbad, CA) according to Dachkevitch and Autran (1989). The chromatograms were analyzed through Spectra-Physics analytical software (San Jose, CA), which permitted integration of the elution curve (Fig. 1). The chromatograms were divided into four main peaks. The first fraction (F1) corresponds to highly aggregated material and elutes at the void volume of the column. Fraction 2 (F2) elutes between 115 and 650 kDa and consists of smaller aggregates than those of F1. Fractions 3 and 4 (F3 and F4) correspond essentially to monomeric proteins—gliadins and salt-soluble proteins, respectively.

Technological Tests

Baking strength determination was based on the *W* index (Chopin alveograph, Tripette et Renaud) according to standard no. 5530/4 of the International Organization for Standardization.

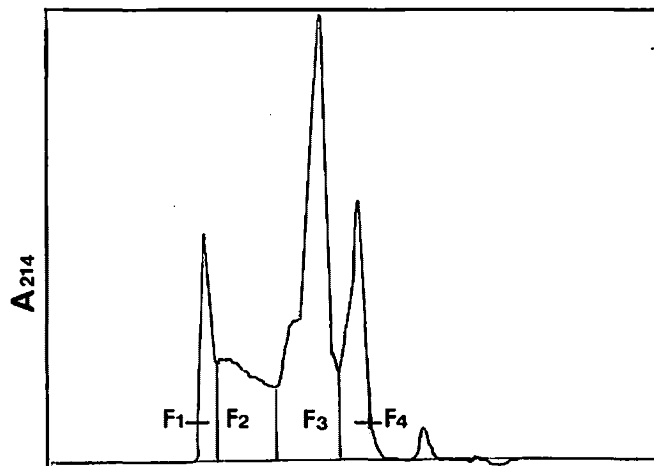

Fig. 1. Elution profile obtained by size-exclusion high-pressure liquid chromatography of unreduced flour proteins extracted with sodium phosphate buffer-sodium dodecyl sulfate from cultivar Camp Rémy. The chromatogram is divided in four fractions, F1–F4 (see Materials and Methods).

RESULTS

Relation Between Protein Content and *W*

For each cultivar, correlations were calculated between baking strength and protein content for the harvests of 1989 and 1990. The results show that the *W* index was significantly linked to protein content (except for Camp Rémy in 1989, $r = 0.58$): $r = 0.94$ for Fandango in 1989; $r = 0.75$ and 0.87 for Camp Rémy and Fandango, respectively, in 1990 (Figs. 2 and 3). The regression slope was significantly higher for Fandango than for Camp Rémy: two times higher for the year 1990 and even four times higher for the 1989 data. This indicates that the level of baking strength of Fandango is more susceptible to changes in protein content modification than the one of Camp Rémy and that Fandango has a lower stability with regard to different levels of soil fertility. Interestingly, the difference in the regression slopes between the 1989 and 1990 data is likely to be explained by the ratio of damaged starch in the flour. The ratio of damaged starch varied from 8 to 9% in 1989, a regular situation, while it was much more important (15–17%) in 1990. Such high ratios were likely to dramatically change the distribution of water between starch granules and proteins during the alveograph testing, resulting in a deviation in the relation between *W* index and protein content.

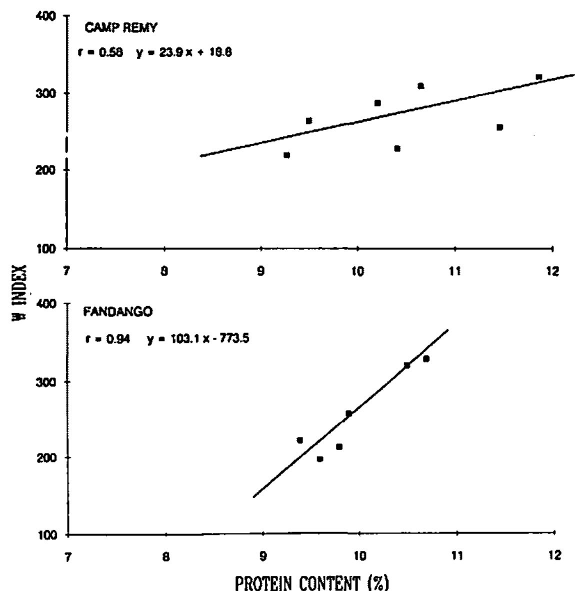

Fig. 2. Correlation between flour protein content (dry basis) and *W* index for cultivars Camp Rémy and Fandango grown in 1989 in different locations in France at different levels of nitrogen supply.

