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Effects of Crop Nutrition on Wheat Grain Composition and End Use Quality

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Increasing applications of nitrogen fertilizer to wheat (from 0 to 288 kg/ha) resulted in an increased proportion of gliadin proteins and increased dough extensibility. Flour from a plot receiving 192 kg/ha N (and no S) was similar to that from a plot receiving 192 kg/ha N and 53 kg/ha S, but the proportion of ω-gliadins was increased and dough strength was more similar to that from plots with lower N. The grain %N from a plot receiving 35 t/ha farmyard manure was similar to that from the plot receiving 144 kg/ha N, indicating that much of the applied N was unavailable. The protein composition and dough properties of flour from this plot were similar to those of grain from conventionally fertilized plots with similar grain N contents. Similar differences in grain N content, protein composition, and functional properties were observed in grain samples from commercial organic and conventional farms.

KEYWORDS: Wheat; grain quality; grain protein; flour functionality; organic farming

INTRODUCTION

The relationship between nutrition and production is of fundamental importance for all crops, but is particularly important for nitrogen nutrition in wheat as the content and composition of the grain proteins determine the suitability and quality for producing bread and other food products. Because a considerable proportion of the 600 million tonnes of wheat that is produced each year is destined for human consumption, it is not surprising that the relationships between nitrogen, grain protein composition, and processing properties have received considerable attention. Genes affecting grain protein content in wheat have been identified and mapped (*1*, *2*), and genetic selection has resulted in differences in protein content of about 2% dry weight between high-protein breadmaking varieties and low-protein varieties suitable for livestock feed or industrial use (*1*, *3*). However, genetic effects on protein content are relatively small compared with the impact of environmental factors, most notably the availability of nitrogen. For example, a recent comparison of a hard breadmaking wheat (Option) and a soft wheat suitable for making biscuits or distilling (Riband) showed increases in protein content from 7.13 to 11.55% dry weight and from 6.61 to 11.51% dry weight, respectively, in samples grown without nitrogen fertilizer and with 240 kg of N/ha (*4*). Similar results have been reported in a number of earlier studies (reviewed in ref *5*).

The increase in grain protein under conditions of high nitrogen fertilization results from greater synthesis and accumulation of storage proteins, which in wheat correspond to the gluten proteins. Gluten proteins are the major determinant of the processing properties of wheat dough, by conferring viscoelasticity. A minimum protein content is therefore required for breadmaking wheat, which for flour millers in the United Kingdom is typically taken as 13% dry basis (db) for the widely used Chorleywood breadmaking process (CBP). Hence, farmers will grow breadmaking wheat with appropriate levels of N fertilization so that grain with the required protein content is delivered to millers. However, strong gluten properties are required for breadmaking as well as high total protein content, and hence the effect of nitrogen on protein composition is also important.

Several studies have shown that increases in grain nitrogen are associated with increased proportions of the monomeric gliadins, resulting in increased dough extensibility (*4*, *6*−*8*). However, Pechanek et al. (*9*) showed that the effect of grain nitrogen on protein composition was not consistent but varied between varieties. Similarly, both increases and decreases in the proportions of high molecular weight (HMW) subunits in the glutenin fraction have been reported (*9*, *10*), whereas Zhu et al. (*11*) reported that cultivars with different HMW subunit alleles showed differential effects of nitrogen nutrition on glutenin polymers and processing quality. Taken together, these studies indicate that increasing N availability tends to have a negative effect on protein quality by increasing the gliadin/glutenin ratio but that this effect may differ between cultivars.

Although the term "organic farming" applies to a whole farming system, one of the key characteristics is the prohibition of inorganic fertilizers and a reliance on traditional organic forms of fertilization, notably animal manures but also clover-rich leys and either undersowing or intercropping with leguminous species. These practices pose a challenge for the production of organic breadmaking wheat, as the nitrogen availability from such organic fertilizers tends to be low (*12*, *13*).

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The Broadbalk long-term experiment at Rothamsted provides an ideal opportunity to determine the relationship between wheat nutrition and breadmaking quality. The experiment was established in 1843 with adjacent strips (each approximately 320 m × 6 m) receiving defined combinations of mineral fertilizer or farmyard manure (FYM) (*14*). These strips are then subdivided into plots (23 m in length) with different wheat rotations or other agronomic treatments. Some modifications have been made to the experiment since 1843, but we were able to compare plots that had received either no N or N in an organic form as FYM since 1843 with plots receiving between 48 and 288 kg of N/ha since 1985 or before. In addition, plots with and without (±) S fertilization since 2000 were compared because of the established relationship between sulfur availability and processing quality (*15*).

The results of these studies, carried out at the biochemical and functional levels, are described in this paper, whereas metabolomic and transcriptomic analyses will be covered in a future publication.

MATERIALS AND METHODS

Growth and Harvesting of Wheat. The breadmaking wheat cultivar Hereward was grown in 2005, 2006, and 2007 on the Broadbalk winter wheat experiment at Rothamsted (N51:48:23; W0:22:21) in southeastern England. Grain samples were taken from plots of continuous winter wheat on seven treatments: N0, N1, N3, N4, N6, FYM, and N4-S, where N indicates nitrogen present in ammonium nitrate and the numbers indicate increments of 48 kg of N/ha. Nitrogen was applied in a single dressing in mid-April. FYM from cattle was applied in the autumn at a rate of 35 t/ha. This application rate is equivalent to the maximum recommended for commercial organic practice and contained approximately 250 kg/ha of N, predominantly in organic form. Plots received sulfur at 53 kg/ha except for the N4-S and the FYM plots, which received none. The N4-S plot received 192 kg of N/ha, that is, the same as for N4.

The Broadbalk plots are not replicated and hence three "replicate sampling areas" (each 0.5 m^2) were selected within each plot to give biological replication. Comparisons were therefore made using three replicate samples per treatment, one from each sampling area. Two or three technical replicates were analyzed from each biological replicate. Commercial samples of certified organically grown (9 in 2006, 5 in 2007) and 30 samples of conventionally grown (20 in 2006, 10 in 2007) wheat cv. Hereward were provided by Centaur Grain (Aylsham, Norfolk, U.K.) (all conventional samples), by F. P. Matthews Ltd. (Chipping Norton, Oxfordshire, U.K.) (2007 organic samples), or directly by farmers (2006 organic samples). Details of the agronomic conditions were not available, but all were grown for breadmaking. The sites were broadly distributed across southern England, from Derbyshire and Lincolnshire in the north to West Sussex and Wiltshire in the south. In this case the samples constituted biological replicates with two or three technical replicates being analyzed for each sample.

Brabender Quadrumat Milling. The water content of the grain was determined using a moisture meter (Marconi Instruments, St. Albans, U.K.) and adjusted to 15% by adding 1 mL of water per 100 g of grain for every 1% increase in moisture required. The grain were fully wetted and then left for 1 h before milling. Samples were milled with a Brabender Quadrumat Junior mill (Brabender GmbH & Co., Duisburg, Germany) for 1 h after the start of conditioning. Only five samples were milled per day at intervals of 90 min to avoid effects of heating of the mill on flour properties.

Chopin Milling. Grain samples were conditioned to a water content of 16% prior to milling using the following method. Water content was measured using a Sinar moisture meter 6060 (Sinar, Newbury, U.K.), using the standard protocol at Campden BRI (TES-CM-011).

