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# Foliar N application at anthesis alters grain protein composition and enhances baking quality in winter wheat only under a low N fertiliser regimen

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## ARTICLE INFO

## ABSTRACT

Wheat is the second biggest staple crop worldwide and is mainly consumed in the form of baked goods, requiring a specific flour quality. Grain protein concentration (GPC) is an underpinning parameter for baking quality and therefore strongly influences the value of wheat. It is a common strategy to increase GPC by combining high protein varieties with the application of a late dose of nitrogen. However, the late use of N fertilisers can cause environmental problems, such as nitrate leaching and gaseous losses. Furthermore, recent studies show, that there is only a weak relationship between GPC and bread volume. The aim of this study was to re-evaluate the merits of a late N application by investigating the effects of a late foliar N application, not only on GPC, but also on the gluten protein composition and on bread volume.

In this study, an increasing GPC did not necessarily lead to improved baking quality. Baking performance rather depended on the grain protein composition. Only at a low N fertiliser level (100 kg N ha$^{-1}$), the foliar N application decreased the HMW-GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio, which led to an improved bread volume. These results imply that a late foliar N application can be used to effectively improve baking quality when the total N uptake was low due to low fertilisation or unfavourable weather conditions. The results also show that quality cannot be evaluated by measuring GPC alone but also needs information of storage protein composition as well as bread volume.

## 1. Introduction

With a worldwide production of 749 million tonnes in 2016, wheat (*Triticum aestivum* L.) is the second biggest staple crop worldwide. After rice, it is the most important food for humans, and by providing 16 g protein capita$^{-1}$ day$^{-1}$ (world average), it is the main source of protein in human nutrition (http://www.fao.org/faostat/en/#compare). Wheat products are mainly baked goods, such as bread, buns, pizza or pastries, and a high baking quality of wheat flour is required. Grain protein concentration (GPC) is an essential parameter for baking quality predictions and therefore often determines the price for wheat grain. In the UK, wheat cultivars are classified into quality groups (NABIM (National Association of British and Irish Millers) groups 1–4) using GPC as one of the main parameters. As wheat quality characteristics are defined by genotype, environment and their interaction, it is a well-known strategy for farmers to increase GPC by combining high protein varieties with the application of a late dose of nitrogen fertiliser. However, recent studies show that there is only a weak relationship between GPC and bread volume, which is a direct measure of baking quality (Kazman and Innemann, 2010; Thanhaeuser et al., 2014). Especially for high protein wheat cultivars with GPCs greater than 12%, only a poor correlation was observed (Gabriel et al., 2017). At present, no better (quickly detectable) parameter other than GPC has been found. The ideal approach would be to combine GPC measurements with the examination of the composition of grain proteins as well as baking performance of the flours to evaluate the end-use quality of wheat grains.

The late use of N fertilisers can cause environmental problems, such as nitrate leaching into the groundwater and gaseous losses in the form of nitrous oxide, which contribute to global warming (Senbayram et al.,

2009). For farmers, these problems represent a monetary risk, particular in the case of unfavourable weather conditions which may limit the uptake of the supplied nitrogen and hence, the required GPC might not be reached. In this case, a large amount of N remains in the soil and increases the environmental risks mentioned above. In this study, the method of foliar N application was chosen as an alternative as it holds advantages over the soil application of N fertilisers. Effects of N fertiliser management on GPC, grain protein composition and baking quality have been investigated previously (Pechanek et al., 1997; Wieser and Seilmeier, 1998; Wan et al., 2014; Schulz et al., 2015; Xue et al., 2016a,b), but the observed changes in protein composition due to different N fertilisation managements and their influence on baking quality are inconsistent. Furthermore, the influence of a foliar N application was considered in none of these studies. Woolfolk et al. (2002); Bly and Woodard (2003) and Tea et al. (2004) studied the influence of a foliar N application on various quality parameters, but not on bread volume. For example Wieser and Kieffer (2001) found a strong influence of the gliadin/glutenin ratio on rheological properties and bread volume, but this could not be confirmed by Pechanek et al. (1997). Another example is the influence of N fertilisation on the quantity of HMW-GS, which was reported to be positive (Wieser and Seilmeier, 1998) and negative (Pechanek et al., 1997). As the relationship of protein composition and baking quality remains unclear, further investigations are needed.

In this context, the aim of this study was to re-evaluate the merits of a late N application by investigating not only the effects of a late foliar N application on GPC, but also on the composition of grain proteins and on the bread volume, in three wheat cultivars, under two N fertiliser regimes.

## 2. Material and methods

### 2.1. Plant cultivation

Two bread-making wheat cultivars (Skyfall and Soissons) and one feed wheat cultivar (Conqueror) were grown in a randomised field experiment, with three replicate blocks at Rothamsted Research (Harpenden, UK) in 2016 and 2017, as part of the Defra-sponsored Wheat Genetic Improvement (WGIN) trials (Barraclough et al., 2010). Nitrogen was applied at two levels, 100 kg N ha$^{-1}$ (N100) and 200 kg N ha$^{-1}$ (N200) and an additional late foliar application of urea (40 kg N ha$^{-1}$) at anthesis (growth stage (GS) 69) was performed on one half of each plot, resulting in the following four treatments: 100 kg N ha$^{-1}$; 100 kg N ha$^{-1}$ + foliar N; 200 kg N ha$^{-1}$; 200 kg N ha$^{-1}$ + foliar N. Nitrogen was applied as ammonium nitrate at two time points for N100 (50-50-0) and at three time points for N200 (50-100-50) during vegetative plant growth (respectively, approximately GS 24, 31 and 32). In 2016, ears were tagged at anthesis and caryopses were harvested from the middle of the ear at 10, 14, 21, 28 and 35 days post-anthesis (DPA) and immediately frozen in liquid nitrogen for protein extraction. Cross sections of the caryopses were taken and fixed (4% paraformaldehyde + 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.4) at each time-point for microscopic analysis. After anthesis, weekly measurements with the HandySpec® Systems (Tec5 AG, Germany) and SPAD meter (SPAD-502, Konica Minolta Sensing Europe B.V.) were carried out to monitor senescence. Plant protection was carried out according to local farming practice. Weather data for the two years of field experiments were obtained from the Rothamsted meteorological station (http://resources.rothamsted.ac.uk/environmental-change-network/rothamsted-weather-charts).