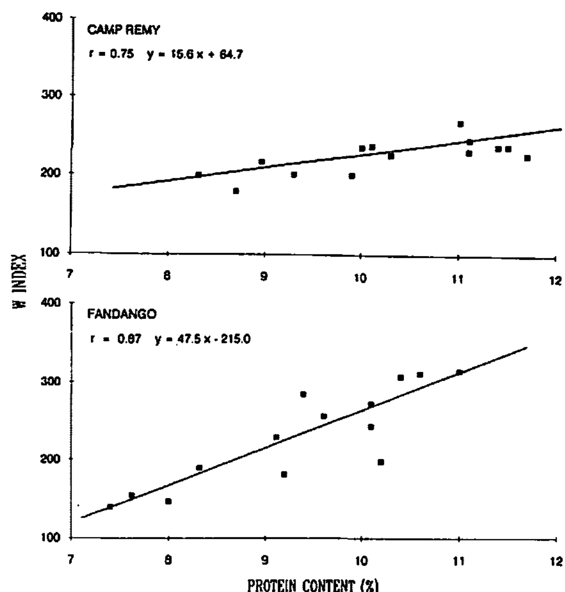

Fig. 3. Correlation between flour protein content (dry basis) and *W* index for cultivars Camp Rémy and Fandango grown in 1990 in different locations in France at different levels of nitrogen supply.

Effect of Soil Nitrogen Level on Protein Content and Baking Strength

From the 1990 harvest, the protein content for both Camp Rémy and Fandango increased linearly with increasing N fertilizer,

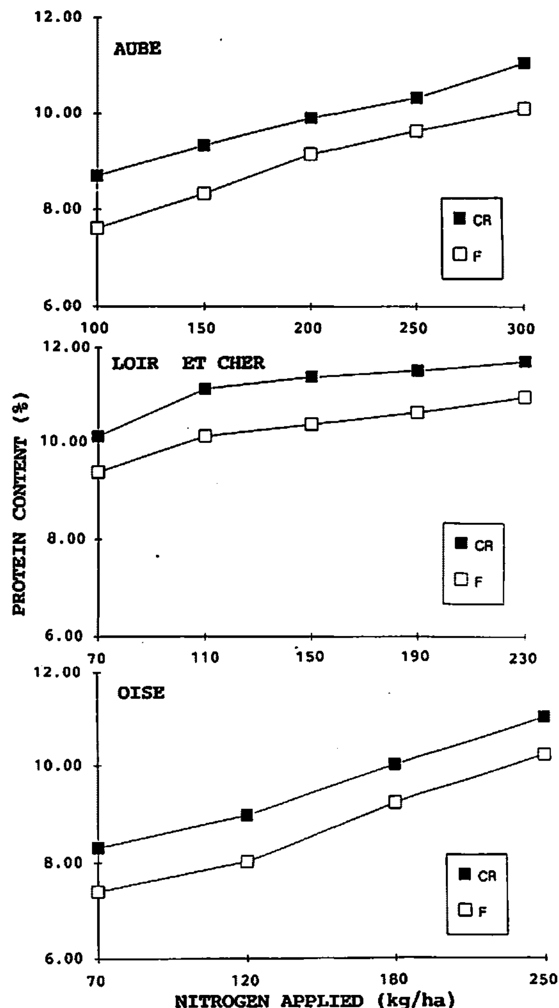

Fig. 4. Flour protein content (dry basis) of the cultivars Camp Rémy (CR) and Fandango (F) grown in 1990 in three locations in France, Aube, Loir et Cher, and Oise, at different levels of nitrogen supply.