Samples with moisture contents of > 16.5% were spread on a metal tray and allowed to dry at room temperature, whereas samples with moisture contents of < 15.5% were conditioned to 16% water. Samples were then milled using a CD1 Chopin laboratory mill (Villeneuve-la-Garenne Cedex, France), which produces flour that approximates to industrial flour in terms of its composition and dough rheological properties. The standard protocol at Campden BRI (TES-CM-011) was used. Conditioned wheat grain was passed once through the break rollers of the mill and twice through the reduction rollers. The reduction and break flours were combined and blended using a Kek-Gardner (Bristol, PA) double-cone blender for 10 min.

Total Nitrogen Determination. Total nitrogen was determined using the Dumas combustion method, using a CNS (carbon, nitrogen, and sulfur) Combustion Analyzer (Leco Corp., St. Paul, MN). Samples of white flour were dried at 80 °C for 8 h prior to analysis. The order of sample analysis was randomized with standards and blanks being included after every 10th sample.

Dough Water Absorption and Rheological Properties (Farinograph). The method is based on a standard Flour Testing Working Group (FTWG) protocol (FTWG method 04, version 1.2) (CCFRA, 2002). A 300 g sample of flour was analyzed using the Brabender Farinograph and the water absorption required to produce dough with a peak development of 600 Brabender units (BU) recorded.

Development time is the time from the beginning of mixing until the peak resistance (i.e., immediately before the dough shows signs of weakening). Stability is the time between the top of the resistance curve meeting the maximum consistency measurement (600 BU) and the point at which it drops below this measurement during dough softening. The degree of softening is the difference between the resistance of the dough at its peak (600 BU) and 12 min later.

Dough Rheological Properties (Extensograph). A 300 g sample of white flour, for which the water absorption value was already determined, was mixed with water containing 6 g of NaCl to a peak resistance of 500 BU in a Brabender Farinograph. The dough was cut in half, molded to a standard shape, and left to rest for 45 min before being stretched on the Extensograph (Brabender GmbH & Co.), using FTWG method 3 version 2.1 (CCFRA, 2002). From this, the peak resistance and the distance the dough piece stretched before breaking (extensibility) were determined from the graph of resistance to extension (expressed in BU) versus stretch distance (cm) (as the rate is constant, distance is proportional to time).

Dough Gluten Content. The Perten Glutomatic (Perten Instruments, Huddinge, Sweden) determines the wet gluten content of a flour sample. This is achieved by washing a piece of dough (made using 10 g of flour and 4.8 mL of salt solution) in a buffered solution of sodium chloride (2% (w/v) sodium chloride, 0.0745% (w/v) potassium dihydrogen phosphate, 0.0246% (w/v) disodium hydrogen sulfate, adjusted to pH 5.95) to remove the soluble components and leave the insoluble gluten matrix. In this case the gluten was weighed when wet and the content was calculated as a percentage of the dough starting weight. This method is based on FTWG method 13 version 2.1 (*16*).

Protein Extraction. Thirty-five milligrams of white flour was extracted with 1 mL of 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 1.5% (w/v) dithiothreitol, 10% (v/v) glycerol, and 0.002% (w/v) bromophenol blue. Prior to electrophoresis, samples were heated at 90 °C for 5 min and then centrifuged for 5 min at 13000 rpm. The supernatant layer was analyzed by SDS-PAGE.

SDS-PAGE. Tris−glycine SDS−polyacrylamide electrophoresis gels were cast according to a modified Laemmli method (*17*) with a 12.5% total acrylamide (pH 8.8; 0.8% present as bis-acrylamide) separating and 4.3% stacking gel. Gels were stained with Coomassie Brilliant Blue R250 and then scanned (Hewlett-Packard Scanjet 3970, Palo Alto, CA). Images were analyzed using phoretix 1D advanced software (Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, U.K.) with an optical density calibration curve calculated from a Kodak T14 control scale (Tiffen LLC, Rochester, NY). Values for band optical density and band percent as a proportion of total lane optical density were analyzed. Three technical replicates were run with gels belonging to the same technical replicate across all of the treatments being run together on the same day or on consecutive days and stained and destained together so that any variability due to time could be removed by blocking into technical replicates during statistical analysis.

SE-HPLC. Size exclusion high-performance liquid chromatography was used to determine the protein polymer size distribution of white flour samples. The analysis was performed at Campden BRI in accordance with the Profiblé method developed jointly by ARVALIS and l'Institut National de la Recherche Agronomique (*18*). A sample of 160 mg of flour was combined with 20 mL of 1% SDS (w/v) in 0.1 M phosphate buffer (pH 6.9) to dissolve the soluble gluten proteins. The solution was then sonicated (Misonix Microson XL2000) to solubilize the polymeric gluten proteins. The solution was then centrifuged for 10 min at 5000 rpm.

Table 1. Mean Grain Yield, Grain %N, and Protein Content from Broadbalk Plots of the First Wheat (After Break Crops) over the Years 2005−2007 (Broadbalk Sections 7, 4, and 5, Respectively) and Mean %N, %S, and N/S Ratios for White Flour Samples Milled from the Same Plots[a]

	application rate (kg/ha)		grain (n = 3)			flour (n = 9)		
treatment code	N	S	yield (t/ha at 85% DM)	%N	% protein (N × 5.7)	%N	%S	N/S ratio
N0	0	53	1.69 (0.19)	1.64 (0.04)	9.35 (0.25)	1.39 (0.04)	0.113 (0.006)	12.31 (0.29)
N1	48	53	3.88 (0.32)	1.37 (0.08)	7.81 (0.46)	1.19 (0.02)	0.093 (0.001)	12.60 (0.25)
N3	144	53	6.99 (0.94)	1.66 (0.08)	9.46 (0.44)	1.46 (0.02)	0.100 (0.005)	14.49 (0.90)
N4	192	53	8.50 (0.65)	2.00 (0.11)	11.40 (0.64)	1.84 (0.12)	0.130 (0.006)	14.19 (0.54)
N5	240	53	8.90 (0.59)	2.22 (0.06)	12.65 (0.35)	2.09 (0.05)	0.137 (0.005)	15.14 (0.85)
N6	288	53	9.08 (0.22)	2.47 (0.06)	14.08 (0.32)	2.22 (0.05)	0.147 (0.010)	15.34 (0.67)
FYM	35 t/ha FYM (approx 250 kg/ha N)	0	5.79 (0.32)	1.56 (0.07)	8.89 (0.40)	1.44 (0.06)	0.107 (0.001)	13.78 (0.46)
N4-S	192	0	8.18 (0.77)	1.98 (0.11)	10.94 (0.64)	1.72 (0.03)	0.103 (0.003)	16.24 (0.67)

[a] See Table S1 of the Supporting Information. All %N and %S values are based on 100% dry matter. Standard errors are given in parentheses.

An aliquot of the supernatant was sealed in an HPLC vial ready for analysis. The SE-HPLC analysis was conducted using a Jasco system operating with a TSK gel G 4000SW column and a TSK gel SW guard column. The flow rate was 0.7 mL/min, and detection was performed at 214 nm.

The chromatograms were integrated using a combination of automated algorithms and manual rules developed as part of the Profiblé method. The resulting SE-HPLC trace has five identifiable peaks, which correlate to different protein fractions of white flour. The first peak to elute from the column is referred to as F1 and consists of HMW polymers enriched in HMW glutenin subunits. The F2 peak comprises low molecular weight (LMW) polymers and is enriched with LMW glutenin subunits. The F3 and F4 peaks are composed principally of ω-gliadins and α-, β-, and γ-gliadins, respectively, and the F5 peak comprises LMW proteins including albumins and globulins. The overall area under the trace is a measure of the total protein content of the flour and is termed AT.