### 2.2. Yield, N, GPC and N-harvest index

Plants were harvested from a sample area (0.5 m$^2$) in each plot at anthesis and at maturity. Mature plants were threshed and grain and straw fresh and dry weight were recorded as well as thousand-grain-

weight (TGW) and Hagberg Falling number (FN). Yields are expressed as 85% dry matter (DM). The weight of flour used for Hagberg Falling Number measurement was adjusted according to the moisture content of the flour. Grain and straw samples were ground with an Ultra Centrifugal Mill (ZM 200, Retsch) and a Hammer Mill (Christy & Norris 8" Lab Mill, Christy Turner Ltd) for further analysis. The nitrogen content of grain and straw samples was measured by the Dumas method using a LECO CN628 Combustion Analyser (LECO Corporation, St Joseph, Michigan, USA) and is expressed in percent of dry matter. Grain protein concentration was calculated by multiplying the N concentration by the factor 5.7. Elemental analyses of grain and straw samples were carried out using ICP-MS (inductively coupled mass spectrometry). Nitrogen harvest index (N-HI) was calculated according to the following equation:

$$NHI \ [\%] = \frac{Grain \ N \ content}{Shoot \ N \ content} * 100$$

### 2.3. Micro baking test

The moisture content of flour samples was calculated from the weights of a given amount of flour before and after drying for 2 h at 110 °C. Afterwards, 10 g of wholemeal flour (14.0% moisture) were mixed with 0.2 g NaCl and then used to determine optimal water uptake and optimal dough kneading time by means of a farinograph (Farinograph-E, Brabender GmbH & Co. KG, Duisburg, Germany). An optimal dough development was defined at a dough consistency of 550 Brabender Units (530–570 BU) (Kieffer et al., 1998). For the microscale baking test, 10 g of flour (14.0% moisture) were combined with 0.7 g fresh yeast and 0.1 g shortening. 1 mL NaCl/sucrose-solution (1% NaCl, 2% sucrose), 0.3 mL 0.004 M L(+)-ascorbic acid solution and the determined amount of water were added and the dough was kneaded for the optimal time required to reach 550 BU. After kneading, the dough was left to rest at 30 °C and 90% relative humidity for 20 min. The dough was reshaped and rounded before secondary proofing for 40 min. Finally, the dough was baked for 10 min at 180 °C increasing to 250 °C on an automated proofing baking line. The bread loaves were weighed after cooling and bread volume was measured by surface scanning, using a Volscan Profiler 300 (Stable Micro Systems Ltd, Godalming, UK).

### 2.4. Protein extraction and SDS-PAGE

Grain samples taken at five time-points after anthesis (10, 14, 21, 28, 35 DPA) were ground using a freezer mill (Freezer Mill 6870, Spex Sample Prep). Aliquots of each sample were freeze-dried for at least 36 h. Total gluten protein fraction was extracted from 10 mg flour using 50% propan-1-ol + 2% dithiothreitol (DTT) at 50 °C, the extraction being repeated and the supernatants combined. After freeze-drying, the protein was dissolved in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 30 mM DTT, 20 mM Tris base). The 2-D Quant Kit (GE Healthcare) was used to quantify the extracted protein. The sample amount containing 10 µg protein was combined with loading buffer (50 mM Tris−HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, 200 mM DTT) and loaded on 7 cm precast gels (4–15% Mini-PROTEAN TGX Precast Protein Gels, Biorad, Munich, Germany) for 1D SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Precision Plus Protein™ Standard (Biorad) was applied to each gel as protein ladder. Running conditions were 40 V for 30 min, then 80 V for 120 min. After completion of the run, gels were fixed in a fixing solution (40% ethanol, 50% H$_2$O and 10% acetic acid) for one hour. Afterwards, gels were stained with staining solution (1 Coomassie tablet (GE Healthcare, Freiburg, Germany) in 1.6 L 10% acetic acid) for one hour and later, destained in 10% acetic acid overnight. Gels were then scanned using an Epson Perfection V700 scanner. Gel image analyses were carried out using the GelAnalyzer 2010a software

(gelanalyzer.com). The protein bands were classified into three groups according to He et al. (2013) and Wan et al. (2014). The first group corresponded to the HMW-GS, the second group to the ω-gliadins and the third group combined a mixture of LMW-GS, and α-/γ-gliadins.

### 2.5. Protein extraction and SE-HPLC

For SE-HPLC (size-exclusion high performance liquid chromatography) analysis, 16.5 mg wholegrain flour were suspended in 1.5 mL of 5% SDS in sodium dihydrogen phosphate (pH 6.9) and then stirred for 5 min. Samples were then sonicated for 40 s, followed by centrifugation (30 min at 10,000$g$) to obtain the proteins in the supernatant. Separation was achieved in 30 min by loading 20 μL of sample into an eluent (50% (v/v) acetonitrile and water, containing 1 mL L$^{-1}$ trifluoroacetic acid (TFA)) at a flow rate of 0.2 mL min$^{-1}$ using a Shimadzu Prominence HPLC and a Phenomenex BioSep™ SEC s4000 column. Proteins were detected by UV absorbance at 210 nm. The SE-HPLC chromatograms were divided into five sections of decreasing molecular size according to (He et al., 2013): large glutenin polymers (F1), small glutenin polymers (F2), ω-gliadins (F3), α-/γ-gliadins (F4) and non-gluten proteins (F5).

### 2.6. Statistical analysis

The GenStat (17th edition, VSN International Ltd., Hemel Hempstead, UK) software was used for statistical analyses. Data were analysed by ANOVA, with the design structure of the experiment (randomised plots) being taken into account. Comparisons of relevant means were made using the least-significant difference at the 5% level (p = 0.05). For the statistical analysis of the gel electrophoresis data, in addition to the field structure, the structure of the laboratory analysis (technical replicates) was included. For statistical analysis, the protein composition data from SDS-PAGE were converted to square root scale to assure variance homogeneity. Regression analysis was used to evaluate the relationships between grain protein concentration, gluten protein or the amount of gliadins and bread volume.

## 3. Results

### 3.1. Grain yield and GPC

Three varieties were grown in a randomised field experiment at Rothamsted, UK at two fertilisation levels of nitrogen (100 kg N ha$^{-1}$ and 200 kg N ha$^{-1}$). An additional dose of foliar N was applied at anthesis to one half of each plot. The three tested wheat varieties belong to different quality groups. Skyfall is a high yielding bread wheat with consistent quality in NABIM (National Association of British and Irish Millers) group 1. Soissons is an early maturing variety from NABIM group 2. Conqueror is a hard-milling feed wheat and belongs to NABIM group 4. Grain yield was enhanced by a higher N fertiliser level in both years except for Soissons in 2017 (Fig. 1A). On average, the higher N fertiliser level resulted in an additional grain yield of 2.9 and 1.2 t ha$^{-1}$ in 2016 and 2017, respectively. An increased grain yield due to foliar N application at anthesis was only observed for Skyfall at N100 and for Soissons at N200. In 2016 at the 100 kg N ha$^{-1}$ level, the grain yield of the feed wheat cultivar Conqueror was significantly (p < 0.05) higher than that of the bread making cultivar Soissons, but there were no cultivar differences observed at the 200 kg N ha$^{-1}$ level. In 2017, Soissons had a lower grain yield than Conqueror and Skyfall at both N fertilisation levels. GPC (calculated as grain N concentration × 5.7) was increased by a higher N fertiliser level as well as foliar N application at anthesis (Fig. 1B). Without foliar N application at anthesis, the average grain protein concentration was 8.9%, which was raised to 10.3% by foliar N application. There were significant (p < 0.05) differences in GPC between cultivars. The lowest grain protein concentration was observed for Conqueror with 8.7%, which was expected as Conqueror is