TABLE I
Grain[a] Yield of Camp Rémy and Fandango Grown in Three Locations at Different Levels of Nitrogen Supply

N Applied (kg/ha)	Grain Yield, t/ha					
	Aube		Loir et Cher		Oise	
	CR[b]	F[b]	CR	F	CR	F
70	...	...	8.1	8.5	8.1	9.4
100	7.3	7.7	...	...	...	...
110	...	...	8.1	9.1	...	...
120	...	...	...	...	9.1	10.4
150	8.7	9.3	7.7	8.9	...	...
180	...	...	...	...	9.4	11.2
190	...	...	8.2	9.5	...	...
200	9	9.3	...	...	...	...
230	...	...	8.5	9.5	...	...
250	8.9	9	...	...	9.7	11.2
300	8.7	8.9	...	...	...	...

[a] Grain humidity = 15%.
[b] CR, Camp Rémy; F, Fandango.

Fig. 3. Correlation between flour protein content (dry basis) and % index for cultivars Camp Rémy and Fandango grown in 1990 in different locations in France at different levels of nitrogen supply.

[a] Grain humidity = 15%.
[b] CR, Camp Rémy; F, Fandango.

except in the Loir et Cher growing location (Fig. 4). Although Camp Rémy always had a greater protein content than Fandango, the rate of increase was almost identical for the two cultivars. In Loir et Cher, the flour nitrogen content was high for the applied 70 kg/ha of nitrogen, and the increase was less apparent than that for the other locations. That result was possibly due to a drought effect combined with a third, too late, nitrogen application for the more important nitrate supplies.

In general, the optimum grain yield was attained for the second or the third level of nitrogen supply according to the location (Table I).

Nitrogen Content of the Various Protein Extracts

The analyses were performed on samples from Aube and Loir et Cher (Fig. 5).

For the two cultivars grown on these locations, the nitrogen content of the sequential extracts generally increased with the N fertilizer level. In all cases, the increase of the ethanol-soluble fraction between the lower and the higher nitrogen supply was high, confirming previous studies in our laboratory. Huebner and