Statistical Methods. The residual maximum likelihood (REML) method was used to analyze the data sets derived from the various analyses described above, by fitting mixed models including fixed (treatment) terms and, where appropriate, random (variance or design) terms (*19*). The REML method was used because of the unbalanced treatment structure of some of the data sets (precluding the use of analysis of variance), specifically where not all treatments were sampled in all years. Following the assessment of statistical significance of treatment model terms (using approximate F tests), biologically relevant pairs of treatment means in significant ($p < 0.05$) terms were compared using the least significant difference (LSD) as derived from the standard error of the difference (SED) between means on the appropriate degrees of freedom.

Multivariate data sets derived for the samples were analyzed using canonical variates analysis (CVA). This method (see, e.g., ref *20*) finds linear combinations (canonical variates) of the responses that maximize the ratio of the variation between treatment combinations (e.g., the nutrient treatments by years) to the variation within treatment combinations, thus performing discrimination between all treatment combinations. The fewest number of canonical variates are retained that take up the most variation in the data and hence make the most discrimination. The data are then visualized on the new dimensions, by plotting the canonical variate scores for each sample. The mean of scores in each dimension for each treatment combination are also plotted. Making the assumption of multivariate normality for the data, 95% confidence circles can be placed around the means. The radius of these circles is

$$\sqrt{\chi^2_{2,0.05}}/\sqrt{n}$$

where n is the replication and $\chi^2_{2,0.05} = 5.99$ is the upper 5% point of a Chi-squared distribution on 2 degrees of freedom. Non-overlapping confidence circles give evidence of significant differences ($p < 0.05$). The magnitude of the coefficients (loadings) on the responses from a multivariate data set can be inspected, for each canonical variate, to consider which responses are important in the discrimination observed.

All analyses were done using the GenStat (2007) (10th ed., Lawes Agricultural Trust (Rothamsted Research), VSN International Ltd., Hemel Hempstead, U.K.) statistical system.

RESULTS AND DISCUSSION

The design of the Broadbalk experiment, with single parallel plots (strips) of each treatment, has remained essentially unchanged since its establishment in 1843. The treatments are not replicated as this concept was developed only in the early part of the 20th century, notably by R. A. Fisher working at Rothamsted (*21*). Hence, a sampling strategy was used in which three "replicate" areas were sampled from within each plot. The treatments selected are listed in **Table 1**. The breadmaking wheat cultivar Hereward has been grown on all Broadbalk wheat plots since 1996. Hereward was released in the United Kingdom in 1992 and is outclassed by >10% in terms of yield by more modern breadmaking varieties. Nevertheless, it is favored by millers and bakers because of its high intrinsic quality and year-to-year consistency of quality, and it continues to be grown on about 2% of the total U.K. area of wheat because it attracts a substantial premium.

The selected treatments allow three comparisons to be made: between N levels ranging from 0 to 288 kg of N/ha, between crops grown with 192 kg of N/ha with and without S, and between crops grown with equivalent levels of N applied in conventional and organic forms. It should be noted that the higher levels of N application are similar to those common in agronomic practice for breadmaking wheat in the United Kingdom.

The analyses were carried out on samples harvested in three successive years (2005, 2006, and 2007). These years differed considerably in their weather conditions [http://www.rothamsted.bbsrc.ac.uk/aen/ecn/]; in particular, 2007 had unusually low rainfall in the spring and high rainfall during grain development. The data presented here are combined from the three years and hence the conclusions should be robust across harvests. Full data for the three years (including means of technical replicates where applicable) are provided in Tables S1 and S2 of the Supporting Information.

Comparison of Grain N and Protein in N0−N6 Samples. The % N contents and calculated % protein contents (N × 5.7) of the wholegrain samples are given in **Table 1**. Because the CBP, which is widely used in the United Kingdom, requires a minimum protein content of 13%, only the N6 sample would be considered appropriate for milling and baking.

The relationship between the N application rate and the %N in white flour fractions is shown in **Figure 1A**. The increase is approximately linear from N1 to N5 and is beginning to reach a plateau by N6. The %N content of the N0 samples is greater than that of the N1 samples, which indicates that low nitrogen fertilization stimulates yield prior to having an impact on protein content. Direct measurement of AT determined by SE-HPLC of total protein extracts (discussed below) gave a similar relationship with respect to application rate (**Figure 1B**). However, the wet gluten content varied linearly with increasing flour %N (**Figure 1C**). This is consistent with the previously reported differential effects on N availability on the synthesis of gluten and non-gluten proteins (reviewed in ref *22*), with the synthesis of

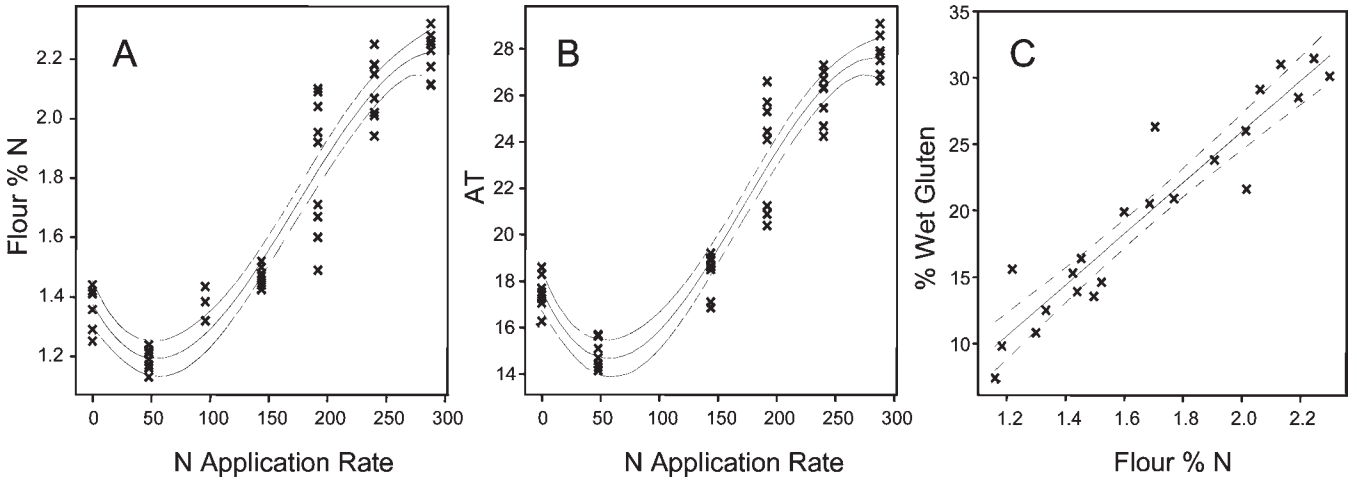

Figure 1. Regression plots of parameters measured on white flour from the Broadbalk experiment from 2005 to 2007 (plots N0, N1, N3, N4, and N6). Fitted polynomial models are shown between N applied and (**A**) flour %N, (**B**) total protein (AT), and straight line model between flour %N and % wet gluten content (**C**). N application rate is presented as kg of N ha^{-1}. The solid line is the fitted model; broken lines are 95% confidence intervals for the model.