a feed wheat. Contrary to the NABIM classification, GPC of Soissons (10.6%) exceeded that of Skyfall (9.6%). However, considering the average GPC, both varieties did not reach the standard specification for bread-making of at least 12%. This minimum GPC for bread-making wheat was only achieved by Soissons in 2017 at the high N level (N200) combined with the foliar N application. GPCs for all varieties and N fertiliser levels were lower in 2016 than in 2017.

GPCs achieved at N200 + foliar N ranged from 9.8% (Conqueror) to 11.9% (Soissons) in year 2016 and from 11.2% (Conqueror) to 13.5% (Soissons) in year 2017. The minimum protein concentration of 12%, required for bread-making in the U.K. (Godfrey et al., 2010; He et al., 2013) was only reached by the two bread-making wheat varieties tested in one of the two years.

### 3.2. Plant N uptake and nitrogen harvest index

Plant N uptake was calculated from N concentration and biomass data at anthesis and maturity; the results are presented in Fig. 1C. At anthesis plant N uptake was equal for all three varieties with higher N uptake at the higher N fertiliser level. For N100, plants absorbed 94 kg N ha$^{-1}$ on average until anthesis and 151 kg N ha$^{-1}$ on average for N200. When no foliar N was applied, the average additional N uptake post anthesis was 16.7 and 42.5 kg ha$^{-1}$ for N100 and N200, respectively. When foliar N was applied, the additional N uptake post anthesis increased to 48.6 kg ha$^{-1}$ at N100 and to 76.3 kg ha$^{-1}$ at N200. An additional foliar N application at anthesis increased N uptake at maturity by 33 kg ha$^{-1}$. This is consistent with the foliar-applied 40 kg ha$^{-1}$ N at anthesis. The higher N fertiliser regimen resulted in significantly (p < 0.05) higher plant N uptake at maturity in all three cultivars.

The N-HI (nitrogen harvest index) was enhanced by a foliar N application at anthesis only in 2016. With an average of 80.1%, the N-HI in 2016 was lower than in 2017 (83.9%) as is shown in Fig. 1D. Statistical analysis showed a three-way interaction of the factors year, cultivar and N level on the N-HI. Part of this interaction is a decrease of the N-HI due to a higher N fertilisation level, but this effect only appeared in the cultivar Soissons and only in year 2016. In that year, Soissons had a higher N-HI than Skyfall and Conqueror at N100, but at N200 the N-HI for Soissons was the lowest. In 2017, the lowest N-HI was observed for Conqueror (82.1%). There was no difference between Skyfall and Soissons at N100, but at N200 the N-HI of Soissons (85.6%) was higher than that of Skyfall (84.5%).

### 3.3. Weather conditions during grain development

Weather conditions after anthesis in 2017 differed from those in 2016. Weather data for the variety Soissons and the varieties Skyfall and Conqueror are presented separately (Figs. 2 and 3 ) because in year 2016 Soissons' development was approximately one week earlier than the development of Skyfall and Conqueror. In a period from 15 to 23 DPA for Soissons and from 17 to 22 DPA for Skyfall and Conqueror, daily average temperatures were higher in 2017 than in 2016 (Fig. 2A). This led to higher accumulated degree days in 2017 from 17 DPA onwards for Skyfall and Conqueror, for Soissons the accumulated degree days in 2017 lay above those in 2016 for the whole period that was monitored (Fig. 2B). The accumulated rainfall and duration of sunshine during grain development are displayed in Fig. 3. Both parameters show considerable differences between the two years of the experiment. In 2017, the total rainfall with 39 mm in a period from anthesis until 35 DPA was less than in 2016 (97 mm for Soissons and 118 mm for Skyfall and Conqueror). The duration of sunshine in the same period was 273 h for Soissons and 287 h for Skyfall and Conqueror in 2017, while in 2016 it was only 171 h (Soissons) and 220 h (Skyfall and Conqueror). For Soissons in 2017 there was 58 mm less rainfall and 102 more hours of sunshine during grain development compared to 2016. For Skyfall and Conqueror, there was 79 mm less rainfall and an additional 67 h of
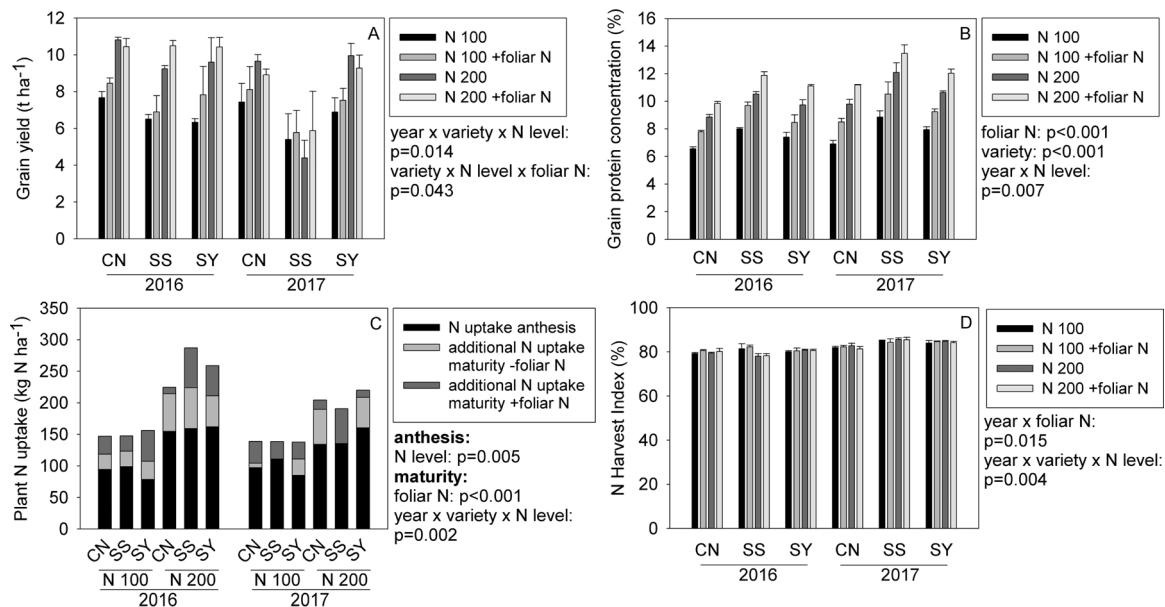
**Fig. 1.** Grain yield (A), GPC (B), plant N uptake (C) and N harvest index (D) of the cultivars Conqueror (CN), Soissons (SS) and Skyfall (SY) at the two N fertilisation levels 100 kg N ha$^{-1}$ (N100) and 200 kg N ha$^{-1}$ (N200), with and without foliar N application at anthesis, in years 2016 and 2017. Error bars represent standard deviations of replicates.

sunshine in 2017 in comparison with 2016.