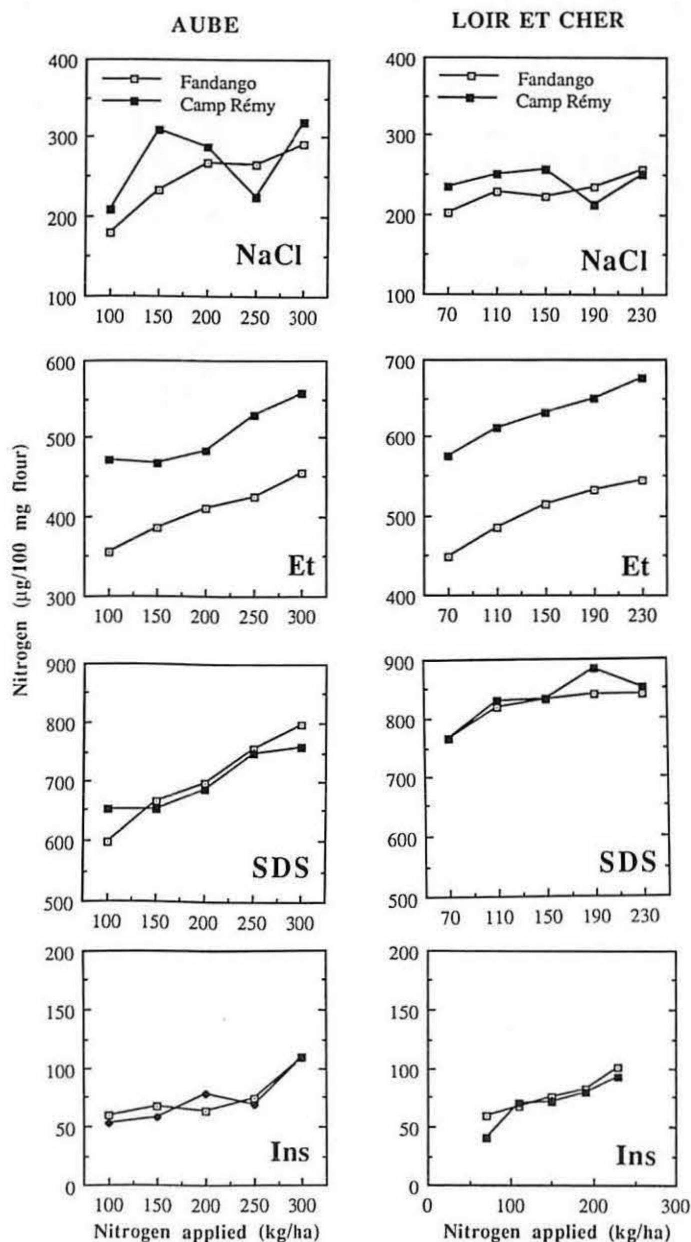

Fig. 5. Nitrogen content of the sequential protein extracts NaCl, ethanol (Et), sodium dodecyl sulfate (SDS), and the insoluble fraction (Ins) obtained from Camp Rémy and Fandango grown in Aube and Loir et Cher, France.

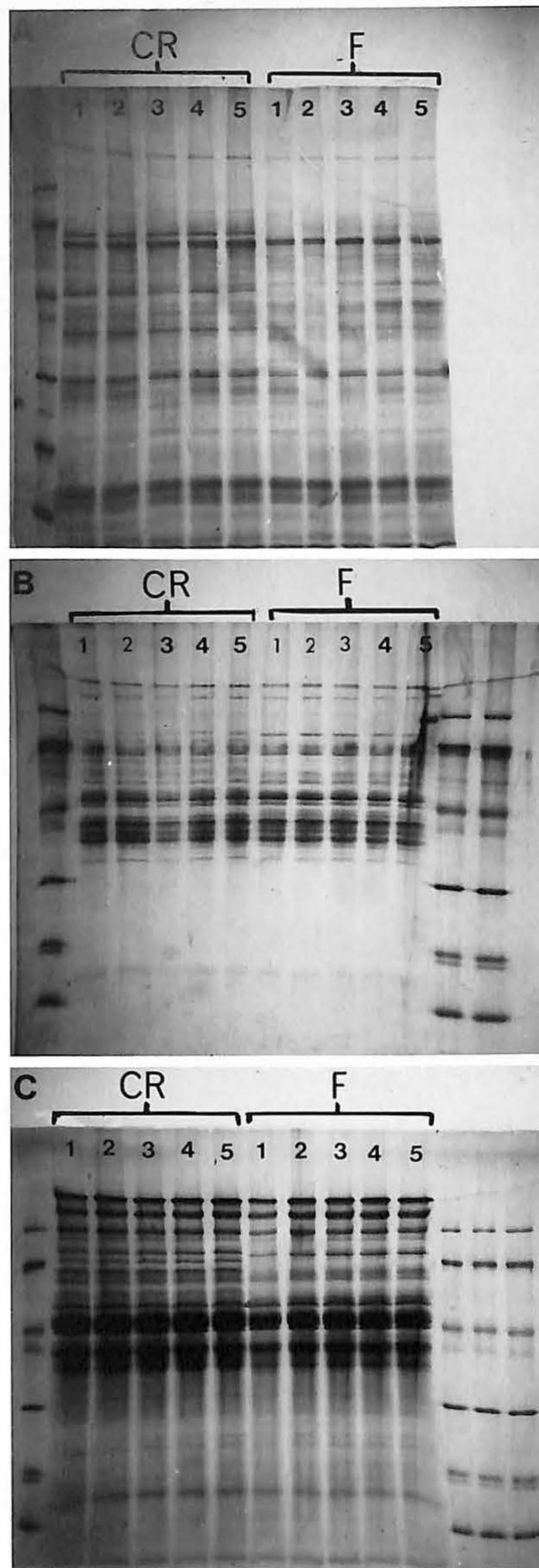

Fig. 6. Electrophoresis patterns of the sequential protein extracts NaCl (**A**), ethanol (**B**), and sodium dodecyl sulfate (**C**) obtained from Camp Rémy (CR) and Fandango (F) grown in Loir et Cher, France, at nitrogen supply levels of 70, 110, 180, 190, and 230 kg/ha for lanes 1–5, respectively. Molecular weight standards were 14, 20, 30, 43, 67, and 94 kDa.