Table 2. Mean Protein Composition of All Samples from Broadbalk Treatment Plots (N0−N6, N4-S, and FYM) and Commercial (Organic and Conventional) Samples, Measured by SE-HPLC and SDS-PAGE[a]

	Broadbalk samples (2005−2007)									commercial samples (2006−2007)		
	mean[b]									mean[c]		
composition parameter	N0	N1	N3	N4	N5	N6	N4-S	FYM	LSD (p = 0.05)	org	conv	LSD (p = 0.05)
						SE-HPLC						
%F1	14.150	14.670	14.430	14.290	14.190	14.120	14.290	14.600	0.293	13.790	13.560	0.284
%F2	26.200	25.790	26.020	25.900	25.920	25.630	25.880	25.700	0.260	25.850	25.510	0.298
%F3	6.725	6.629	6.911	7.342	7.704	8.095	7.670	6.855	0.216	7.488	8.351	0.255
%F4	32.710	31.190	33.660	35.890	37.220	38.050	36.640	33.480	0.508	35.950	38.290	0.625
%F5	20.190	21.710	18.990	16.590	14.980	14.090	15.530	19.350	0.622	16.910	14.280	0.788
%F1/%F2	0.540	0.569	0.555	0.552	0.547	0.551	0.552	0.568	0.012	0.534	0.532	0.013
(%F3 + %F4)/%F1	2.785	2.590	2.816	3.043	3.172	3.257	3.118	2.789	0.094	3.164	3.466	0.096
(%F3 + %F4)/ (%F1 + %F2)	0.978	0.936	1.004	1.077	1.121	1.161	1.105	1.003	0.024	1.097	1.195	0.028
						SDS-PAGE						
band 1	9.574	9.016	9.251	9.589	9.919	10.314	9.694	9.690	0.468	9.284	9.978	1.603
band 2	10.760	10.870	10.450	10.330	10.280	10.590	10.000	10.750	0.484	10.260	10.625	1.972
band 3	6.355	6.152	6.383	6.751	7.015	7.411	6.850	6.514	0.227	6.805	7.423	0.604
band 4	22.660	23.120	21.990	21.080	20.370	19.990	20.190	21.890	0.524	23.280	22.190	5.255
band 5	33.670	32.990	34.040	34.600	34.870	34.600	34.910	33.910	0.368	35.860	36.295	6.187
(B2 + B3 + B5)/B1	5.305	5.585	5.509	5.383	5.253	5.065	5.466	5.276	0.295	5.709	5.450	1.414
(B2 + B3 + B5)/ (B1 + B4)	1.573	1.558	1.626	1.680	1.719	1.726	1.748	1.618	0.058	1.630	1.690	0.481

[a] HPLC fractions are % of total protein (AT). Least significant differences (LSD) between means at the p = 0.05 level are given. [b] SE-HPLC, n = 8 or 9; SDS-PAGE, n = 3. [c] SE-HPLC, n = 14 for organic and n = 30 for conventional; SDS-PAGE, n = 14 for organic and n = 29 for conventional.

non-gluten proteins being favored at low N availability and that of gluten proteins at high N availability.

The composition of the gluten proteins was determined using two systems (**Table 2**). SDS-PAGE under reducing conditions allowed the relative proportions of five groups of bands to be determined. These correspond to HMW subunits of glutenin (band 1), ω-5 gliadins (band 2), other ω-gliadins (band 3), and two complex mixtures corresponding largely to LMW subunits of glutenin (band 4) and α-type/γ-type gliadins (band 5). The identities of these bands are discussed in more detail in a recent study of grain development in cv. Hereward (*23*). SE-HPLC provided complementary information on the size distribution of the unreduced grain proteins, separating fractions corresponding to large glutenin polymers (F1), small glutenin polymers (F2), mainly ω-gliadins (F3), mainly α-type/γ-type gliadins (F4), and non-gluten proteins (F5) (*18*). As the extraction of proteins for SE-HPLC is quantitative, the combined areas under the peaks provide estimates of total protein content (F1−F5, usually called AT and measured in arbitrary units) and total gluten protein content (F1−F4). The ratio of gliadin to glutenin proteins is given by (%F3 + %F4)/(%F1 + %F2). The well-established relationship between the proportion of large glutenin polymers and dough strength means the size of the F1 peak may be used as an estimate of dough strength and hence breadmaking potential, depending on the flours used (*24*). However, Millar (*25*) showed that more accurate estimates of dough strength were provided by comparing the ratio of large to small glutenin polymers (%F1/%F2) and the ratio of gliadins to large glutenin polymers ((%F3 + %F4)/%F1).

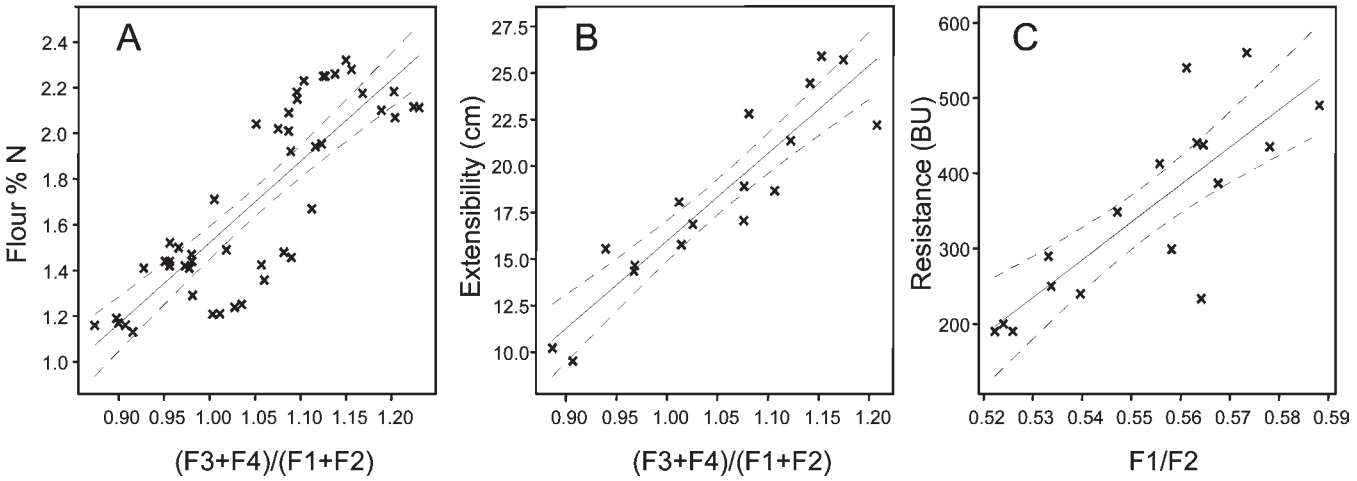

Figure 2. Relationship between protein fractions separated by SE-HPLC and functionality properties of white flour from the Broadbalk experiment from 2005 to 2007 (plots N0, N1, N3, N4, and N6). Fitted straight line models are shown between the gliadin/glutenin ratio ((%F3 + %F4)/(%F1 + %F2)) and (**A**) flour %N and (**B**) dough extensibility (Extensograph) and (**C**) between the %F1/%F2 ratio and Extensograph resistance.