### 3.4. Thousand-grain-weight and Hagberg falling number

Grain size was measured as thousand-grain-weight (TGW). The TGW was increased by the foliar N application at anthesis at both N fertiliser levels (N100 and N200) as is shown in Fig. 4A. When more N was supplied (N200 vs. N100) TGW was decreased, though this effect was only present when foliar N was applied at anthesis. The highest TGW was realised with 45.7 g at N100 when foliar N at anthesis was applied. When comparing cultivars, an increased N fertiliser level reduced grain size for Conqueror and Soissons but not for Skyfall. At both N fertiliser levels, Soissons had the smallest grains with an average

TGW of 37.72 g. TGW for Conqueror and Skyfall did not differ at N100, but at N200 Skyfall with a TGW of 46.37 g, had bigger grains than Conqueror (43.47 g). As TGW varied between varieties but yield was similar, it may be concluded that those varieties with smaller grains (lower TGW), such as Soissons, produced more kernels to compensate for the smaller size of grains.

Hagberg falling number (HFN) is a measure of the starch quality of flour produced from wheat grain. A falling number of less than 220 s is recognised as low, whereas a falling number greater than 300 s is high. The HFN was improved by 23.5% due to the foliar N application at anthesis when N supply was low (N100) but at high N supply (N200) there was no effect of foliar N on HFN observed (Fig. 4B). A higher N fertiliser level only improved HFN when no foliar N was applied at
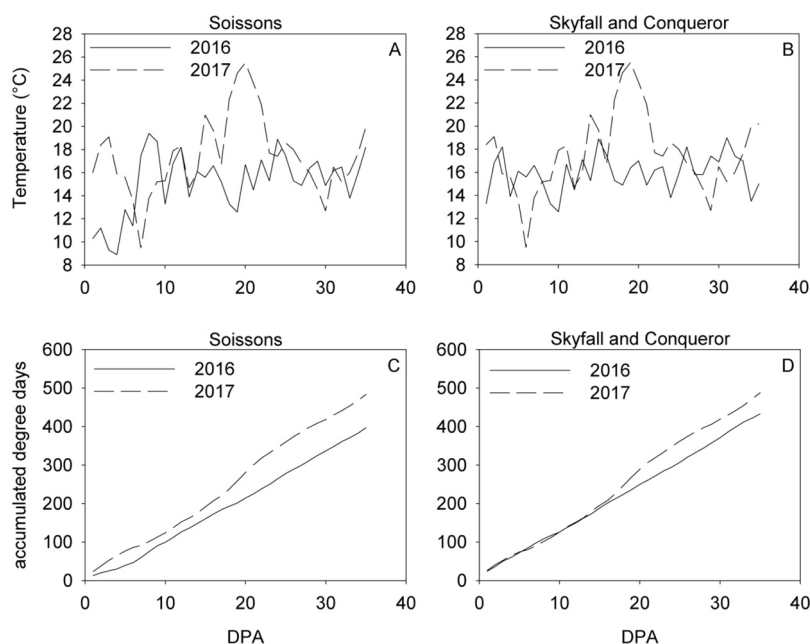


**Fig. 2.** Daily average temperature (A and B) and accumulated degree days (C and D) during grain development (1–35 days post anthesis (DPA)) of the cultivars Conqueror, Soissons and Skyfall in the years 2016 and 2017.
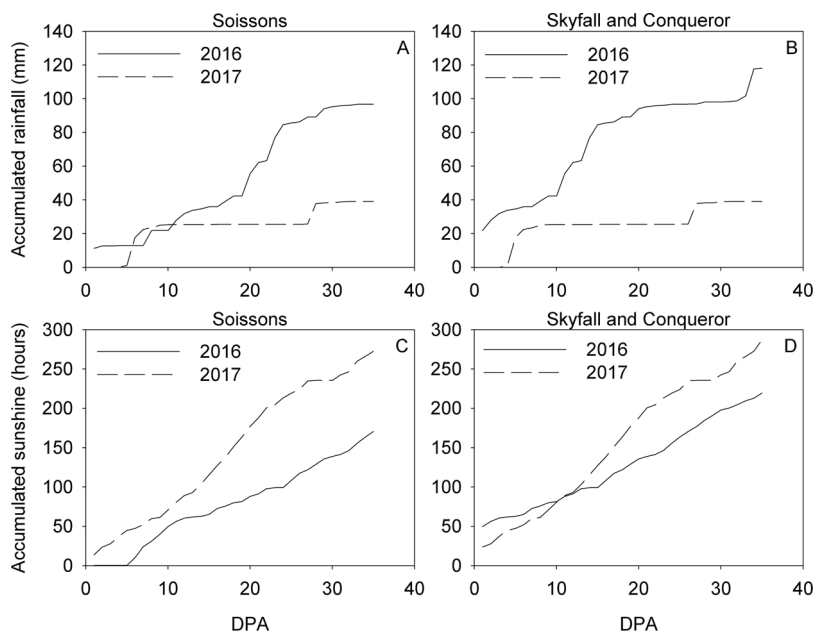
**Fig. 3.** Accumulated rainfall (A and B) and sunshine (C and D) during grain development (1–35 days post anthesis (DPA)) for the cultivars Conqueror, Soissons and Skyfall, in the years 2016 and 2017.

anthesis. There was a strong gradient within the three tested varieties, with flour from Skyfall having the highest HFN (350.7 s), followed by flours from Soissons (318.1 s) and Conqueror (221.4 s).