Bietz (1988) also noticed that considerable quantitative variation among gliadins could result from environmental factors. Camp Rémy shows a greater nitrogen content for these extracts. A higher increase of the nitrogen content of the NaCl and the SDS sequential extracts was found for Aube than for Loir et Cher in relation to the evolution of the total protein content (Fig. 4). The curve evolution was quite similar for the two cultivars; the increased nitrogen content of the SDS extract was slightly lower for Camp Rémy in Aube.

Electrophoresis

The aim of the electrophoretic study was to determine whether new proteins could be detected in kernels as a result of the increase in the level of fertilizer. The same protein bands were present in all samples of each cultivar, even in the case of the lowest doses of fertilizer (Fig. 6). This confirms previous studies based on reversed-phase HPLC (Huebner and Bietz 1988). The only difference that could be noted was a slight increase of the band intensities upon application of higher doses of nitrogen fertilizer in the Fandango pattern of the SDS-mercaptoethanol extracts.

SE-HPLC

SE-HPLC is a powerful tool for studying native protein aggregates and the physicochemical basis of baking strength (Dachkevitch and Autran 1989). It is likely that SE-HPLC is a more accurate tool than sequential extraction for investigating the aggregative level of the various protein subunits. In this study, the major peaks of the elution curve, referred to as F1–F4, were quantified for Camp Rémy and Fandango grown in Aube, Loir et Cher, and Oise. The results were expressed either as absolute quantities (peak area) or as relative quantities (ratio of peak area to total chromatogram area). When expressed as relative quantities, the different fractions of the two cultivars showed similar variation as the level of fertilizer increased (Figs. 7 and 8). These results are in agreement with those of Tanaka and Bushuk (1972), who observed that the proportions of soluble and gluten proteins remain essentially constant with increasing total protein contents. On the other hand, when expressed on a peak area basis, F1 and F2 did not show the same evolution between the two cultivars (Figs. 9 and 10). With Camp Rémy, the areas of peaks F1 and F2 were nearly identical, irrespective of the level of fertilizer. With Fandango, the amount of each fraction increased steadily to reach a maximal value for the higher fertilizer application.

Regarding F3 and F4, expressed by either area or ratio, the same variation was observed for the two cultivars in response to increasing nitrogen supplies (results not shown).

DISCUSSION

As reported above, Camp Rémy and Fandango were selected for this study on the basis of their similar average *W* index and their different behavior in response to changes in the level of nitrogen supply. Because only these two cultivars were investigated in detail, our conclusions are tentative. However, to the extent that our experimental approach permits, several clear results may be reported. For instance, the more constant amount of HMW aggregates was found with the more stable cultivar (Camp Rémy), suggesting that the amount (and not the percentage) of HMW aggregates (F1 and F2) present in the grain may be directly involved in the stability of quality in response to variable levels of nitrogen supply. This develops further previous reports suggesting that baking quality depends not only on the composition