In the present study the proportions of glutenin subunits separated by SDS-PAGE (bands 1 and 4) were correlated with the combined proportions of glutenin polymers separated by SE-HPLC (%F1 + %F2) ($r = 0.901$, $p < 0.001$), as were the proportions of gliadins determined by the two types of analysis (corresponding to bands 2 + 3 + 5 by SDS-PAGE and to peaks %F3 + %F4 by SE-HPLC) ($r = 0.830$, $p < 0.001$).

SE-HPLC showed clear increases in the proportion of gliadins (%F3 + %F4) with increasing N and a corresponding decrease in the proportion of nongluten proteins (%F5) (**Table 2**). Consequently, there was an increase in the gliadin/glutenin ratio ((%F3 + %F4)/(%F1 + %F2)), which was strongly correlated with flour %N (**Figure 2A**).

Comparison of the protein composition measured by SE-HPLC using CVA allowed all of the treatments to be clearly distinguished (**Figure 3A**), with the first canonical variate (CV1) accounting for 83% of the variance (and discrimination) and separating flours derived from plots ranging from low N to high N. The position of the N0 sample in this analysis falls between the N1 and N3 samples, as observed when total %N and total protein (AT) are plotted against N application rate in **Figure 1A**,**B**. The N4-S sample is most similar to the N4 sample in the direction of CV1 as they both received the same N application, whereas the FYM sample is most similar to N3. CV2 accounts for only 12% of the variance and may relate specifically to sulfur nutrition.

Comparison of Dough Functional Properties in N0−N6 Samples. The functional (processing) properties of dough made from white flour were determined using three standard laboratory test instruments: the Farinograph, Extensograph, and Reomixer. The Farinograph measures the water absorption, which is determined by the composition and physical properties of the grain as well as their interactions with the milling process used (consistent for all samples reported here). In particular, milling of hard grain results in a greater degree of starch damage than milling of soft grain and, hence, greater water absorption. All three instruments also measure aspects of dough behavior such as development during rinsing (Farinograph and Reomixer), rheological properties (resistance and extensibility) (Extensograph), and stability (Farinograph).

CVA allowed the treatments to be separated on the basis of functional properties (**Figure 3B**) in the same way as they could be separated on the basis of protein composition (**Figure 3A**). In this analysis 91% of the variance is attributed to CV1, which relates to the N fertilizer treatment with the lower N treatments (N0−N3) and FYM forming one cluster and the high N fertilizer treatments (N4−N6 and the N4-S treatment) forming a second cluster.

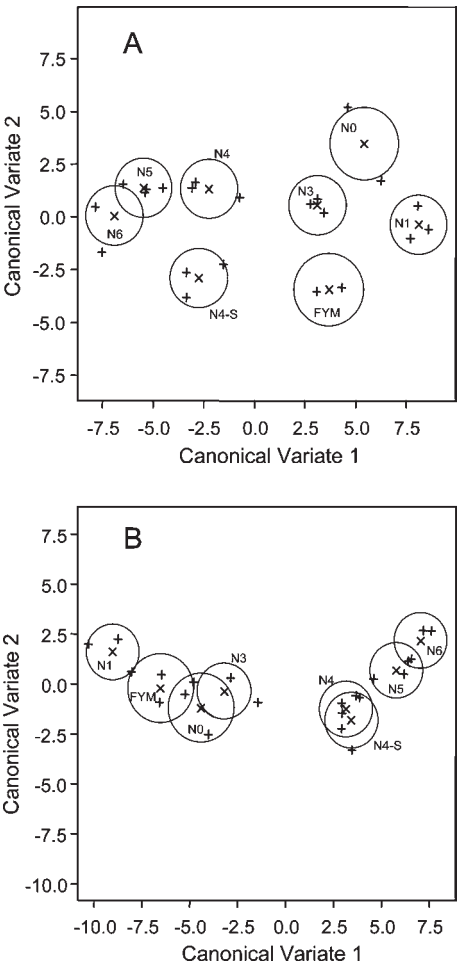

Figure 3. Canonical variates analysis plots using values for protein composition (**A**) and flour functional properties (**B**) for wheat grown on a range of N treatments and without S. Samples are from the Broadbalk experiment from 2005 to 2007 (plots N0, N1, N3, N4, N4-S, N6, and FYM). Central points (×) are the means of canonical variate scores, and each is surrounded by a 95% confidence circle. Individual year values are indicated (+).

Table 3. Correlation Coefficients (r) and Their Probabilities of Significance (p) for Correlations between Functional Properties and N Application Rate, %N in White Flour, and Gliadin/Glutenin Ratio Measured by SE-HPLC and SDS-PAGE on Samples from Broadbalk 2005−2007[a]

	Farinograph (n = 23)								Extensograph (n = 44)				Reomixer (n = 48)			
	development time		degree of softening		stability		water absorption		extensibility		resistance		maxcenter		maxtime	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
N application rate	**0.668**	**0.003**	−0.351	0.168	**0.830**	**<0.001**	**0.757**	**<0.001**	**0.756**	**<0.001**	−0.053	0.820	**0.879**	**<0.001**	**-0.804**	**<0.001**
% N in white flour	**0.641**	**0.001**	−0.399	0.066	**0.816**	**<0.001**	**0.798**	**<0.001**	**0.818**	**<0.001**	−0.175	0.448	**0.945**	**<0.001**	**-0.822**	**<0.001**
AT	**0.647**	**0.001**	−0.348	0.112	**0.795**	**<0.001**	**0.821**	**<0.001**	**0.850**	**<0.001**	−0.233	0.310	**0.955**	**<0.001**	**-0.840**	**<0.001**
% wet gluten	**0.648**	**0.001**	−0.270	0.225	**0.816**	**<0.001**	**0.839**	**<0.001**	**0.877**	**<0.001**	−0.233	0.309	**0.852**	**<0.001**	**-0.734**	**<0.001**
%F1	−0.119	0.598	**-0.503**	**0.017**	−0.078	0.731	**-0.523**	**0.012**	**-0.603**	**0.003**	**0.744**	**<0.001**	**-0.324**	**0.025**	**0.412**	**0.004**
%F2	−0.057	0.800	−0.374	0.087	0.033	0.884	−0.333	0.130	−0.278	0.210	**0.539**	**0.012**	−0.227	0.121	**0.389**	**0.006**
%F3	**0.441**	**0.040**	0.002	0.992	**0.560**	**0.007**	**0.753**	**<0.001**	**0.788**	**<0.001**	−0.431	0.051	**0.801**	**<0.001**	**-0.792**	**<0.001**
%F4	**0.599**	**0.003**	−0.115	0.611	**0.691**	**<0.001**	**0.895**	**<0.001**	**0.909**	**<0.001**	**-0.446**	**0.043**	**0.927**	**<0.001**	**-0.793**	**<0.001**
%F5	**-0.625**	**0.002**	0.278	0.210	**-0.760**	**<0.001**	**-0.838**	**<0.001**	**-0.846**	**<0.001**	0.272	0.233	**-0.943**	**<0.001**	**0.773**	**<0.001**
%F1/%F2	−0.119	0.599	**-0.473**	**0.026**	−0.099	0.662	**-0.505**	**0.017**	**-0.614**	**0.002**	**0.688**	**<0.001**	−0.281	0.053	**0.324**	**0.025**
(%F3 + %F4)/%F1	**0.443**	**0.039**	0.185	0.410	**0.486**	**0.022**	**0.832**	**<0.001**	**0.876**	**<0.001**	**-0.635**	**0.002**	**0.772**	**<0.001**	**-0.744**	**<0.001**
(%F3 + %F4)/ (%F1 + %F2)	**0.507**	**0.016**	0.064	0.778	**0.570**	**0.006**	**0.864**	**<0.001**	**0.886**	**<0.001**	**-0.563**	**0.008**	**0.846**	**<0.001**	**-0.792**	**<0.001**
band 1	0.209	0.350	**-0.755**	**<0.001**	0.419	0.052	−0.005	0.982	−0.045	0.843	**0.641**	**0.002**	0.210	0.324	0.121	0.575
band 2	−0.059	0.796	**-0.705**	**<0.001**	0.080	0.723	**-0.451**	**0.035**	**-0.487**	**0.022**	**0.762**	**<0.001**	−0.252	0.235	**0.520**	**0.009**
band 3	**0.461**	**0.031**	−0.315	0.153	**0.609**	**0.003**	**0.577**	**0.005**	**0.542**	0.009	−0.004	0.985	**0.742**	**<0.001**	**-0.664**	**<0.001**
band 4	−0.309	0.162	**-0.425**	**0.049**	−0.303	0.171	−0.741	**<0.001**	**-0.755**	**<0.001**	**0.658**	**0.001**	**-0.636**	**<0.001**	**0.798**	**<0.001**
band 5	0.336	0.126	**0.447**	**0.037**	0.227	0.311	0.698	**<0.001**	**0.706**	**<0.001**	**-0.748**	**<0.001**	0.387	0.062	**-0.595**	**0.002**
(B2 + B3 + B5)/B1	−0.098	0.664	**0.706**	**<0.001**	−0.291	0.190	0.155	0.491	0.196	0.383	**-0.681**	**<0.001**	−0.059	0.785	−0.278	0.188
(B2 + B3 + B5)/ (B1 + B4)	0.243	0.276	**0.571**	**0.006**	0.161	0.474	**0.647**	**0.001**	**0.614**	**0.003**	**-0.711**	**<0.001**	**0.488**	**0.018**	**-0.613**	**0.002**