### 3.5. Bread volume

Bread volume was investigated by micro baking tests and results are summarised in Fig. 5. The volume of breads baked from the flour of the variety Soissons, with 30.14 mL (mean over all N levels), was significantly higher than those of Skyfall and Conqueror (24.28 and
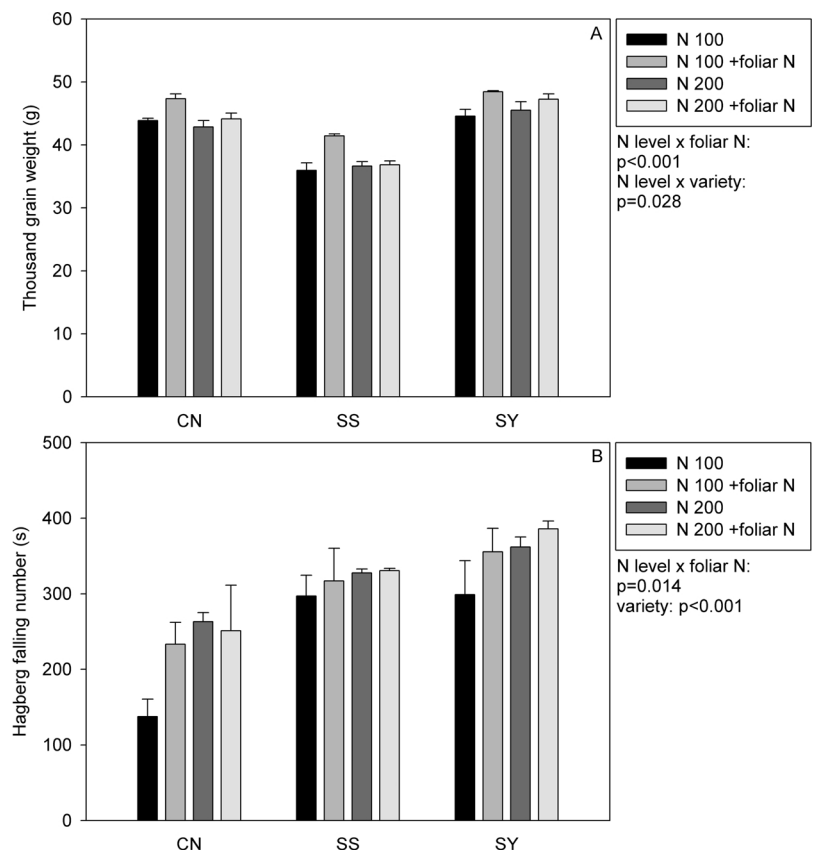


**Fig. 4.** Thousand grain weight (A) and Hagberg falling number (B) of the cultivars Conqueror (CN), Soissons (SS) and Skyfall (SY), at the two N fertilisation levels 100 kg N ha$^{-1}$ (N100) and 200 kg N ha$^{-1}$ (N200), with and without foliar N application at anthesis. Error bars represent standard deviations of replicates.
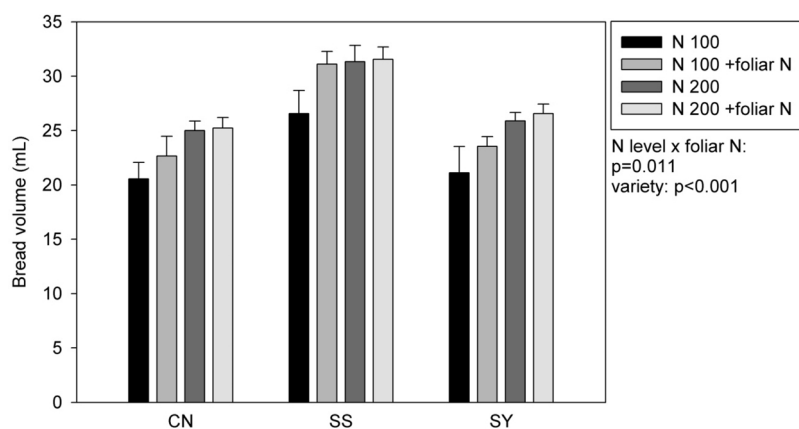
**Fig. 5.** Bread volume in the cultivars Conqueror, Soissons and Skyfall as affected by the two N fertilisation levels 100 kg N ha$^{-1}$ (N100) and 200 kg N ha$^{-1}$ (N200), with and without foliar N application at anthesis. Error bars represent standard deviations of replicates.

23.36 mL, respectively). A foliar N application at anthesis improved bread volume only at the low N fertilisation level (N100). In general, bread volumes were higher at the high N fertilisation level (N200). The average bread volume at N100 with an additional dose of N applied via the leaves at anthesis (25.78 mL) was comparable with the average bread volume at N200 without a foliar N application at anthesis (27.41 mL).

### 3.6. Protein composition, measured by SDS-PAGE

Extracted grain proteins were separated by size on the basis of their mobility using SDS-PAGE to analyse the gluten subunits. The protein bands were classified into three groups. The first group corresponded to the HMW-GS, the second group to the ω-gliadins and the third group combined a mixture of LMW-GS, and α-/β-/γ-gliadins. The SDS-PAGE results are shown in Fig. 6A.

Considering the general increase in GPC with higher N fertiliser level and foliar N application at anthesis, the alterations in the composition of the storage protein fractions given as percentage from total extracted protein is of particular interest. The LMW-GS and α-/β-/γ-gliadins were the major group of gluten proteins with an average of 90.7%. The HMW-GS made up for 6.5% whereas the ω-gliadins accounted for 2.8%. The percentage of ω-gliadins was not changed by foliar N application at anthesis. The percentage of HMW-GS was increased by foliar N application while the percentage of LMW-GS and α-/β-/γ-gliadins was decreased. The gluten protein composition was also influenced by variety. Soissons showed higher percentages of HMW-GS and ω-gliadins than Skyfall and Conqueror. The percentage of LMW-GS and α-/β-/γ-gliadins was lowest in Soissons (82.6%) and highest in Conqueror (96.6%).

### 3.7. Protein polymer composition, analysed by SE-HPLC

Storage proteins were analysed by size using SE-HPLC technique to analyse polymers. The results from this analysis are presented in Fig. 6B. The SE-HPLC chromatograms were divided into five sections, representing different storage protein fractions: large glutenin polymers (high molecular weight glutenin subunits (HMW-GS)) (F1), small glutenin polymers (low molecular weight glutenin subunits (LMW-GS)) (F2), ω-gliadins (F3), α-/γ-gliadins (F4) and non-gluten proteins (F5). (F1 + F2 + F3 + F4 + F5) represents the total protein amount, whereas (F1 + F2 + F3 + F4) represents the amount of gluten protein.

While the total amounts of HMW-GS, LMW-GS, ω-gliadins and α-/γ-gliadins were increased by a foliar N application at anthesis at both, low and high N fertilisation level, the amount of the non-gluten proteins was increased solely at the low N fertiliser regime (Fig. 6B). Only the LMW-GS and α-/γ-gliadins were enhanced by a higher N fertilisation

level. For all storage protein groups, there was a gradient between the three tested cultivars, with Conqueror showing the least amounts of each protein group, followed by Skyfall and then Soissons with the highest amounts. The HMW-GS are the only exception from this pattern, with Skyfall and Soissons showing similar amounts, both higher than Conqueror.