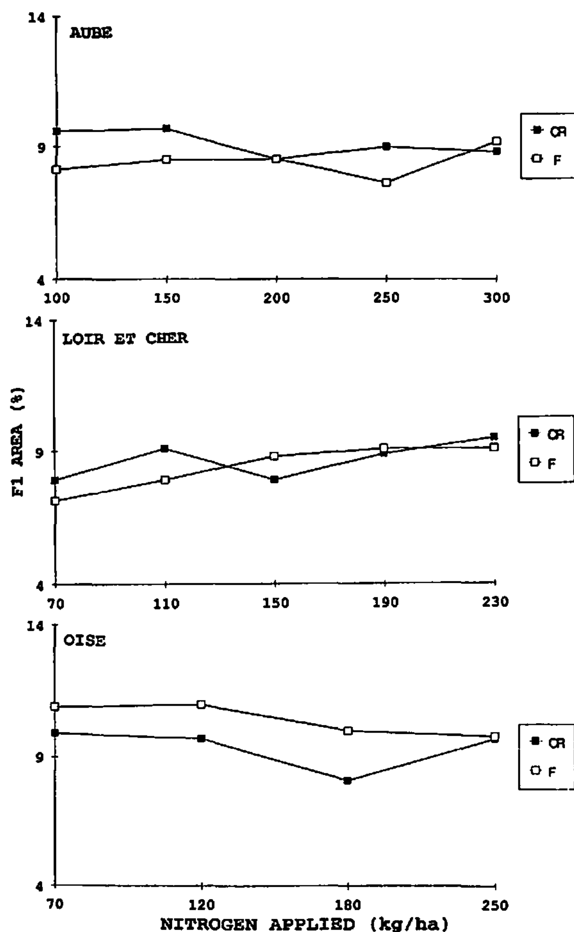

Fig. 7. Evolution of the fraction 1 (F1) peak expressed as the percentage of the total area as a function of the nitrogen applied. Cultivars Camp Rémy (CR) and Fandango (F) were grown in 1990 in Aube, Loir et Cher, and Oise, France.

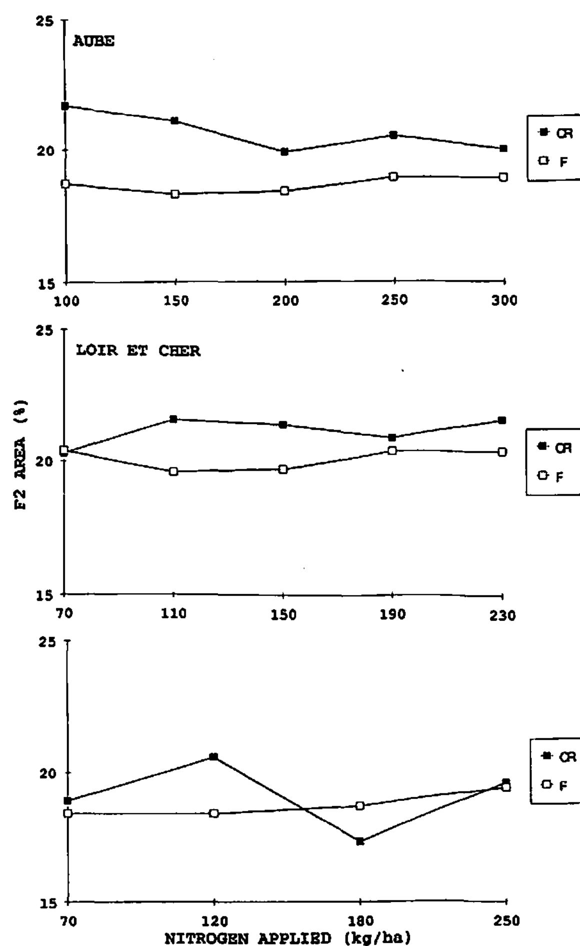

Fig. 8. Evolution of the fraction 2 (F2) peak expressed as the percentage of the total area as a function of the nitrogen applied. Cultivars Camp Rémy (CR) and Fandango (F) were grown in 1990 in Aube, Loir et Cher, and Oise, France.

in glutenin subunits but also on the amount of aggregative fractions (Payne et al 1987, Sutton 1990). Whereas nitrogen fertilization has no effect on the composition of protein subunits (Fig. 6), increased nitrogen supplies clearly influence the aggregation level of the proteins.