[a] Significant ($p < 0.05$) correlations are given in bold.

It is important to distinguish between effects related to the increase in grain protein content that occurs with increasing N applications and effects that relate to the changes in protein composition which also occur (notably the increased ratio of gliadin/glutenin). **Table 3** therefore shows correlations between the functional properties, N application rate, flour %N content, and protein composition measured by SE-HPLC and SDS-PAGE. Water absorption was positively correlated with N application, flour N, and gliadin/glutenin ratio. There is a well-established relationship between flour protein content and water absorption (*26*), and it is thought that this is the dominant effect, rather than the associated change in gliadin/glutenin ratio.

Dough development was measured using two instruments, the Farinograph (development time) and the Reomixer (maxtime). These showed positive and negative correlations, respectively, with N application and flour N, probably due to the different mixing actions of the two machines: the relatively fast pin mixer in the Reomixer and the relatively slow z-blade mixer in the Farinograph. In addition, dough stability (measured in the Farinograph) was strongly positively correlated with N application rate and flour %N (i.e., total protein content) and more weakly positively correlated with the gliadin/glutenin ratio (measured as (%F3 + %F4)/(%F1 + %F2)).

Positive correlations occurred between dough extensibility measured by the Extensograph, the N application rate, and flour %N (**Table 3**), implying that a change in dough rheology occurred with a shift toward more extensible, less resistant dough properties in the high-N treatments. Extensibility was also positively correlated with the gliadin/glutenin ratio ((%F3 + %F4)/(%F1 + %F2)) (**Figure 2B**), which is consistent with the generally accepted view that gliadins contribute particularly to dough extensibility (*27*) and glutenins to strength (elasticity) (*28*).

Dough strength (resistance) measured by the Extensograph was negatively correlated with the gliadin/glutenin ratio ((%F3 + %F4)/(%F1 + %F2)) and positively correlated with the %F1/%F2 ratio (**Table 3**; **Figure 2C**). The latter corresponds to the ratio of high molecular mass to low molecular mass glutenin polymers, and the correlation is therefore consistent with the generally accepted role of these polymers in determining dough strength (reviewed in ref *29*).

In contrast, the dough strength (maxcenter) measured by the Reomixer was positively correlated with N application rate and flour %N, indicating a relationship to protein amount rather than composition. This confirms previous results from an evaluation of the Reomixer (*30*) in which protein content was shown to correlate positively with peak height, whereas differences in protein strength between varieties affected the mixing time as well as the overall shape of the trace.

Effect of S Fertilization on Grain N and Protein. Starting in 2000, selected plots on Broadbalk received N at 192 kg/ha (equivalent to N4) but without applied sulfur, whereas the conventional plots (including the N0 treatment and the control plot N4) received S at 53 kg/ha per year in the form of potassium sulfate and kieserite (predominantly $MgSO_4$).

The %N contents of white flour fractions from the N4 (i.e., +S) and N4-S samples were similar in 2005, but the %N contents of N4-S samples were lower and significantly different ($p < 0.05$) from those of the N4 samples in 2006 and 2007 (**Table 1**; Tables S1 and S3 of the Supporting Information). The N4-S flour also had a lower and significantly ($p < 0.05$) different S content (1071 ppm) and a higher ratio of N:S (16.25:1 when compared with the N4 flour (1298 ppm and 14.20:1) for S content and N:S ratio, respectively). However, the N:S ratio was also affected by N supply, with the lowest ratio (12.31:1) being in the N0 sample. This is consistent with a requirement for a balance between the availability of N and S to support the synthesis of proteins and other S-containing components.

CVA (**Figure 3A**) showed that the N4 and N4-S samples had broadly similar protein compositions by SE-HPLC, forming groups in similar positions on CV1 (accounting for 83% of the total variance). However, the two samples were separated on CV2 (12% of the total variance), which was related to increases in the ratios (%F3 + %F4)/(%F1 + %F2) and (%F3 + %F4)/F1% in the N4-S samples ($p < 0.05$) (**Table 2**). An increased proportion

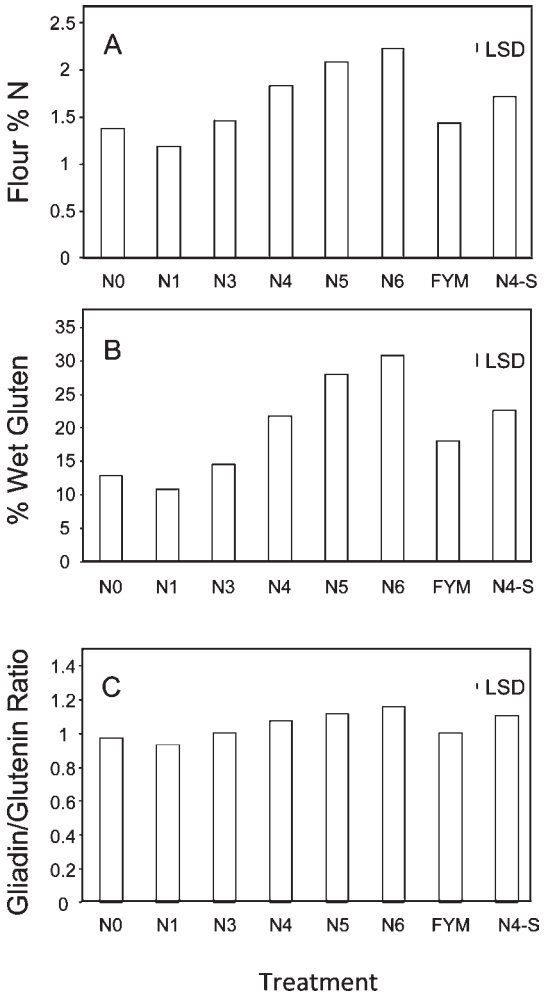

Figure 4. Comparison of the compositions of flours from conventionally fertilized plots with the FYM plot harvested from the Broadbalk experiment in 2005−2007 ($n = 3$): (**A**) %N in white flour; (**B**) % wet gluten; (**C**) gliadin/glutenin ratio measured by SE-HPLC ((%F3 + %F4)/(%F1 + %F2)). LSD at $p = 0.05$.

of ω-gliadins determined by SE-HPLC (%F3 of 7.670 and 7.342, respectively) was also demonstrated in the N4-S treatment compared with N4 (**Table 2**). This is consistent with previous studies that have shown increased proportions of the S-poor ω-gliadins with increasing N:S ratios (*31*).