Ratios between certain storage protein fractions, such as HMW-GS/LMW-GS ratio, gliadin/glutenin ratio and gliadin/HMW-GS ratio ((F3 + F4)/F1), are said to indicate for the baking quality (Godfrey et al., 2010; Millar, 2003). The ratio of HMW-GS to LMW-GS is given by F1/F2, the gliadin/glutenin ratio is calculated by (F3 + F4)/(F1 + F2) and the ratio of gliadin to HMW-GS is represented by (F3 + F4)/F1. Both, HMW-GS/LMW-GS ratio and gliadin/HMW-GS ratio were changed due to the foliar N application at anthesis, but only at the low N fertilisation level (Fig. 7). The HMW-GS/LMW-GS ratio was decreased, whereas the gliadin/HMW-GS ratio was increased by the foliar N application. At the low N fertiliser level (N100), the amount of gliadins was increased by 33% as a result of foliar N application at anthesis, whereas the amount of HMW-GS was increased by 19%. As the increase in gliadins was stronger than that of HMW-GS, the ratio of gliadins to HMW-GS was enhanced by foliar N application at anthesis under a low N fertiliser regime (Fig. 7B). The gliadin/glutenin ratio remained unaffected.

### 3.8. Relationships between some protein-related parameters and bread volume

Regression analyses were carried out to evaluate the relationships of all gluten protein fractions and subunits measured by SE-HPLC, results obtained from farinograph measurements, and in addition GPC, with bread volume. The correlations of the most promising three parameters (GPC, total amount of gliadins and total amount of gluten protein) are presented in Fig. 8. The best correlation was found between bread volume and the amount of gliadins in the flour with a correlation coefficient of 0.718. When regression analyses were performed for the varieties separately, the correlations were weakest for Soissons and strongest for Conqueror.

## 4. Discussion

### 4.1. Plant N uptake effects on grain yield and GPC

As nitrogen is an essential component of grain proteins, plant N uptake is crucial for grain yield production as well as storage protein accumulation during grain development. When more N (N100 vs. N200) was supplied, plants assimilated more N until anthesis. This plant N uptake until anthesis results in both a higher grain yield and a
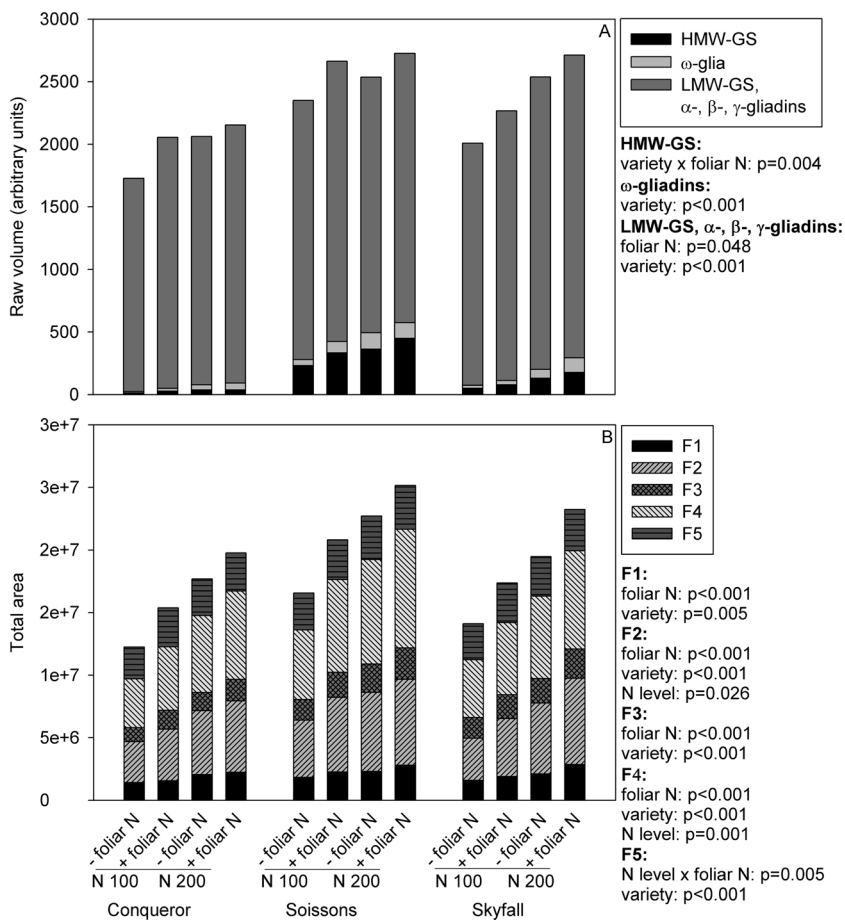
**Fig. 6.** Proportions of protein fractions at maturity, measured by SDS-PAGE (A) and composition of protein polymers analysed by SE-HPLC (B) (displayed as area underneath the chromatogram), as affected by the two N fertilisation levels 100 kg N ha$^{-1}$ (N100) and 200 kg N ha$^{-1}$ (N200), with and without foliar N application at anthesis in the cultivars Conqueror, Soissons and Skyfall. **F1**: large glutenin polymers (containing HMW-GS), **F2**: small glutenin polymers (containing LMW-GS), **F3**: fraction enriched in ω-gliadins, **F4**: fraction enriched in α-/γ-gliadins and **F5**: containing non-gluten proteins.

greater GPC. The increased plant N uptake at maturity due to a foliar N application at anthesis influenced grain yield only for Skyfall at N100 and for Soissons at N200, but led to an increased GPC in all three tested varieties at both N fertiliser levels (Fig. 1). Therefore, it can be assumed that the additional N, taken up after foliar N application at anthesis, was used primarily for grain protein synthesis. This finding goes along with results from Kichey et al. (2007) and Taulemesse et al. (2016), showing that major proportions of N absorbed during the post-anthesis period are translocated to the grain. Also, Martre et al. (2006) showed that especially under low N supply, the N accumulation in the wheat grain is sink-regulated. Plant N uptake is an important measure, but under high N fertiliser regimes, grain N concentration usually reaches a maximum and then remains stable, whereas the N concentration in the straw fraction continues to rise (Barneix, 2007; Kong et al., 2016; Pask et al., 2012; Triboi and Triboi-Blondel, 2002). In this case, the N harvest index would be reduced. In this experiment, the higher N level only decreased N-HI in Soissons in one of the two years. For the cultivars Skyfall and Conqueror there was no such effect observed. These findings demonstrate that even a dose of 200 kg N ha$^{-1}$ can still be used effectively by wheat plants by translocating the absorbed N to the grains. The changes in GPC under different N fertiliser treatments also support this statement because GPC was enhanced by both a higher N fertiliser level and a foliar N application at anthesis.