On the other hand, differences in the amounts of aggregated fractions between the two cultivars could be related to the grain yield level. For instance, when the grain yield reaches its maximal value (Table I), the amounts of F1 and F2 are still increasing for Fandango, except for Loir and Cher samples, for which the total protein content was more important. This suggests that, at lower fertilization supplies, the nitrogen supply to the grain was certainly insufficient. The fact that the grain nitrogen content was always lower in Fandango than in Camp Rémy (Fig. 4) strengthens this hypothesis. As suggested by Kasarda (1989), the aggregate composition of a wheat sample could be related to changes in the amount or ratio of the LMW and HMW subunits during the grain development, so that the amount and physicochemical characteristics of the protein aggregates could be influenced by the mechanisms that regulate the expression of the protein synthesis. A significant variation of the amount of some HMW subunits between cultivars during maturation was demonstrated by Huebner et al (1990). It can be assumed that a significant variation also exists in the amount of LMW subunits. Whereas the qualitative composition of protein subunits within HMW or LMW group can be used for fingerprinting wheat genotypes, our results tend to indicate that this composition could not determine the ability of the proteins of a wheat sample to form HMW

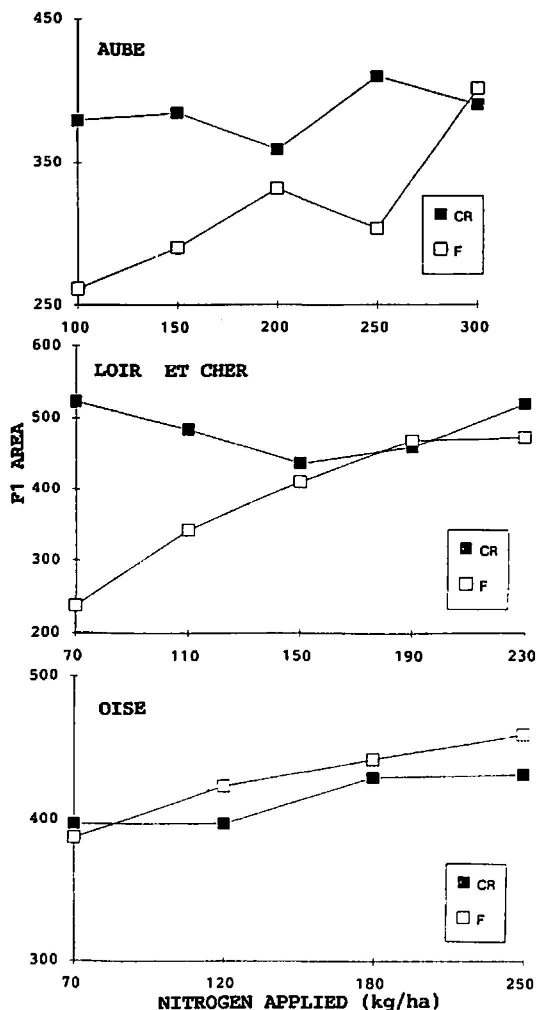

Fig. 9. Evolution of the fraction 1 (F1) area as a function of the nitrogen applied. Cultivars Camp Rémy (CR) and Fandango (F) were grown in 1990 in Aube, Loir et Cher, and Oise, France. The chromatogram area is expressed as Spectra-Physics arbitrary units.