Effect of S Fertilization on Dough Functional Properties. CVA (**Figure 3B**) showed that the N4 and N4-S treatments had broadly similar functional properties, with the two 95% confidence circles largely overlapping. Consistent with this was the observation that the only statistically significant difference between the functional properties of the two samples was the dough strength measured by the Extensograph (resistance) ($p < 0.001$) (Table S2 of the Supporting Information).

Comparison of Organic and Conventional Fertilizers. The "organically" fertilized plot was treated with 35 t/ha of farmyard manure (FYM), which contained about 250 kg/ha of total N. However, not all of this N is readily available to the crop, and variation in the weather can affect the rate of FYM decay and hence the release and losses (for example, by leaching) of available N. Such variation in release of N probably accounted for much of the observed year-to-year variation in the grain grown on the FYM plots, with analysis of material grown in 2006 indicating that more N was available to the plants than in the other two years. When the data from all three years were combined, the

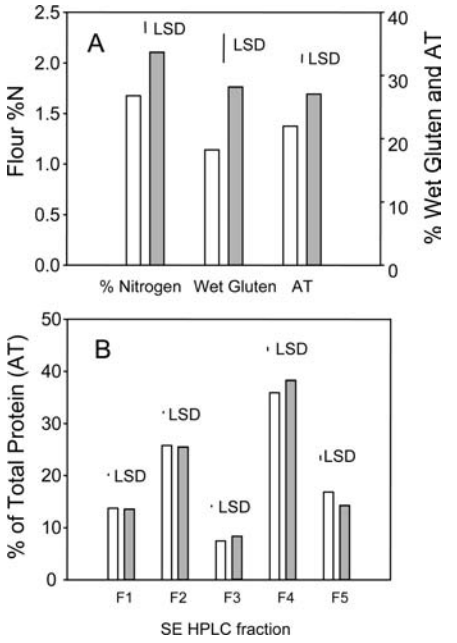

Figure 5. Comparison of conventionally grown (gray bars) ($n = 30$) and organically grown (white bars) ($n = 14$) commercial samples of wheat cv. Hereward: (**A**) mean values of protein, flour % N, % wet gluten, and total protein (AT) by SE-HPLC; (**B**) mean values for individual peaks in SE-HPLC profiles (expressed as a % of AT). LSD at $p < 0.05$.

FYM treatment was most similar to the N3 treatment in flour %N, wet gluten content, and protein composition including the gliadin/glutenin ratio measured by SE-HPLC ((%F3 + %F4)/(%F1 + %F2)) (**Figures 3A** and **4**; **Table 2**). The functional properties of the FYM samples were more similar to those of the low-N plots (N0, N1, and N3), as illustrated by the CVA plot in **Figure 3B**.

It is therefore concluded that the available nitrogen in the FYM plot was equivalent to or below that in the N3 treatment (144 kg/ha).

Comparison of Commercially Grown Organic and Conventional Wheats. Low gluten content is recognized as a problem in producing organic wheat for breadmaking in the United Kingdom. Until recently, Hereward was the most widely grown cultivar on organic farms for breadmaking, although it is now largely replaced by the spring cultivar Paragon, which allows farmers to produce grain with a higher N content. We therefore compared grain of Hereward from organic and conventional farms across England to determine whether the differences in composition and quality determined on the Broadbalk samples were representative of those occurring between commercially grown samples. A total of 14 samples of organic wheat (9 in 2006, 5 in 2007) and 30 samples of conventionally grown wheat (20 in 2006, 10 in 2007) were obtained directly from farmers or grain merchants. No details of the agronomic conditions were available, but all were grown for breadmaking and would therefore have received high levels of nitrogen (organic practices or conventional fertilizers). The sites ranged from Derbyshire and Lincolnshire in the north to West Sussex and Wiltshire in the south. Full analyses of these samples are presented in Tables S4 and S5 of the Supporting Information, and the results are summarized in **Figures 5** and **6** and **Table 4**.

The results show agreement with those reported above for the Broadbalk samples, with the organically fertilized grain being consistently lower in grain %N, wet gluten, and AT measured by SE-HPLC (**Figure 5A**). Measurements of the gliadin/glutenin

ratio by SE-HPLC ((%F3 + %F4)/(%F1 + %F2)) and SDS-PAGE ((B2 + B3 + B5)/(B1 + B4)) also agreed with this comparison (data in Table S4 of the Supporting Information).

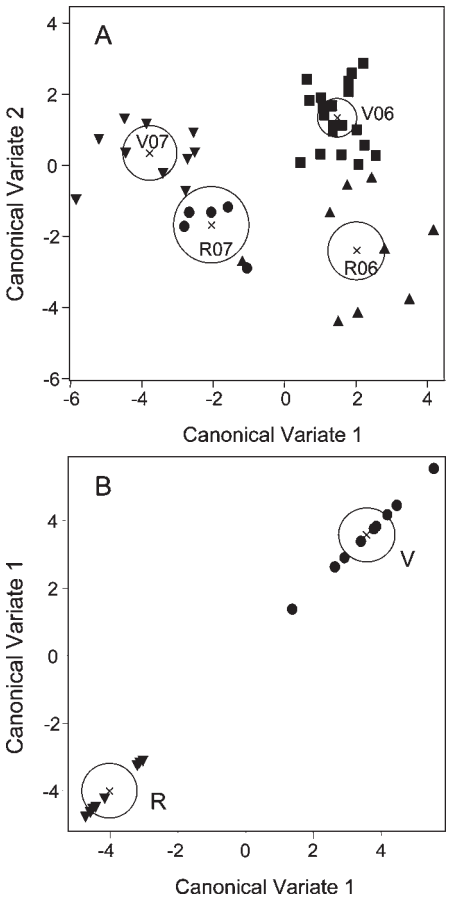

Figure 6. (**A**) Canonical variates analysis plot for SE-HPLC data showing the canonical variate scores for each combination of regimen (conventional (V, ▼, ■) or organic (R, ●, ▲)) by year (2006, ■, ▲; or 2007, ▼, ●). The means of canonical variate scores (×) are also shown surrounded by 95% confidence circles ($n = 9$ for R06, $n = 20$ for V06, $n = 5$ for R07, and $n = 10$ for V07). (**B**) Canonical variates analysis plot for flour properties and AT measured by SE-HPLC showing the canonical variate scores for each combination of regime, (conventional (V, ●) or organic (R, ▼)) for samples from 2006. The means of canonical variate scores (×) are also shown surrounded by 95% confidence circles ($n = 9$ for both R and V).