### 4.2. Influences of the weather conditions during grain development on yield and protein concentration

Weather conditions strongly affect grain yield as well as grain quality of wheat (Johansson and Svensson, 1998; Schulz et al., 2015). The higher grain yield in 2016 can be explained by the lower temperature during grain development, which slows down the process of

senescence and therefore extends the phase of grain filling. Johansson and Svensson (1998) observed a positive correlation between the hours of sunshine in May and June and the GPC as well as a negative correlation between the amount of rainfall in June and the GPC. These findings are supported by the results of our experiment which show less GPC in consequence of fewer hours of sunshine and more rainfall during grain development (Figs. 1 and 3).

### 4.3. Thousand grain weight and Hagberg falling number as influenced by N fertiliser management

Grain yield is composed of parameters, such as seed rate, shoots per plant and grain size. TGW was increased by foliar N application at anthesis but there was no effect of foliar N application on grain yield (Figs. 4 and 1). It can be concluded that due to the foliar N application the wheat plants produced fewer but larger grains. Demotes-Mainard et al. (1999) found that the number of grains is usually determined before anthesis, which contradicts the conclusion indicated above. During sampling in the field, there were more sterile florets observed in plots which had received a foliar N application. This could be the reason for the impact of foliar N application on TGW even if the number of florets (potential grains) has been determined earlier. The foliar N application at anthesis only improved HFN at the low N fertilisation level (N100). This finding is consistent with the statement of Gooding and Davies (1992) that foliar application of urea can reduce α-amylase activity and therefore improve HFN.

### 4.4. Grain protein composition as influenced by N fertiliser management

The composition of grain protein determines the end-use quality of wheat flours and was analysed in samples from year 2016.
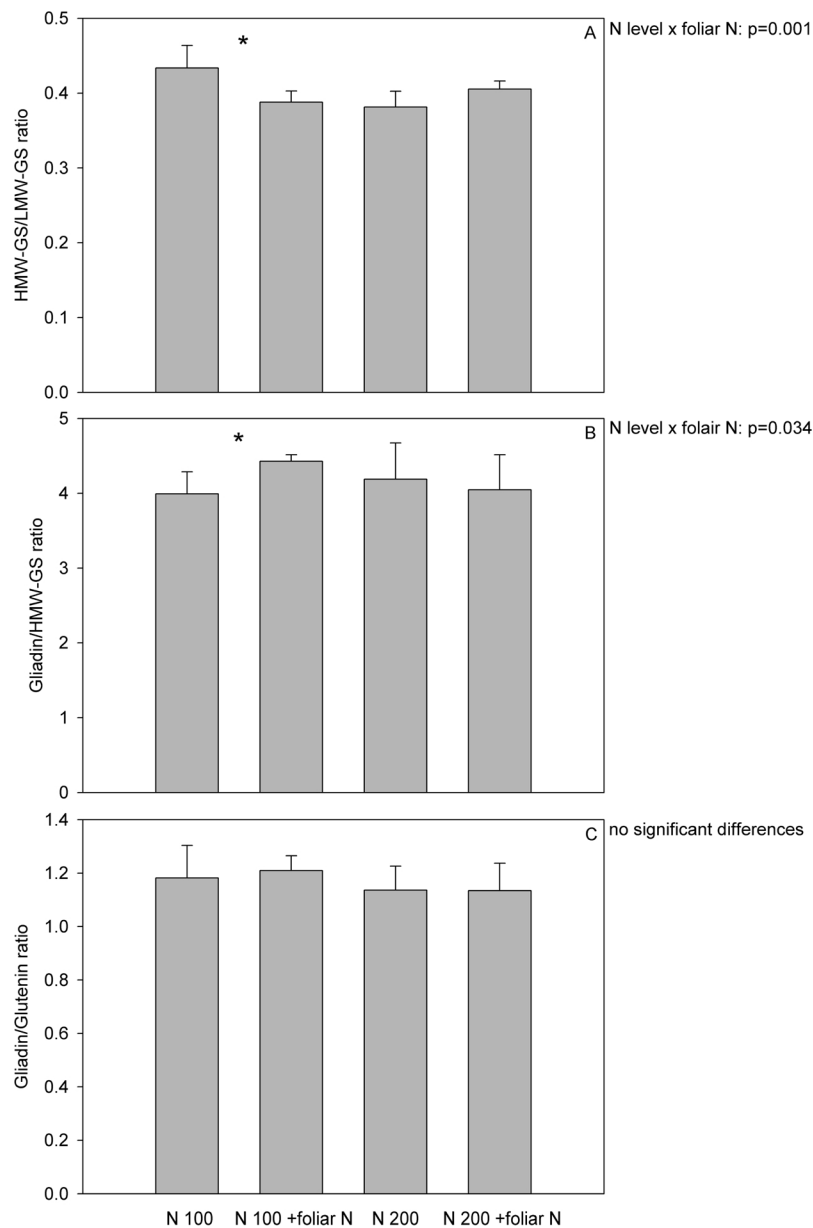
**Fig. 7.** HMW-GS/LMW-GS ratio (A), gliadin/HMW-GS ratio (B) and gliadin/glutenin ratio (C) at the two N fertilisation levels 100 kg N ha$^{-1}$ (N100) and 200 kg N ha$^{-1}$ (N200), with and without foliar N application at anthesis. Error bars represent standard deviations of replicates. Asterisks mark significant effects of a foliar N application (p < 0.05).

The results of SDS-PAGE show an increased amount of HMW-GS due to the foliar N application at anthesis in two of the three tested varieties (Skyfall and Soissons). The amounts of LMW-GS and α-/β-/γ-gliadins were also positively affected by the foliar N application (Fig. 6A). The ω-gliadins were the only group that remained unaffected by the application of foliar N. Daniel and Triboi (2000) and Hurkman et al. (2013) also report an increase of HMW-GS in response to N fertilisation but they found that ω-gliadins increase as well and amounts of LMW-GS decrease.

Considering the results from the SE-HPLC analysis, it is noteworthy that even though the N fertiliser level increased GPC, the effect on the composition of grain proteins was rather small. The N fertiliser level only affected the amounts of the fractions F2 (LMW-GS) and F4 (α-/γ-gliadins) but these changes caused a significant (p < 0.05) influence of N fertiliser level on both total gliadins and total glutenins (Fig. 6B). This confirms the results from Xue et al., 2016b; and Fuertes-Mendizábal et al., 2010, who also reported extended amounts of gliadins and

glutenins due to a higher N fertilisation rate. The amounts of gliadins and glutenins were also influenced by an additional foliar N application at anthesis. Since both protein fractions were enlarged by 21% as a result of a foliar N application, the ratio of gliadins to glutenins remained unchanged (Fig. 7C). Triboï et al. (2000) also observed equal increases of gliadins and glutenins in response to N supply and therefore, no changes in their ratio. On the basis of protein composition modelling, Martre et al. (2006) suggest a transcriptional regulation of the accumulation of protein fractions.