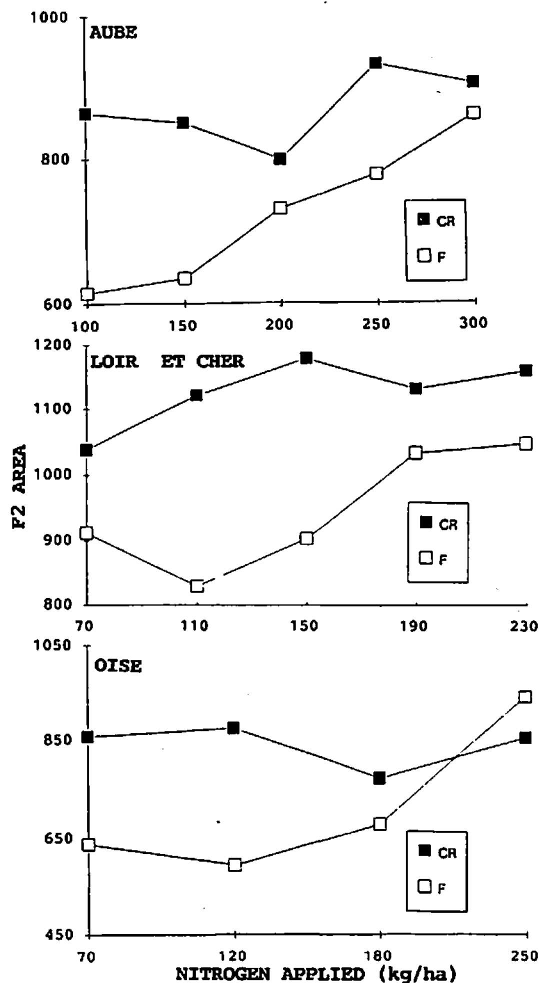

Fig. 10. Evolution of the fraction 2 (F2) area as a function of the nitrogen applied. Cultivars Camp Rémy (CR) and Fandango (F) were grown in 1990 in Aube, Loir et Cher, and Oise, France. The chromatogram area is expressed as Spectra-Physics arbitrary units.

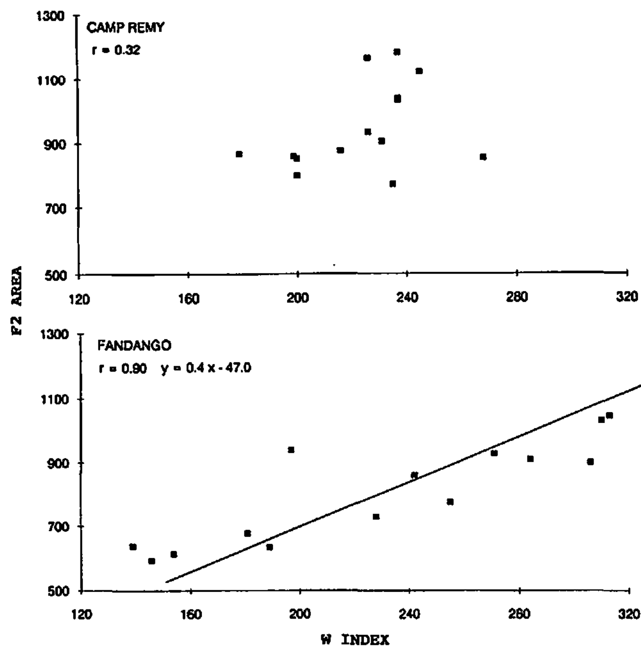

Fig. 11. Relation between chromatogram fraction 2 (F2) area and *W* index for cultivars Camp Rémy and Fandango grown in 1990 in three locations in France at different levels of nitrogen supply. The chromatogram area is expressed as Spectra-Physics arbitrary units.

aggregates. Effectively, whereas the subunit nature did not change in response to increased nitrate supplies, the amount of HMW aggregates increased. The nitrogen content of the SDS extracts was quite similar for the two cultivars; but the amount of gliadins (ethanolic extracts) was more important for Camp Rémy flours, suggesting that the gliadins may play a part in aggregate formation during the grain development.

The relationship between the area of F2 and baking strength is shown in Figure 11. Although the baking score is linearly associated with the area of F2 for Fandango, there is no significant correlation in the case of Camp Rémy. Again, the higher stability in quality observed for Camp Rémy seems associated with a stable amount of protein aggregates. This is probably due to both the higher total protein content in Camp Rémy and the different composition of the HMW and LMW subunits of glutenin between the two cultivars (Branlard et al 1990). This agrees with the fact that both the amount and composition of HMW subunits influence baking quality (Payne et al 1987, Sutton 1990) and that the ability to form large aggregates may depend on these two factors.

Our results may partially explain the origin of differences between cultivars in their response to changes affecting grain protein content. Consequently, SE-HPLC could be a tool to evaluate the stability of quality in response to changes in the level of nitrogen supply. Although these results have to be extended over a larger number of cultivars and over several years, they provide a basis for further investigations of associations between the aggregate composition and the stability of technological quality in response to environmental factors other than nitrogen nutrition.

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[Received October 15, 1991. Revision received March 17, 1992. Accepted April 29, 1992.]

NOVEMBER–DECEMBER 1992

VOLUME 69 NUMBER 6
ISSN 0009-0352
CECHAF 69(6):587–696

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