CVA of the SE-HPLC data clearly distinguished between the conventional and organic samples in both years (**Figure 6A**), with CV1 (due mainly to F3) separating the years and CV2 (due mainly to F1 and F2) separating the treatments. This is illustrated in **Figure 5B**, which shows that the organically fertilized samples contained a lower proportion of gliadin proteins (F3 and F4), with the gliadin/glutenin ratio ((%F3 + %F4)/(%F1 + %F2)) being 1.195:1 for the conventionally fertilized samples and 1.097:1 for the organically fertilized samples (significantly different at $p < 0.001$). This is consistent with the lower protein content (as discussed above).

The functional properties of the samples were determined on nine organic and nine conventionally grown samples from 2006 only, using the Farinograph, Extensograph, and Reomixer, and the results were analyzed using REML. It is clear that the higher protein content of the conventionally fertilized grain resulted in stronger dough, which is shown by the higher values for development time ($p < 0.001$), stability ($p < 0.009$), water absorption ($p < 0.001$), extensibility ($p < 0.006$), and maxcenter ($p < 0.001$) and in the lower values for maxtime ($p < 0.001$) and degree of softening (not significant, $p > 0.05$) (see Table S5 of the Supporting Information). CVA of the functionality data also clearly distinguished between the two sets of samples (**Figure 6B**).

The means of responses for the Broadbalk data for 2005−2007 show that the N4, N5, and N6 treatments have functional properties which are intermediate between those of the commercial conventional and commercial organically fertilized samples (**Table 4**). Moreover, the conventionally fertilized commercial samples (data from 2006) gave stronger dough even than the N6 treatment, showing a longer development time, greater stability, lower degree of softening, higher maxtime, and lower maxcenter. This may appear surprising as the N6 application rate of 288 kg of N/ha is similar to commercial application rates for breadmaking wheat in the United Kingdom. However, the Broadbalk experiment was not designed to study grain protein content and quality, the present study representing the first time in which this has been determined in its 166 year history.

Conclusions. We have used material from the Broadbalk continuous wheat experiment at Rothamsted to determine the relationships between plant nutrition, grain protein composition, and processing quality, focusing on the amount and form of applied nitrogen and the effects of sulfur fertilization. Grain samples from plots with N applications ranging from 0 to 288 kg/ha could be clearly distinguished on the basis of their gluten protein composition, with increases in N application resulting in an increased proportion of monomeric gliadins and a decreased proportion of large glutenin polymers. These changes are consistent

Table 4. Mean Functional Properties of Commercial Organically and Conventionally Grown Samples of Wheat Cv. Hereward (2006) Compared with Samples from Broadbalk (2005−2007)[a]

	commercial samples ($n = 9$)				Broadbalk samples					
	org	conv	LSD ($p = 0.05$)	F probability	FYM	N4	N5	N6	LSD ($p = 0.05$)	F probability
Farinograph										
water absorption (%)	47.94	50.79	1.51	0.001	46.03	47.54	49.13	50.01	1.63	<0.001
development time (min)	1.78	3.06	0.65	<0.001	1.17	1.67	2.00	2.83	0.79	0.008
stability (min)	2.72	4.39	1.18	0.009	1.33	2.16	3.17	3.659	0.73	<0.001
degree of softening (BU)	163.9	134.4	44.49	0.180	186.7	163.10	153.30	144.8	45.42	0.348
Extensograph										
resistance (BU)	496.20	495.00	57.35	0.964	386.20	332.10	343.00	288.60	76.14	0.110
extensibility (cm)	14.51	18.53	2.65	0.006	13.14	20.05	22.38	23.35	2.04	<0.001
Reomixer										
maxcenter (V)	7.08	4.73	1.20	0.013	4.81	6.84	7.60	8.028	0.19	<0.001
maxtime (min)	6.30	7.91	0.56	<0.001	9.25	5.90	4.77	3.95	1.22	<0.001

[a] Least significant differences (LSD) between means at the $p = 0.05$ level are given. Farinograph, $n = 3$; Extensograph and Reomixer, $n = 4-6$. BU, Brabender units.

with previous reports of increased gliadin content with increasing N fertilization (*4*, *6*−*8*).

The increased proportion of gliadin proteins was reflected in the higher dough extensibility of the samples grown with high N fertilization, as measured using the Extensograph. However, only the N6 application gave grain with sufficiently high protein content (13%, ≈2.28% N) to meet flour mill intake criteria for bread wheat in the United Kingdom.

Comparison of the N4 treatment with grain grown with the same N application (192 kg/ha) but without applied sulfur (N4-S) showed similar functional properties, except that the dough strength of the N4-S samples measured using the Extensograph was more similar to that of the low-N samples.

The results presented here are consistent with previous studies showing that a balance of N and S is required for optimum gluten protein composition and functional properties. Because a significant proportion of the land used for bread wheat production in the United Kingdom is now deficient in sulfur, many farmers routinely apply S-containing fertilizer at levels of 15−20 kg/ha (*32*).

The availability of samples from plots that had received only organic manure for over 160 years also allowed comparisons to be made with samples which had received conventional fertilizer. Although the amount of N applied to the FYM plots was estimated to be about 250 kg/ha, the grain %N content was similar to that of the N3 plots, which received 144 kg/ha, indicating that only about half of this was available to the plant. This was associated with wet gluten content and gluten protein composition similar to those of the N3 sample, including a lower ratio of gliadins/glutenins. Although the dough strength was greater than that of the N4−N6 samples, the low protein content (below 11%) would render it unsuitable for breadmaking using the CBP.

The agronomic regimen used on the Broadbalk FYM plots differs from organic systems used by commercial farmers with conventional control of pests, pathogens, and weeds being permitted. The relevance of the data obtained to commercial organic production was therefore evaluated by comparing conventionally and organically produced commercial samples of the same cultivar. Although the samples were grown across the United Kingdom over two years and would be expected to differ widely in their agronomy and environmental conditions, they nevertheless exhibited differences similar to those observed between the organically and conventionally fertilized Broadbalk samples. In particular, the organically grown samples had lower grain %N and protein contents, which resulted in reduced suitability for breadmaking despite their higher proportions of glutenin proteins and acceptable dough strength.

However, the quality of the Broadbalk samples was lower than those of the corresponding commercial samples. This reflects the fact that the agronomic inputs into the Broadbalk experiment have not been optimized for grain processing quality, whereas the commercial organic and conventional farmers target their whole systems to the production of grain of the appropriate quality. Thus, although similar differences are observed between the conventional and organic samples from both Broadbalk and commercial farms, in both cases the quality was greater from the commercial production systems.

ABBREVIATIONS USED

AT, total area under SE-HPLC peaks P1−P5; BU, Brabender unit; CBP, Chorleywood breadmaking process; CV, canonical variant; CVA, canonical variate analysis; FTWG, flour testing working group; FYM, farmyard (cattle) manure; HMW, high molecular weight; LMW, low molecular weight; N, nitrogen; REML, residual maximum likelihood; S, sulfur; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high-performance gel electrophoresis.

ACKNOWLEDGMENT

Commercial wheat samples were generously provided by Centaur Grain (Aylsham, Norfolk, U.K.) (all conventional samples), by F. P. Matthews Ltd. (Chipping Norton, Oxfordshire, U.K.) (2007 organic samples), and by individual farmers (2006 organic samples).

Supporting Information Available: Tables S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

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