### 4.5. Concentration and composition of grain protein and their connection to bread volume

Grain N concentration was enhanced by N level as well as foliar N application (Fig. 1B). As the bread volume only increased due to a foliar N application at a low N fertilisation rate (N100), it can be concluded that an increasing grain protein concentration does not necessarily lead
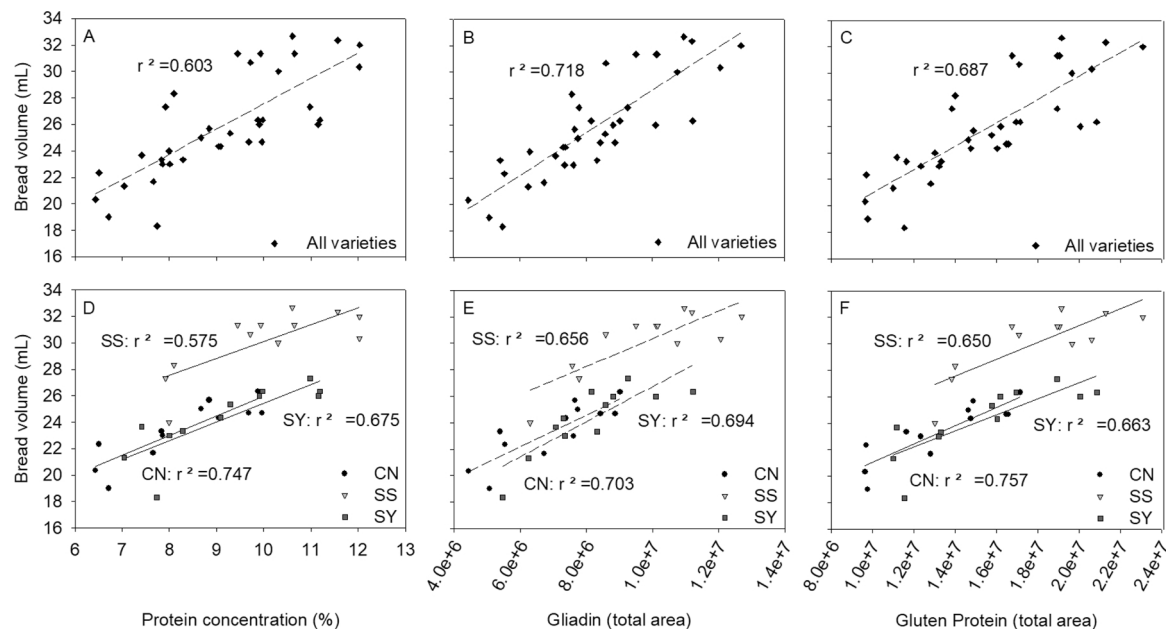
**Fig. 8.** Correlations between bread volume and protein concentration (A and D), bread volume and the amount of gliadins measured by SE-HPLC (B and E), and bread volume and the amount of gluten protein measured by SE-HPLC (C and F) for all three varieties (A, B and C) and for each of the three varieties (Conqueror (CN), Soissons (SS) and Skyfall (SY)) (D, E and F).

to an improved baking quality. Baking performance rather depends on the composition of grain proteins. According to Triboi et al. (2003), the storage protein composition strongly depends on the quantity of N in the grain, which is in contrast to the present findings, as increased GPCs due to a foliar N application at a high N fertilisation level (N200) did not lead to a further improvement of baking quality. Millar (2003) states that bread-making varieties generally tend to have higher HMW-GS/LMW-GS and lower gliadin/HMW-GS values. However, the results of our experiment show the opposite effect. At a low N fertiliser level (N100), the foliar N application at anthesis decreased the HMW-GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio. These alterations led to an improved bread volume. Interestingly, the foliar N effect on the ratios of HMW-GS/LMW-GS and gliadin/HMW-GS only occurred when N fertiliser supply was low (N100). There was no such effect observed at high N fertilisation level. The changes in bread volume follow the same pattern: bread volume was increased by foliar N application at low N fertiliser level (N100) but not when N supply was high (N200). The increased GPC due to foliar N application at the high N fertilisation level did not lead to further improvements in bread volume.

Surprisingly, the baking performance of the bread-making variety Skyfall was comparable to that of the feed wheat variety Conqueror. A possible explanation for the poor bread volume of Skyfall could lie in the flour type used for the micro-scale baking test. As wholemeal flour was used for the baking tests and Skyfall is a variety designed for the British market, where there is mainly bread consumed which is produced from white flour, this might have particularly affected the results of Skyfall. It can be speculated that Skyfall is unsuitable for being processed in the form of wholemeal flour but might show a good baking performance when used in form of white flour.

The parameter which could explain variance in bread volume in our experiment best was the total amount of gliadins measured by SE-HPLC ($r^2 = 0.718$). Grain protein concentration only accounted for 60% of the variance in bread volume when all three varieties were considered. When regression analysis was performed separately for the varieties, the correlation was weakest for Soissons ($r^2 = 0.575$) which had the highest GPC and strongest for Conqueror ($r^2 = 0.747$) which had the lowest GPC. This finding confirms the weaker relationship between GPC and bread volume in high protein varieties discovered by Gabriel

et al. (2017). This demonstrates that the evaluation of bread making quality of wheat flour should not depend on GPC alone but needs consideration of storage protein composition as well.

## 5. Conclusion

In this study, an increasing GPC did not necessarily generate an improved baking quality, as the increased GPC due to foliar N application at the high N fertilisation level did not lead to further improvements in bread volume. Baking performance rather depends on the composition of grain proteins. At a low N fertiliser level (N100), the foliar N application at anthesis decreased the HMW-GS/LMW-GS ratio and increased the gliadin/HMW-GS ratio, which led to an improved bread volume. No such effects were observed at high N fertilisation level (N200). In this study, the parameter which could explain variance in bread volume best was the total amount of gliadins.

These results imply that a late foliar application of nitrogen can be used to effectively improve baking quality in case the total N uptake was low due to unfavourable weather conditions during growing season until anthesis. The findings also show that this effect cannot be observed by measuring GPC alone but also needs a combined inquiry of storage protein composition as well as bread volume. For the future, a quickly detectable parameter for precise bread making quality predictions still needs to be developed. One possibility is that this parameter should be based on the total amount of gliadins.

## Declaration of interest

None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.eja.2019.04.004.

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